CRANFIELD UNIVERSITY

CARLA CRISTINA PEREIRA SALGUEIRO CATARINO RATO

THE LIFE MARKER CHIP: POTENTIAL USE OF APTAMERS AGAINST SMALL MOLECULES AND CONSIDERATION OF INSTRUMENT PLANETARY PROTECTION

CRANFIELD HEALTH

PhD THESIS

Academic Year: 2009 - 2012

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This thesis is submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

To my family

It always seems impossible until it is done.

Nelson Mandela

ABSTRACT

The Life Marker Chip (LMC) instrument was developed with the aim to detect evidence of life on Mars. The detection was based on an inhibition immunoassay. In this work aptamers were evaluated as potential alternative to antibodies for the LMC. Aptamers were synthetic oligonucleotides able to bind specifically with high affinity to a wide range of target molecules, and have been also integrated as bioreceptors in several detection instruments. The generation of new aptamers against two small molecules using the FluMag-SELEX method was tested and was verified the adaptability of pre-existing aptamers against small targets to the LMC assay type. Based on the fact that the LMC was going to be integrated into the space programme ExoMars, it was also implemented into a small scale experiment the Planetary Protection and Contamination Control requirements found on a life-search mission. In addition to that aptamers compatibility with a sterilisation procedure used in life-search missions was also tested. Furthermore because of the nature of the small molecules studied, multiple analytical chemistry techniques were assessed to verify covalent chemistry surface immobilisation.

Within the project timeline it was not possible to achieve a full aptamer generation process but it was possible to understand the methodology behind the procedure and give input for future work. It was found that the direct implementation of existing aptamers against small molecules into the LMC assay was not successful. It was also seen that in the case of aptamer integration onto the LMC some assay changes would probably have to be made. This information was very useful to understand if aptamers could be an alternative to antibodies and be implemented directly into the LMC. It was found that aptamers survived the preliminary sterilisation method applied, which might open the possibility of making aptamers convenient space bioreceptors, reducing time and costs of instrument Planetary Protection implementation. In conclusion aptamers were not straightforward alternatives to antibodies for the LMC because aptamers interacted differently with their targets in comparison to antibodies, particularly with small molecules. Also the biochemical simplicity of the small molecule targets introduced difficulties in aptamers generation that more complex targets would have not. Although aptamers shown incompatibility with the LMC assay format against small targets, they presented resilience to a sterilisation procedure implemented on space missions which could lead to the development of more robust bioreceptors for space missions. This information was helpful in understanding which assay formats were

better for detection of small molecules using aptamers and that might contribute for future assay choices applied in detection instruments. It was also possible to make recommendations for the LMC regarding design and validation methods used in life-search missions based on the lessons learn from the developed of a small scale experiment.

The developed work was presented at conferences and mentioned in an article journal, and in that way contributed to the knowledge of the space community in general.

Keywords: ExoMars, Mars, life-search, bioreceptor, FluMag-SELEX, ELAA, surface immobilisation, PP&CC, CASS·E

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LIST OF ABBREVIATIONS

AIV	Assembly, integration and verification
At.	Atomic
ATP	Adenosine triphosphate
BB	Binding buffer / Biobarriers
BEXUS	Ballon Experments for University Students
Bio	Biotin
CASS·E	Cranfield Astrobiological Strastospheric Sampling Experiment
COSPAR	Committee on Space Research
CU	Cranfield University
DHMR	Dry heat microbial reduction
DLR	German Aerospace Center
DNA	Deoxyribonucleic acid
ECSS	European Cooperation for Space Standardization
ELAA	Enzyme linked aptamer assay
ELISA	Enzyme linked immunoassay
EPSRC	Engineering and Physical Sciences Research Council
ESA	European Space Agency
E-SEM	Environmental scanning electron microscopy
FAM	Fluorescein
FTIR	Fourier Transform Infra-Red
FWHM	full width at half maximum
HEPA	High Efficiency Particulate Air
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IPA	Isopropanol
ISO	International Organisation fos Standardisation
IR	Infra-Red
LMC	Life Marker Chip
LPS	lipopolysaccharides
NASA	National Aeronautics and Space Administration
NMR	Nuclear magnetic resonance
OTA	Ochratoxin A
PAH	polycyclic aromatic / aliphatic hydrocarbons
PEG	Polyethylene glycol
PP&CC	Planetary Protection and Contamination Control
qPCR	Real-time Polymerase chain reaction

REXUS	Rocket Experiment for University Students
RLU	relative luminescence units
RNA	Ribonucleic acid
RT	Room temperature
RU	Resonance Units
SA	streptavidin
SAL	Sterility Assurance level
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SPR	Surface Plasmon resonance
SSC	Swedish Space Corporation
STFC	Science and Technology Facilities Council
TAE	Tris-Acetate-EDTA
TBD	To be defined
TBE	Tris-Borate-EDTA
UCZ	Ultra clean zone
USSR	Union of Soviet Socialist Republic
XPS	X-ray spectroscopy

Chapter 1. Introduction – Aptamers and Planetary Protection in the context of the Life Marker Chip

1.1. Preamble

The current thesis was performed as part of the on-going Science and Technology Facilities Council (STFC) funded Life Marker Chip (LMC) project. The LMC consortium aims to develop an instrument able to detect the presence of biomarkers that could reveal evidence of extinct or extant life on the planet Mars.

Cranfield is a major partner of the LMC consortium and is responsible to develop the biological detection system used for the biomarkers search on Mars. The LMC integrates a biological assay where antibodies are used as bioreceptors. This work tested the ability of aptamers to be alternative bioreceptors to the antibodies. In order to understand if this was possible the following tests were made: test the possibility of generating aptamers against LMC biomarkers; the integration of aptamers into the existing LMC assay format; and preliminary tests to the aptamer's survival to one of the sterilisation procedures required in this type of space mission. Also in this work it was used a small scale experiment to demonstrate the implementation of specific design, validation and clean procedures in a life-search mission type. The experiment was conducted in order to contribute at an early stage to the overall LMC required Planetary Protection and Contamination Control (PP&CC) plan, essential to a life-search mission.

1.2. Context for thesis – Search for life on Mars and the Life Marker Chip instrument

The life on Earth as we know it could be simply characterised by the presence of the Carbon atom, the existence of complex metabolic pathways based on oxidation-reduction chemistry, the passage of genetic information to the subsequent generations and the ability to adapt and evolve, (Schart 2009). It would be also necessary to address the critical need for liquid water in the biological systems. The presence of liquid water was justified by the fact that the Earth was around 150 million Km distance from the Sun giving origin to a mean planet temperature below the water freezing point. Also the existence of an atmosphere helped to keep the evaporated water and its cycle, (Plaxco, Gross 2006).

The actual scientific knowledge and understanding of Earth as well as the beginning of the space exploration in the 20th century, allowed the possibility of using the advances in technology for searching for life on other planets. Mars was traditionally a well observed planet and became the object of study for Astrobiology based on factors such as its location in the solar system, the fact that it was a solid terrestrial planet (as the Earth), the existence of frozen water and similar biochemistry to Earth´s. Based on this Mars could present similar life conditions to the ones found on Earth.

Over the past years several missions have had the role of studying Mars and Life search missions such as the Viking were made with the aim of detecting organic compounds that could prove the presence of extant life. Currently missions, as for example Curiosity (NASA´s rover), are exploring the geochemistry of the planet in order to detect geological evidences of an environment that could have had or has life. The LMC instrument was developed to detect the presence of life organic molecules markers on Mars and it was planned to be sent as part of the ExoMars missions, led by the European space agency (ESA). The LMC instrument detection was based on an immunoassay and this work studied the possibility of using aptamers as alternative bioreceptors for the LMC, as well as their ability to survive a sterilisation procedure used in the implementation of Planetary Protection in life-search missions.

1.3. Problems to be addressed

1.3.1. Need for alternative molecular receptors for planetary exploration

Antibodies were used as bioreceptors in life-search instruments such as the LMC, the Signs of Life Detector (SOLID) and the Modular Assay for Solar System Exploration (MASSE) (Parnell, Cullen et al. 2007). The choice of antibodies relied on their wide use as bioreceptors, their implementation in detection systems able to function with complex matrices and their ability to bind to epitopes common to family molecules. Those characteristics allowed detection of a specific group of molecules and in real / complex samples, (Cullen, Mark 2007, Parro, Rodríguez-Manfredi et al. 2005, Warmflash, McKay et al. 2002).

Disadvantages of using antibodies were that antibodies generation was dependent on animal hosts, which made generation of antibodies difficult against toxic components and small biological molecules with low or no immunogenicity. Besides that it was a

long and expensive process and the obtained antibodies varied from batch to batch. Finally antibodies did not recover once denatured, losing their binding ability. Based on those limitations it was necessary to search for alternative bioreceptors to see if it was possible to overcome those issues.

Aptamers were initially reported by Gold and Tuerk, and by Ellington and Szoztak in 1990. Both groups reported the *in vitro* generation of synthetic oligonucleotides able to bind specifically to their target, (Tuerk, Gold 1990, Ellington, Szostak 1990). The advantages of using aptamers were that aptamers as synthetic oligonucleotides did not require animal use, were generated in shorter periods of time and presented reproducibility between batches, allowed chemical modifications and refinement and could be generated against toxic compounds or those with low immunogenicity. Also aptamers were compatible with high-throughput methods such as automation, and importantly, could suffer brief denaturation processes that would recover their initial conformation and binding ability. Because the size of aptamers was between 5 and 15 kDa, which was significantly smaller than antibodies (IgG with 158 KDa, and scFvs antibodies varied from 26 to 28 kDa); it could allow better binding access than the ones available for antibodies, (Burtis, Ashwood 2001, Mascini 2009, Weisser, Hall 2009). For all those reasons aptamers were tested as alternative bioreceptors to antibodies for the LMC.

1.3.2. Need for Planetary Protection and Contamination Control for Life Marker Chip type instruments

The LMC aimed to detect the presence of life biomarkers on Mars. In order to do so the LMC would be integrated into the ExoMars rover and suffered PP&CC implementation. The implementation of PP&CC would diminish the probability of contamination of Mars with Earth microbial and organic content, prevent instrument detection of false positives, and in return missions protect Earth from extraterrestrial contaminants.

As it will be explained in detail later on this document the implementation of PP&CC was required for space missions and its implementation was based on the mission aim and destination. The LMC as part of the ExoMars and being a search for life experiment had to comply with the PP&CC requirements. In order to give preliminary input to the LMC PP&CC plan, a small scale experiment was used to demonstrate the PP&CC implementation. This also enabled lessons learnt from the experiment to be taken to the LMC. Based on the fact that aptamers were tested as alternative

bioreceptors to the LMC their resilience to sterilisation by dry heat microbial reduction (a common method and one which antibodies are not compatible with) was also tested.

1.4. Approaches to address the problems

Based on the need to understand if aptamers could perform as alternative bioreceptors to antibodies and to give preliminary input on the LMC PP&CC plan, as well as to verify if aptamers could resist to a sterilisation method by dry heat microbial reduction, the following approaches were taken:

- It was tested if aptamers could be generated against the LMC targets
- It was tested if aptamers could be implemented into the LMC assay format
- It was tested if the PP&CC implementation could be demonstrated
- It was tested if aptamers could be compatible to sterilisation by dry heat microbial reduction

1.5. Thesis structure

The work presented in this thesis was structured in a total of seven chapters. Briefly the content of each chapter was explained to give an overview of the thesis organisation.

Chapter 1 consisted of a brief introduction to give a general overview of the project and to address the problems that this thesis' work aimed to answer.

Chapter 2 presented an overall literature review, in which the major concepts and background for this work were given. At the beginning of the chapters describing the experiments (chapters 4, 5, and 6), a more detailed literature review was presented.

Chapter 3 described the overall aims and objectives of this work.

Chapter 4 was the first of three experimental chapters and addressed the issue of the generation of aptamers against the LMC targets.

Chapter 5 was the second experimental chapter and addressed the question about the compatibility of aptamers integration as bioreceptors into the LMC assay format.

Chapter 6 was the third experimental chapter and covered the need of LMC implementation of PP&CC and the response of aptamers to a sterilisation method used commonly on space missions.

Chapter 7 presented the overall thesis discussion and conclusions, and the further work that could be done on the subject in the future.

Chapter 2. Literature review – Life on Mars, the Life Marker Chip, Aptamers and Planetary Protection

2.1. Aims and objectives

The aims of this literature review were to give an overall background about life search on Mars and the LMC instrument part of a life-search mission.

The objectives that arose from the aims included an overview on life biomarkers that could be searched for on Mars, the background about the LMC instrument that has been developed in order to search for evidence of life on Mars, the aptamers as synthetic bioreceptors, and the required PP&CC implementation in Life-search space missions.

2.2. Life on Mars

The definition of life was a challenging subject and was briefly introduced in section 1.2 (Chapter one). Earth´s life obeyed to the biology dogma where DNA was transcribed into RNA, which was translated into proteins. This simplistic dogma was responsible for generation of molecules with high specificity and functionalization in the life systems. Life, as we know it was the product of the Darwinian evolution because it reacted to environmental stimuli and sustained itself during time and reproduced into new better adapted generations, while it was made of the same basic components and had the same requirements such as the need for liquid water. Based on that and to understand how life arose on Earth several theories were developed. These were known as life theories and included the Chemical Evolution (based on the Miller-Urey model where the Earth´s pre-biotic conditions were re-created and simple amino acids were biosynthesized), the Protein World (proteins had the ability to support life because of their role in the biochemical reactions present in all living systems), the RNA World (RNA was a good template for storage and replication of genetic information in the early Earth´s development stages), and others such as Panspermia,.which supported the presence of life on other planets besides ours, for example on Mars, and that life could have been transported between planets by meteorites carrying life forms,(Sullivan III, Baross 2007, Rauchfuss 2008, Miller 1955, Dworkin, Lazcano et al. 2003).

Mars´ characteristics for life support

Mars presented several similarities with Earth; it was a rocky planet with a thin atmosphere consisting majorly of CO_2 and N_2 but also with water in a concentration enough to cover the planet surface with 1 µm depth. It had rotation and translation with 39 more minutes in rotation than the 24 hours of Earth's and the planet presented 25 degrees of inclination. The inclination gave origin to seasons as on Earth and influenced the insolation on the equator regions with the south hemisphere hotter than the north. The internal structure of the planet was believed to be similar to the Earth; from meteorite studies it was possible to conclude that it had three regions, the crust, the mantle and the core. From the observation of the irregular surface and the absence of a magnetic field it was deduced that the planet had an active core and probably lost its properties as volcanoes calderas were observed which proved the existence of volcano activity at some period of the planet history. It is not understood, at the moment, whether Mars has volcanic activity or whether the volcanoes are inactive. The surface presents a vast number of characteristics besides the mentioned volcanoes, such as valleys, mountains, channels, canyons, and depressions that could be caused by lakes and oceans based on the fact that sediment deposits and solid water (and possibly liquid in the past) were found. This information was of major importance in considering that life biomarkers were preserved on Mars, they could be found conserved at the subsurface, in a salt form or in the ice frozen areas. Also the similarity in the planet geological history between Mars and Earth (both had four major eons located more or less in the same geologic time), might had led to similar environmental conditions which might have been translated into alike biochemical structures that probably generated life forms or at least organic molecules with equivalent structural and functionality. The major differences between Mars and Earth were the environmental conditions and size and mass: Mars was colder and drier, smaller and with ten times lower mass than Earth's, (Carr 2006, Pudritz, Higgs et al. 2007, Sullivan III, Baross 2007, Forget, Costard et al. 2008).

Considering the Earth life forms, it was verified that life existed even in extreme conditions such as temperatures values below -2 ºC or above 50 ºC, salinity values above 8 %, pH below 4 or above 8 and atmospheric pressure up to 20 MPa, (Natural History M. 2010). The existence of extremophiles on our planet maintained the possibility of similar life forms in twin extreme environments, such the ones found on Mars. Another interesting point was the analysis of Martian meteorites. It was found a

particular meteorite (ALH84001) which presented small hydrocarbons denominated polycyclic aromatic and aliphatic hydrocarbons (PAH) such as phenanthrene and pyrene. The organics origin was attributed to Mars and considered evidence of early life forms on Mars. It was assumed that Martian origin because similar organics were not found in another meteorites assuming that it was not Earth contamination That was a controversial statement and so far it was not possible to verify that assumption and prove that it was not pre-existent from Earth, (Sullivan III and Baross, 2007; Pudritz, 2007).

Brief review of Mars exploration made to date

Since 1960 more than twenty spacecraft missions flew by, orbited or landed on Mars but until now only two missions were sent to Mars in search for life. Those were the Viking (1975) and the Beagle 2 (2003). Of these the only successful mission was the Viking, which sent data that did not support evidence of life on Mars by not detecting the presence of organic material at parts per billion (ppb), (Pudritz, Higgs et al. 2007, Navarro-Gonzalez, Enrique Iniguez et al. 2009). Besides those missions others gave more information about Mars geochemistry and the environment. Examples of those were the Pathfinder lander (1997), which gave information about atmospheric conditions and soil composition, the Odyssey orbiter (2002), which allowed observing ice water at the poles, or the Phoenix lander (2007), that revealed that atmosphere was rich in CO2 and accessed possible habitable zones. Currently the Curiosity rover was sent to study the geochemistry of Martian soil and find if it could sustain life forms (NASA 2010).

2.3. Biomarkers on Mars

The choice of biomarkers to search for life evidence on Mars using the LMC, were discussed by Parnell et al (2007). The biomarkers list included representatives of existing life forms, extinct biomolecules found on fossils (to understand if life was present on Mars before but not currently), biomarkers representative of organic compounds found on meteorites, and molecular contaminants that could be present on the LMC instrument for assay and contamination control. The extant biomarkers were representative of the basic components of the living cell; it included fatty acids (used also as extinct life biomarker), metabolic molecules involved in energy production based on oxidation-reduction reactions as ATP or phosphoenolpyruvate, and molecules that encoded genetic information as DNA. The chosen biomarkers for

searching for evidence of extinct life were simple biomolecules with a stable biological structure that resisted biochemical degradation, examples of them were stearanes or hopanoids. The chosen biomarkers for extinct life were elemental units of complex biomolecules as amino acids or PAH as pyrene. In the contamination control biomarkers, used for assay validation, it was chosen molecules signature of bacterial presence such as lipopolysaccharides (LPS) or dipicolinic acid, Table A-1. For assay development purposes the LMC targets (life Biomarkers) were organised according to their biochemical properties: small apolar acyclic aliphatic (e.g. phytane, squalane, hexadecane), small apolar polycyclic aliphatic (e.g. 5β-cholanic acid, hopanoids), small polycyclic aromatic (e.g. pyrene, phenanthrene, naphthalene), and small polar (e.g. amino acids), (Parnell, Cullen et al. 2007, Sims, Cullen et al. 2012).

2.4. Life Marker Chip and other related instruments

Other sensing instruments were developed with the aim of detecting life biomarkers, as reported in the previous chapter (section 1.3.1, Chapter one) they were the SOLID and MASSE. Those were tested on Earth´s extreme life condition environments that were considered analogues to Mars´ and succeeded in demonstrating the principle by detecting the presence of extremophile life forms. The referred instruments presented similar detection methods, both based on fluorescent detection and immunoassays. The LMC instrument was also developed under the same background, using an immune inhibition assay and fluorescence optical detection. The LMC instrument was made to detect the presence of life biomarkers (organic molecules) on Martian regolith or rock samples. The LMC instrument was organised in four individual sub-systems that allowed the samples analysis at Earth´s standard pressure and temperature and also the verification of the assay pH values. Associated to each system was a fluid cartridge, a pump and a laser diode. Each system had a single-use microfluidic channel where the microarray spots were located. The full system allowed independent and in parallel analysis of four Martian samples and the search of 25 targets on each collected sample, (Sims, Cullen et al. 2012).

The LMC project was designed and developed with the aim of being integrated into the ExoMars mission flying in 2018 to Mars. The current status (late 2012) of the LMC was the de-selection of the mission due to payload mass and volume restrictions. Nevertheless it remains a candidate for integration in other life-search missions.

2.4.1. Assay Format

The LMC assay format was an inhibition ELISA, where the fluorescently labelled antibody was mixed and incubated in solution with the free target and then exposed to surface immobilised target derivatives. In the LMC the drilled Martian sample was mixed with extraction solvents, and then the obtained fluid put in contact with the fluorescently labelled antibodies. The new mixture was put in contact with the surface immobilised target derivatives, so that the unbound labelled antibodies could bind to the surface. The detection would be done by optically detection of the amount of fluorescence present on the chip surface. The lower the signal obtained, the higher the presence of target in the Martian soil sample, Figure 2-1.

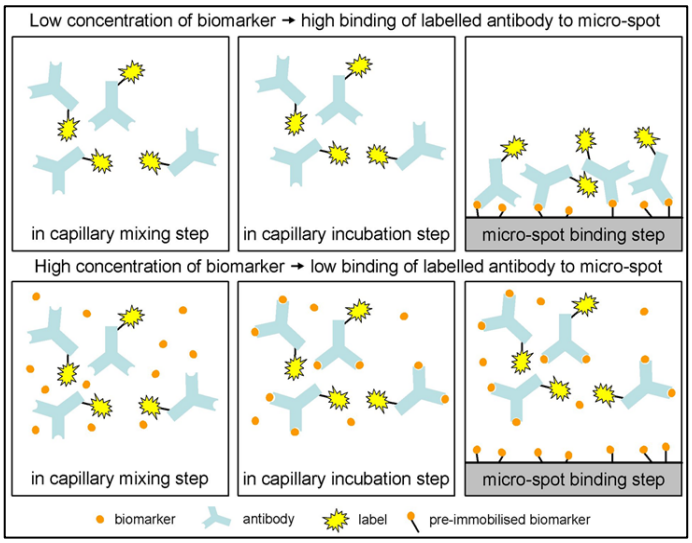

Figure 2-1 LMC inhibition immunoassay describing the scenario of low existence of biomarkers on Martian sample (above figure line), and the scenario of high presence of biomarkers on the sample (lower figure line), (Sims, Cullen et al. 2012).

The reagents for the array were freeze-dried packed and conserved until sample analyses, being solubilised in assay buffer solution before the assay detection, so that could maintain biochemical integrity during the flight. The current assay sensitivity was reported at ~10 ppb, but further work was under development, (Cullen, Mark 2007, Sims, Cullen et al. 2012).

2.5. Aptamers

As mentioned before, aptamers presented several advantages over antibodies. Those included the capability of recovering their shape, folding and binding ability after

suffering denaturation, their smaller size, the fact that were synthesised in vitro, being reproducible between batches and had the potential of automation. Aptamers required less time consuming in its creation and were cheaper than the production of antibodies. Aptamers could be generated virtually against any type of target. In addition to this aptamers have been used as bioreceptors in diagnostics, therapeutics, environmental analysis, food industry and biosecurity, (Tombelli, Minunni et al. 2007, Karkkainen, Drasbek et al. 2011). Furthermore several patents were registered giving author rights to aptamer development and use, in combination with that, several biotechnology companies started focusing their product on aptamers, developing aptamer based therapeutic drugs and detection methods using aptamers, (Missailidis, Hardy 2009).

Based on the presented advantages over antibodies and already demonstrated applications in similar assays to the LMC, aptamers were chosen to be tested if could be used as alternative bioreceptors to antibodies in the LMC instrument.

2.5.1. Aptamer generation

Aptamers were synthetic oligonucleotides sequences that due to adopting a specific three-dimensions folding bound to target molecules, were classified as affinity probes. They could be DNA or RNA aptamers and compete with other bioreceptors in the choice of the most appropriate molecule to capture targets of interest. The principle of aptamer generation was based in well-known scientific pillars such as the biology central dogma, the Darwin's evolution theory, the Watson-Crick base- pairing principle, and the RNA world theory. In the first, the nucleic acids had the ability to be templates in the replication and transcription processes for the generation of other nucleic acids; this allowed artificial amplification by polymerase chain reaction (PCR) and molecular interaction between oligonucleotides and targets. In the second, the Darwinian evolution principle was mimicked by the *in vitro* selection, where the strands that bound to the target were selected and amplified in each iteration, until it was obtained a low diverse pool with high specificity from the original pool that had high diversity but low specificity. Aptamer generation was also based on the theory that RNA in the primordial world had the ability of self-replication and catalytic properties such as seen in ribozymes and riboswitches in gene expression mediation. Based on that principle RNA was considered able to bind cofactor molecules (Gold, Tuerk 1995, Jayasena 1999, Klussmann 2006, Roth, Breaker 2009).

The aptamers generation method was named SELEX (systematic evolution of ligands by exponential enrichment) and consisted simplistically on successive iterations of the steps selection, elution, amplification and positive strand separation. The selection step was the mixing of the target of interest and a random ssDNA library with around 10^{13} to 10^{15} different strands[1].The library ssDNA strands presented two fixed regions at its extremities for primer annealing in the amplification step. During the target and library mixing some ssDNA strands interacted with the target and bound to it. The elution was the step in which it was separated from the initial mix the complexes formed between target and strands, and the ssDNA that showed target affinity was recovered after the preliminary separation. Gopinath et.al, (2007) reviewed the separation methods used within the SELEX method, (Gopinath 2007). The following step was the amplification by PCR of the eluted ssDNA. In the PCR amplification it was possible to introduce any desired modifications to the ends of the strands by annealing of modified primers. Once amplified the ssDNA became dsDNA. Because it was used only the positive strand (ssDNA pool) in the selection, and since it was those strands that showed affinity, the positive strand was separated from the negative strand by a denaturing procedure and recovered. A new ssDNA pool was made with the recovered positive strands. That pool was used in the following iteration. The method was repeated until the pool affinity showed no improvements in its binding ability to the target. In order to reduce the iterations number, the stringency conditions could be increased during the cycles by changing slightly the selection conditions or doing counter-selection with biochemically similar molecules to the targets of interest. Once the ssDNA pool presented the enriched ssDNA, the selected strands were cloned by bacterial vector transformation and the achieved colonies sequenced. The sequences could show similar conserved regions that indicated the sequence that gave origin to a specific binding secondary structure. Subsequently the potential aptamers were implemented in binding assays and the sequences that showed better affinity and specificity were classified as aptamers against the initial target. In some cases the molecular interaction between target and aptamer was studied by X-ray crystallography or NMR, (Stoltenburg, Reinemann et al. 2007). In the case of generating RNA aptamers, it was necessary to

[1] The random ssDNA library complexity could be calculated as 4^N, where N was the length of the sequences. That information helped to understand which amount of ssDNA library should be used in the selection, considering nevertheless that the maximum experimental screened amount will not exceed the given range of 10^{13} to 10^{15} different strands, (Sampson 2003).

do *in vitro* transcription before the selection step, and the process was the same as explained.

The generation of aptamers since its beginning had many variations (more than 20 different SELEX methods exist nowadays) but kept the explained initial SELEX principle. That showed the adaptability and high ability of modelling the creation *in vitro* to the demands of each experiment and scientific work, making aptamers a versatile bioreceptor and suitable for applications in almost every area. Besides modifying the method to specific requirements it also introduced the possibility of making chemical modifications to aptamers after their generation. That was mainly a response to prevent enzymatic degradation provoked by RNAses or DNAses. The modifications could be in the phosphodiester backbone or in the nucleotide bases, usually these last were in the sugar skeleton 2´position or at the pyrimidine C5. Another way of overcoming the enzymatic cleavage was the use of aptamers enantiomers, known as spielgelmers. Spielgelmers were chemical mirror images of aptamers generated against a target mirror image. The final spielgemer bound to the original target and was not sensitive to degradation, (Eulberg, Klussmann 2003).

2.5.2. Aptamers for small molecules

Small molecules were considered more difficult targets to generate bioreceptors against. This was because naturally they had a lower number of charged groups or complex structures, compared to larger molecules such as proteins, which stimulated molecular interactions such as hydrogen bonds, π-π stacking or van der Waals interactions, all necessary to occur molecular binding, (Roth, Breaker 2009). Nevertheless based on the fact that RNA should present natural binding ability to small molecules such as cofactors, it was attempted with success to generate RNA and DNA aptamers against small molecules. It was seen however that in their majority were small molecular targets that presented complex structures such as aromatic rings or were rich in charged groups. Several reviews were made regarding the existing aptamers generated against small molecules, (Stoltenburg, Reinemann et al. 2007, Klussmann 2006, Famulok 1999), and the latest reviewed the existence of more than 40 aptamers against small molecules. The authors organised the targets according to their classification which included examples of cofactors, nucleotides, antibiotics, drugs, carcinogenics, organic dyes, amino acids, carbohydrates and other biocompounds. The same authors referred that the lowest reported affinity value was

regarding the RNA aptamer to its target tobramycin (K_D of 0.77 nM), and that the general binding affinities presented by aptamers against small molecules were located in the μM level. It was also reviewed by the same authors examples of excellent specificity of aptamers (the theophylline RNA aptamer did not bind to caffeine), and ability to discriminate between enantiomers (the thalidomine DNA aptamer bound preferentially to the (R) form), (Lau, Li 2011). In some cases it was reported that after generation of aptamers those went by a second selection in order to improve the binding affinity performance, (Stoltenburg, Reinemann et al. 2007). Another example of increment of affinity and also changing of specificity was reported by Mannironi et al., (2000) who changed the affinity of an aptamer by inserting mutations up to 30%. From the initial dopamine binding aptamer pool, the authors generated an aptamer with affinity to L-tyrosine. (K_D of 35 μM),(Mannironi, Scerch et al. 2000). This could be advantageous in the case of the LMC instrument in order to obtain aptamers with detection limits from the nM to the pM range.

2.5.3. Aptamer applications in assays

Aptamers ability to recover their conformation after denaturation allowed the development of reusable biosensors, also properties such as being stable molecules with low molecular weight, allowed standard biochemical modifications; presented versatility in transduction systems implementation, and their low affinity values made them adaptable and robust bioreceptors, (Baldrich 2011, Cass, Zhang 2011). Both RNA and DNA aptamers were implemented as bioreceptors in detection systems used in diagnostic, (Hesselberth, Robertson et al. 2000), therapeutics, (Lassalle, Marchal et al. 2012), biosecurity,(Fischer, Tarasow et al. 2007) general detection of microbes and viruses, (Torres-Chavolla, Alocilja 2009), or in separation and purification processes, (Ravelet, Grosset et al. 2006). Aptasensors used optical, electrochemical, mass change and oligonucleotides amplification as detection methods. Those included aptamer incorporation of fluorophores that changed their fluorescence initial characteristics after aptamer binding conformation changed; the use of nanoparticles as quantum dots, whose fluorescence allowed cells *in vivo* visualisation, and gold nanoparticles which gave quick assessment by colour change according to the level of molecular binding. Other options included the use of electrochemical detection methods, which detected the electron flux according to oxidation-reduction reactions caused by aptamer binding interaction, and label free systems as the surface plasmon resonance (SPR), which detected mass changes that occurred with aptamer binding.

Cho et al (2009) described the technique where binding confirmation was made by amplifying the bound aptamers with real-time PCR, (Cho, Lee et al. 2009). The majority of the detection assays used a solid platform to which was immobilised one of the assay components. Also the demonstrated assays presented different formats, as single-site binding (direct), double-site (sandwich) binding or competition binding. Examples of each format assay using aptamers was reviewed by Mascini et al (2012), the authors also mentioned that the most applied aptamers in aptasensors were the aptamers against thrombin, vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), immunoglobulin E (IgE), lysozyme, theophylline, cocaine and ATP, (Mascini, Palchetti et al. 2012).

Sassolas et al (2011) reviewed the use of aptamers in assays occurring only in solution. The detection was based in colorimetric, fluorescence, electrochemical and magnetic resonance imaging (MRI) changes that occurred with the aptamer-target binding. The detection format was similar to the ones referred previously with the exception that the obtained signal occurred because it was depended in specific aptamer conformation or on a linker molecule that would work as a binding reporter, not requiring separation of the formed target-complex from the initial mixture. An example was the structure-switch in which a fluorophore and a quencher were present in complementary sequences to the aptamer, after the aptamer bound to target the quencher strand was released and the fluorophore gave a stronger signal. The better affinity value reviewed by the authors was of $4x10^{-5}$ nM, regarding adenosine detection using gold nanoparticles, (Sassolas, Blum et al. 2011).

Small molecules detection with aptasensors was reviewed by Walter et al (2012). The authors presented five types of detection based on the aptamers´ ability of target binding and conformation change. The detection methods were the ones already described but with a different perspective, based on the aptamers interaction with the target. The binding interaction could be made in a sandwich, in a competition, based on the target-induced dissociation mode, on the target-induced structure switching, and on the target-induced re-arrangement methods. The lower limit of detection reviewed was also regarding adenosine aptamer but used in an electrochemical detection assay with $2x10^{-5}$ nM, (Walter, Heilkenbrinker et al. 2012).

Besides the mentioned methods, aptamers were also introduced in microarrays for simultaneous detection of several targets. In that case the binding signal was detected by fluorescence and was developed against proteins with detection limit in the nM - pM

range, (Cho, Collett et al. 2006, Baldrich 2011). A microarray was also developed which allowed the simultaneous detection of more than 800 proteins at once present in blood serum. In that case the aptamers were initially generated with a 5´-end modified bases library (each base was side-chain modified with simple amino acids structures) which gave origin to a new version of aptamers denominated SOMAmers. Those were presented as having increased binding ability to previously considered difficult targets for aptamers to successfully bind. The microarray was reported to detect protein concentrations in the pM range, (Gold, Ayers et al. 2010).

2.6. Planetary Protection and Contamination Control

Planetary Protection importance in space missions was reviewed by Bergstrom, S. et al (2004), in which it was mentioned that the principal objectives of PP were to keep the explored planet initial conditions as unspoiled as possible to allow future missions, to avoid compromising the exploited planet by minimising its contamination with Earth microbial or organic material, and also to preserve the planet Earth from possible extraterrestrial contamination present in returning missions´ material. In order to regulate the procedures required to reach those objectives the Committee on Space Research (COSPAR) was created in 1958 (Bergstrom, Rummel 2004). COSPAR classified the different space missions into categories based on their mission type and destination, and suggested that PP&CC implementation was made, Table 2-1.

Table 2-1 Missions categories based on mission type and destination, adapted from Office of planetary protection, NASA, (NASA 2013).

Planet targets/ locations	Mission Type	Mission Category
Undifferentiated, metamorphosed asteroids; Io; others TBD.	Flyby, Orbiter, Lander	I
Venus; Earth's Moon; Comets; non-Category I Asteroids; Jupiter; Jovian Satellites (except Io and Europa); Saturn; Saturnian Satellites (except Titan and Enceladus); Uranus; Uranian Satellites; Neptune; Neptunian Satellites (except Triton); Kuiper-Belt Objects (< 1/2 the size of Pluto); others TBD.	Flyby, Orbiter, Lander	II
Icy satellites, where there is a remote potential for contamination of the liquid-water environments, such as Ganymede (Jupiter); Titan (Saturn); Triton, Pluto and Charon (Neptune); others TBD.	Flyby, Orbiter, Lander	II*
Mars; Europa; Enceladus; others TBD (Categories IVa-c are for Mars).	Flyby, Orbiter	III
	Lander, Probe	IV(a-c)
Venus, Moon; others TBD: "unrestricted Earth return"	unrestricted Earth-Return	V (unrestricted)
Mars; Europa; Enceladus; others TBD: "restricted Earth return"	restricted Earth-Return	V (restricted)

It was defined several subtypes of category IV, according to whether it was or not a life-detection mission and what zone of the planet Mars was intended to be explored, Table 2-2.

Table 2-2 Sub-categories dedicated for missions to Mars, adapted from Office of planetary protection, NASA, (NASA 2013).

Planet targets/ locations	Mission Type	Mission Category
Lander systems not carrying instruments for the investigations of extant Mars life.	Lander, Probe	IVa
Lander systems designed to investigate extant Martian life.	Lander, Probe	IVb
Missions investigating Martian Special Regions, even if they do not include life detection experiments. Martian Special Regions include those within which terrestrial organisms are likely to replicate and those potentially harboring extant Martian life.	Lander, Probe	IVc

The COSPAR recommendations were transformed in standards to be performed, by the European Cooperation for Space Standardization (ECSS) The LMC instrument within the PP&CC context was classified as part of a mission category IVb, because it aimed to search for evidence of life on Mars. According to that mission type, the LMC needed to present microbial burden less than 300 bacterial spores per square meter, while the external landing system needed to present per square meter less than $2x10^5$ spores. Besides microbial contamination organic contamination had also to be

controlled and kept at 1 ng of total organic carbon per gram of analysed sample, (Pillinger 2009).

In order to reduce the microbial presence, it was necessary to implement contamination control procedures. The contamination control included all the procedures that reduced the total spacecraft microbial presence, until it reached the desired level, in order to meet the COSPAR guidelines. Those were achieved by the implementation of cleaning and sterilisation procedures during the assembling, integration and verification (AIV) of the spacecraft, by following the ECSS standards, and by adopting a spacecraft design compatible with PP&CC implementation and prevention of re-contamination (Debus 2006).

2.7. Summary and conclusion

Mars was considered a planet with similar characteristics to Earth in which might be present or had existed simple life forms similar to the ones found on Earth. In order to discover that, the LMC instrument was developed; the instrument was designed to detect evidence of life on Mars. It was an instrument in which antibodies were used as bioreceptors in an inhibition assay. These were developed against a group of biomarkers which were chosen with the aim of covering the essential biochemical groups present on extant and extinct life forms found on Earth, in meteorites, and in molecules that could be used as contamination control. Based on that it was concluded that aptamers presented examples of applications in similar detection assays instruments and that had been generated against similar molecules to the chosen biomarkers, and for that reason could be tested as possible bioreceptors for the LMC. Another conclusion was the need to test early on the aptamers compatibility with PP&CC requirements and also to understand PP&CC basic procedures and implementation into an instrument for life detection missions.

Chapter 3. Thesis Aims and Objectives

3.1. Aims

The overall aim of the PhD study was to consider specific development aspects of the LMC instrument and development programme which could then be used to guide work and technology choices with the LMC programme.

That overall aim broke down into two major parts:

- To determine if aptamers could be used in an LMC instrument and development programme.
- To discuss, at an early stage, design and protocols for PP&CC implementation that could aid in the LMC development process, and also to have a preliminary understanding whether aptamers could contribute to the improvement of the LMC PP&CC implementation.

Those two parts were divided into more detailed aims:

- To understand if aptamers could successfully be developed against LMC low molecular weight targets.
- To ascertain whether aptamers could be developed within a timescale and resource level that was compatible with the LMC development programme.
- To comprehend if aptamers could be integrated into the existing LMC assay format.
- To recognise if there were aspects of instrument design and protocols needed to allow an LMC instrument to meet the PP&CC requirements in the development process.
- To understand if the use of aptamers could be the easy route to produce an LMC instrument that would meet PP&CC requirements.

3.2. Objectives

Resulting from the project aims, the following thesis objectives arose:

- To choose an aptamer generation method and test it against specific LMC targets to understand the process and its limitations within the context of the LMC programme.

- To test implementation of existing aptamers against small molecule targets in the LMC assay format.
- To use a small scale experiment to apply design modifications for PP&CC requirements, to implement standard cleaning PP&CC protocols for microbial reduction, a sterilisation procedure and validation methods, and to demonstrate resultant experiment in flight conditions.
- To test aptamers compatibility with a sterilisation procedure required for PP&CC implementation in the LMC.
- To make recommendations in terms of the development and use of aptamers and PP&CC implementation in the context of the LMC development programme for the ExoMars 2018 mission.

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Chapter 4. Can aptamers be easily generated against small-molecule LMC targets?

4.1. Chapter introduction

The LMC baseline bio-receptors were antibodies; however it was desired to explore the potential of aptamers as alternative candidates. It was decided to verify if aptamers could be generated against LMC targets. These were molecules with low molecular weight, the majority were hydrophobic and all presented minimal functionality[2]. When searching for already existing aptamers generated against targets with similar physiochemistry to the LMC targets, only a few examples were found. It was for this reason that it was decided to test if it would be possible to generate aptamers against LMC targets, within the restricted time frame of approximated three years allocated for the LMC part of the ExoMars mission, and resources structure of a typical planetary exploration flight programme.

It was chosen to test aptamer generation against representative LMC targets. The method involved immobilising the targets to a surface. Due to the simple nature of the targets it was necessary to use derivatives with reactive groups that allowed covalent chemistry. An aptamer generation method was implemented and the details of the process evolved dependent on the on-going findings.

4.2. Literature review on aptamer generation and techniques to detect small molecules during aptamer generation

4.2.1. Chemical properties of LMC targets and LMC target derivatives

The LMC target molecules had low molecular weight and their simple chemistry and absence of functional/reactive groups made them atypical targets for aptamer generation this was reviewed in detail in section 2.3 (Chapter 2). Aptamer targets were

[2] By “minimal functionality” –within the context of this thesis meant low presence of chemical groups able to form hydrogen bonds, electrostatic interactions, etc.

commonly proteins or other macromolecules and in the case of small molecules, these were rich in charged groups or in aromatic rings, (Klussmann 2006).

The LMC targets derivatives were versions of the LMC targets that presented reactive groups as carboxylic acids or primary amines, for example, at one side of the molecule, but were equal at the rest of the structure. The derivatives allowed surface immobilisation via the functional end, exposing the LMC target structure. The derivatives reactive groups served as linkers for covalent chemistry of the LMC targets.

Although derivatives had reactive functional groups (to allow immobilisation to a solid-support), these were not sufficient for simple detection using standard analytical chemistry techniques. The LMC targets derivatives did not present heavy ionic charge, secondary or tertiary structures and had less overall reactivity than traditional targets, such as proteins. Some derivatives were also hydrophobic which increased incompatibility with biological aqueous buffers. This was critical when working with ssDNA that required this type of matrix.

4.2.2. Aptamer generation against small molecules

It was found in literature only few examples of aptamers against small molecules, which were reviewed in section 2.5.2 (Chapter 2). This occurred despite the fact that small molecules were desirable targets in most of scientific fields, and because of their simplicity are present in the most degraded forms of the compounds or are usually products difficult to eliminate that nevertheless might have an unwanted high impact, (Tombelli, Minunni et al. 2007).

This fact showed that potentially generating aptamers against small molecules could be more difficult than generating aptamers against large molecules as proteins.

The approaches found in literature, taken for aptamer generation against small molecules, were modifications of the initial standard SELEX method, developed by Ellington et al (1990). The SELEX involved immobilising the target onto the surface of agarose beads packed in an affinity chromatography column. The bound oligonucleotides were eluted by addition of free target to the column, (Ellington, Szostak 1990).

Another method used to generate aptamers against small molecules was the FluMag-SELEX. This was a variation of the initial method where instead of using a column,

magnetic beads were used as solid surface, (Stoltenburg, Reinemann,C., Strehlitz,B. 2005).

In the previous methods, the targets were always surface immobilised. An example was found where selection was performed in solution. The structure-switch method allowed selection in solution with an unmodified target. It consisted in a selection with a ssDNA pool with three fixed regions, the flanked ends and a middle region. After selection the central fixed region would be bound to a biotinylated complementary sequence and in that way free the bound target into solution. The separated strands would be amplified and used in the following iteration, (Nutiu, Li 2005). A new method is under development for aptamer generation against small molecules and consisted in using gold nanoparticles as solid surface during the SELEX, (personal communication of Y.S. Kim). The gold nanoparticles allowed an easy visual detection of surface immobilised molecules because they presented different colours according to the light path distance to the nanoparticle surface, (Yang, Wang et al. 2011).

4.2.3. Importance of target immobilisation and confirmation of immobilisation to solid-phase supports during aptamer generation

As reviewed in the last point, in the majority of aptamer generation, targets were surface immobilised. The immobilisation was based on covalent chemistry.

When using Dynabeads® as solid phase, the target was directly immobilised while in some examples, when using agarose beads, was mentioned the use of carbon spacers to add length from the surface to the target, and in both cases, counter-selection to increase specificity and affinity, (Ellington, Szostak 1990).

The most common approaches used were the formation of a carbodiimine to promote the bond between a carboxyl groups and an amino group. This was used in attaching proteins to surfaces which have naturally available primary amine and carboxylic groups in their structure. The immobilisation chemistry of small molecules depended on their functional groups, (Thermo Scientific Pierce 2010). The LMC targets derivatives chemistry was simple, which lead to reduced options of covalent chemistry and made them difficult to detect and quantify.

Those characteristics made immobilisation confirmation even more important given the likely difficulty of generating aptamers against the LMC target derivatives. Aptamer

generation depended on how efficient the target immobilisation was, and for this reason it would be important to always confirm the quality of this step. Knowing that immobilisation occurred would prevent the use of resources in aptamer generation and would decrease the chance of selecting aptamers against unmodified surfaces. Confirming target immobilisation should be done systematically because of the previous point but also to validate the selection step.

Despite this only one example was found in literature, where aptamers were generated against a small target using the FluMag-SELEX and the Dynabeads® immobilisation was confirmed. In that case the target chemistry allowed UV detection and the immobilisation was verified by quantification in solution of the initial and final target concentrations and the difference was assumed covalently attached, (Kim, Hyun et al. 2010). In the case of the traditional SELEX it was more common the quantification of bound target into agarose beads, also by calculating the difference of target prior and after column passage (Klussmann 2006).

4.2.4. Detection methods for confirmation of target immobilisation to solid-phase supports during aptamer generation

As mentioned before, only one example was found in literature, when using FluMag-SELEX, of a routine analytical technique that confirmed target immobilisation, (Kim, Hyun et al. 2010).

It was considered testing if it would be possible to detect directly immobilisation on the Dynabeads® surface. It was then necessary to search for analytical chemistry techniques that could confirm the presence of specific functional groups on their surface.

This was first based on the nature of the targets analogues. It was tough that the same functional groups that allowed covalent chemistry would also confirm immobilisation. This could be done either by reacting free reactive groups that would be present only after immobilisation or by decrease of magnetic beads reactive functional groups. Indirect detection was also considered but as a second choice due to lower liability.

Direct Analytical Chemistry detection methods of amino groups

The chosen working Dynabeads® were either surface modified with primary amine groups or with carboxylic groups. This was according to the desired immobilisation chemistry to covalently bind the targets.

It was chosen to use a spacer that would allow the target exposure further distant of the surface, and in that way, expected to decrease the surface interference in the aptamer generation.

To confirm immobilisation it was tested using direct analytical chemistry reaction methods. Because the majority of Dynabeads® and targets had at some point of the immobilisation primary amine groups, it was tested methods that would react directly with those. This was tested either by reacting un-coupled primary amines in the Dynabeads® surface before and after immobilisation or by reacting immobilised material that would have available primary amine groups.

The standard methods found in literature for primary amine detection were trinitrobenzenesulfonic acid (TNBS), fluorescamine, σ-phthaldialdehyde (OPA) and ninhydrin, (Cooper, Packer et al., 2001, Wallace, Fox 1998). The products obtained with these chemicals, after reaction with primary amines, allowed for either colorimetric or fluorescent detection.

TNBS reacted in solution with primary amines (trinitrophenylation), giving origin to a yellow product named N-trinitrophenyl-protein (TNP-NH-R). The product was detected by absorption spectroscopy and was directly proportional to primary amine concentration, Figure 4-1, (Cayot, Tainturier 1997).

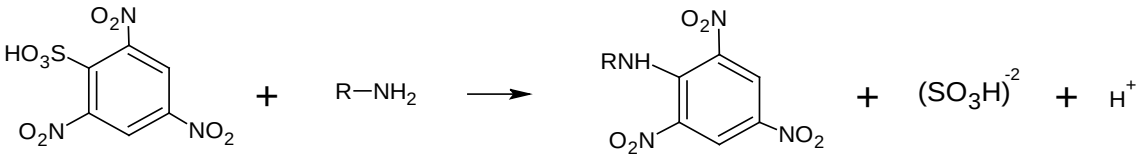


Figure 4-1 TNBS reaction mechanism with primary amino groups, (Cayot, Tainturier 1997).

Fluorescamine, widely used for protein quantification, when reacting with primary amines gives origin to a fluorescent product, fluorophore, Figure 4-2. Chen et al (2010) patented a method for quantification of biomolecules immobilised onto the surface of nanoparticles based on the fluorescamine reaction, (Chen, Bieniarz et al.

2010). Stein et al (1973) reported that fluorescamine allowed amino acids detection down to the picomolar concentration, (Stein, Bohlen et al. 1973).

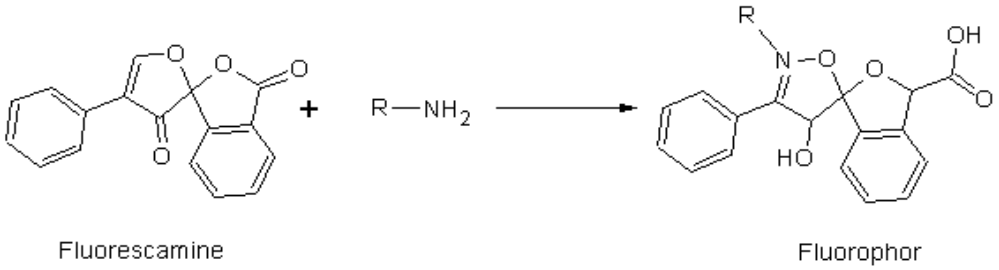


Figure 4-2 Chemical structure of Fluorescamine and the reaction product with primary amines, adapted from Stein et al (1973), (Stein, Bohlen et al. 1973).

OPA also, when reacting with primary amines, gives origin to a fluorescent product, Figure 4-3. Both Fluorescamine and OPA are known for being very sensitive detection methods, with detection limits reviewed by Danielson, et al (2000), of 0.3 ng and 200 fmol respectively, (Danielson, Gallagher et al. 2000).

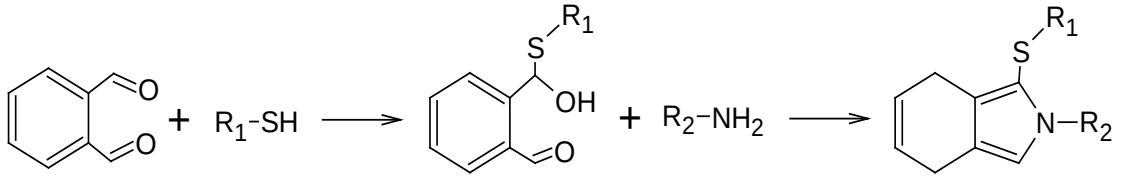


Figure 4-3 OPA reaction with primary amines, adapted from Kyprianou et al (2010), (Kyprianou, Guerreiro et al. 2010).

Ninhydrin was also a well-known colorimetric method that has been used to confirm surface function. Sano et al (1993), used ninhydrin to access polyethylene solid surface modification with proteins, (Sano, Kato et al. 1993).

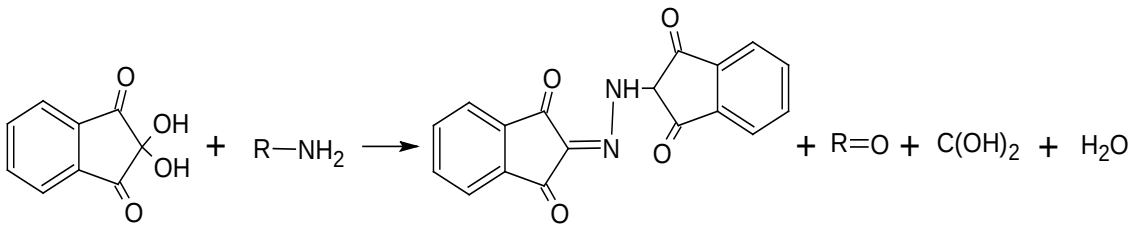


Figure 4-4 Ninhydrin reaction with primary amines, adapted from Sano et al (1993), (Sano, Kato et al. 1993).

There were alternative methods for detection of primary amine groups and also other techniques that could be used to confirm surface immobilisation that did not necessary react with primary amines.

Indirect detection methods of amino groups and other relevant techniques

It was considered the targets specific chemistry and in one case there was a thiol group which allowed the test of Ellman's reagent. The Ellman's reagent reacted with thiol groups giving origin to a chromophoric product,Figure 4-5 (Aslam, Dent 1998).

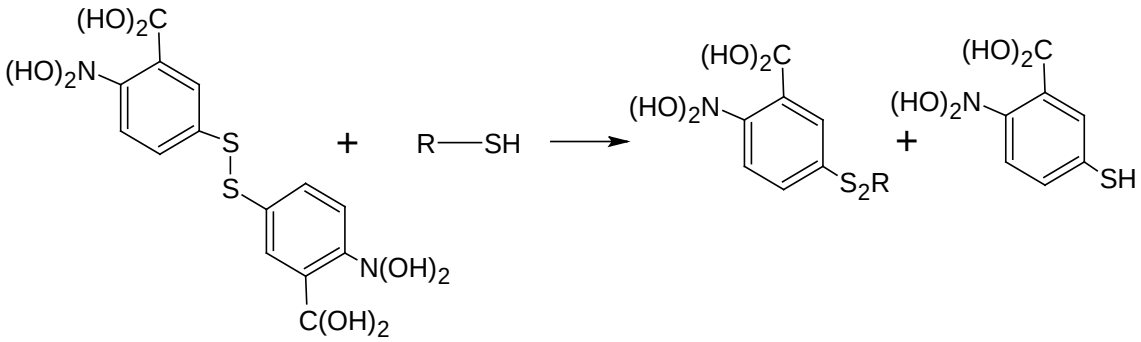


Figure 4-5 Ellman´s reaction with thiols giving origin to the chomophoric 2-nitro-5-mercaptobenzoate anion, adapted from Aslam et al (1998), (Aslam, Dent 1998).

When using HPLC, to detect small molecular weight molecules, usually chemical derivatisation was used, as explained by Danielson et al (2000), (Danielson, Gallagher et al. 2000). Another example where small targets were detected using HPLC was the detection of amines as cadaverine, bilic acids, cysteine or ibuprofen, (Chiavari, Galletti et al. 1989, Amarnath, Amarnath et al. 2003, Battu 2009, Iida, Shinohara et al. 1988). In the majority of the cases the detection was done using reverse phase HPLC and, as mentioned before, for simpler targets derivatisation was often mentioned. This was found to be used in food and environmental analysis, where the targets of interest were similar in size and properties to the LMC, (Corradini, Philips 2011). In the case of the LMC targets, derivatisation was not considered for a routine side-technique due to its complexity.

In alternative to the previous detection methods there were infra-red (IR), Fourier transform spectroscopy (FTIR), elemental analysis and x-ray photoelectron spectroscopy (XPS).

IR and FTIR were spectroscopy techniques where according to the absorbed wavelength was possible to characterize compounds. Gaber et al (2013) used IR to

confirm the chemical modification of nanoparticles, (Gaber, El-Sayed et al. 2013) and Saikia et al (2013), applied FTIR to confirm the presence of functional groups in polyphenols, (Saikia, Konwarh et al. 2012).

Elemental analysis consisted on determining the atomic composition of a sample by its combustion products. Yan et al (1998) used it to quantify a surface modification, (Yan, Jewell Jr. et al. 1998). In that case, elemental analysis was used in parallel to other techniques such as IR, FTIR or X-ray. Also elemental analysis was used for the characterization of modified cellulose surface, for the characterization of Cu-nanoparticles, and in the synthesis of cadmium ligands, (Yu, Tong et al. 2013, Zhang, Zheng et al. 2013, Gaber, El-Sayed et al. 2013).

The XPS was a surface analysis technique where, by counting emitted electrons, it was possible to get information about the chemistry and space arrangement of the sample atoms. The sample was placed under vacuum and exposed to X-ray photons. In result the atoms present on the surface of the sample released core electrons. These electrons were collected, separated and quantified according to their energy level. This technique was optimal because the electrons energy was signature of the element and its surrounding molecular environment. In that way it was possible to collect information about the chemical composition of the surface material, (Fairley 2009).

Several groups have used XPS to access surface quality and chemical modifications. Like in the case where nanoparticles were build and modified, (Wang, Zhou et al. 2008), or where surface modification of iron particles was confirmed, (Chen, Peng et al. 2007). Graf et al (2009) did a study where amino groups were immobilised onto surfaces and accessed by XPS, (Graf, Yegen et al. 2009), and lysine was also studied after surface adsorbed by Eralp et al (2011), (Eralp, Shavorskiy et al. 2011). XPS was also used in bioreceptors where a caffeine MIP was surface analysed, (Ebarvia, Cabanilla et al. 2005).

It was found several examples in literature that were used to analytically detect the presence of specific functional groups (thiol, primary amines and carboxylic acids) but it was necessary to test if they were adequate to confirm the targets derivatives immobilisation onto the surface of Dynabeads®.

4.3. Chapter Aims and Objectives

The aim of the work presented in this chapter was to determine if aptamers could be generated against small-molecule LMC targets within the context of the LMC programme.

That aim lead to the following objectives:

- To select and produce LMC targets derivatives immobilised on solid-phase for subsequent aptamer generation.
- To decide upon an analytical methodology that allowed the direct detection of the small hydrophobic molecule LMC targets analogues on beads or to indirectly show that they have been immobilised.
- To implement method(s) to produce beads with immobilised targets for subsequent aptamer generation.
- To choose an aptamer generation method and test it to understand the process and its limitations within the context of an LMC programme.

4.4. Chapter experimental rationale

In order to understand if aptamers could be generated against the LMC targets of interest this chapter was divided in three major sections. The first section described the choice of the aptamer generation method and the targets to generate aptamers against. Because the targets were very simple, with low molecular weight and had to be surface immobilised, in order to apply the chosen generation method, a second section of work was developed. The second section described the search for an appropriated side thecnique to confirm targets Dynabeads® surface immobilisation. The initial approach taken was to identify the presence of primary amino groups attached to the Dynabeads®, and in that way confirm the surface immobilised spacer or target rich in amino groups, The first assay was performed with TNBS because it was a well-known technique used in protein detection. The obtained data pointed out that it was not indicated for the detection of Dynabeads® surface immobilised targets. It was then tested Fluorescamine and OPA because were more sensitive techniques. It was expected to be possible to detect the presence of immobilised targets or spacers rich in primary amino groups. And also to assume immobilisation by signal difference in Dynabeads® surface modified with primary amines that were afterwards reacted with targets that did not present amine groups. In both cases it was shown to be impossible

to obtain significant data due to light scattering. For that reason these two techniques were not incorporated in the main experimental part and only described here and at the chapter discussion and summary. Afterwards the approach taken was changed and Ellman´s reagent was used to detect the presence of thiol groups in solution and in that way extrapolate if target surface immobilisation was successful or not. The obtained data was inconclusive and HPLC was used to quantify in solution the unbound target and extrapolate if immobilisation had occurred or not. It was discovered with HPLC that secondary reactions could interfere with the detection and that most of the targets were too simple to be detected by that technique. Once again a new approach was taken and it was analysed the elemental composition of the immobilised surfaces versus the unmodified ones to search for differences. The same comparison was made using IR and FTIR. Because it was not obtained any clear difference between immobilised and unmodified surfaces those three techniques were not expanded in the main experimental section and only mentioned here, at the chapter discussion and summary. The following step taken was to test Ninhydrin which was used to detect the presence of primary amines that could be present on the Dynabeads® surface. Because the detection was made by quantifying the obtained product in solution and not bound to the surface it was possible to understand that this technique could be used as a side-technique to verify the surface immobilisation of rich in primary amine targets and spacers. Despite that it was found that it was only useful for surface direct immobilisation and not to the immobilisation of spacers and targets as desired. The final technique tested was XPS for surface analysis, and that indicated the atomic neighbourhood, allowing the understanding if covalent bonds had been made or not. That technique proved to be the most appropriated as side-technique to verify the LMC targets and spacers immobilisation on Dynabeads® surface.

The third part of this chapter was dedicated to the aptamer generation method and the need to have full working steps prior to perform the full generation system. It was necessary to develop in special the amplification step and the separation step. In the first a new approach was taken, which was the implementation of real-time PCR instead of traditional PCR. This was done to improve the knowledge of the amount of strands selected from each round and also to verify the quality of the amplified product. In the separation step it was tested two different published methods to choose the most appropriated one. Because the confirmation of targets surface immobilisation took longer than expected, only a few rounds of aptamer generation were made to each target. Also it was found that the chosen method presented small but important details

that became challenges in the iterative process. In this chapter it was seen the challenges that generating aptamers against small targets presented and the lessons learned from that.

4.5. Choice of aptamer generation method and LMC targets

4.5.1. Choice of method to test aptamer generation and LMC targets

It was decided to test the generation of aptamers against the LMC derivatives using one of the methods reviewed in section 4.2.2. All the methods had the same standard methodology and were either the standard SELEX or adaptations of it.

In the most common method in literature, (SELEX), the target was immobilised onto the surface of agarose beads. The second method found was the FluMag-SELEX, which used Dynabeads® as solid surface and modification of the positive and negative DNA strands so quantification and separation were controlled during the generation process, (Stoltenburg, Reinemann,C., Strehlitz,B. 2005). A third method was a less established procedure named structure-switch. This was very interesting because it did not require target immobilisation, (Nutiu, Li 2005).

Aptamer generation was tested using the FluMag-SELEX method. This method presented several advantages as allowing interaction in solution between ssDNA and immobilised target, easy magnetic partition, easy washing steps for stringency and easy visualization and quantification of positive strand at each iterative round due to fluorescein modification after first round, (Stoltenburg, Reinemann,C., Strehlitz,B. 2005). Furthermore LMC target derivatives presented, in the majority of times, low solubility in aqueous matrices and the use of magnetic beads allowed change of buffer matrices from the targets chemistry immobilisation to a biological matrix compatible with DNA for aptamer selection.

This method was chosen also due to the number of examples found in literature when in comparison with the structure-switch, and because it was seen as an updated version of the standard SELEX. FluMag-SELEX, in similarity to SELEX, consisted of an iterative process of rounds of selection, elution, amplification, and separation. An initial ssDNA pool was incubated with Dynabeads® immobilised targets derivatives. The complexes target-ssDNA formed were separated from the original pool using the

magnetic properties of Dynabeads®. The selected ssDNA was eluted and amplified with polymerase chain reaction and during this process the positive strands were modified with fluorescein and the negative strands with either a biotin or a poly A tail. These modifications were subsequently used to separate the positive strands. Afterwards the positive strands were used as a new selection pool and the method repeated. The iterations were made until at least 70% of the strands showed affinity to the immobilised target. Afterwards the selected strands were amplified E-coli transformation. The strands were sequenced and tested for target affinity and specificity in order to determine the best aptamer candidate against the target, Figure 4-6.

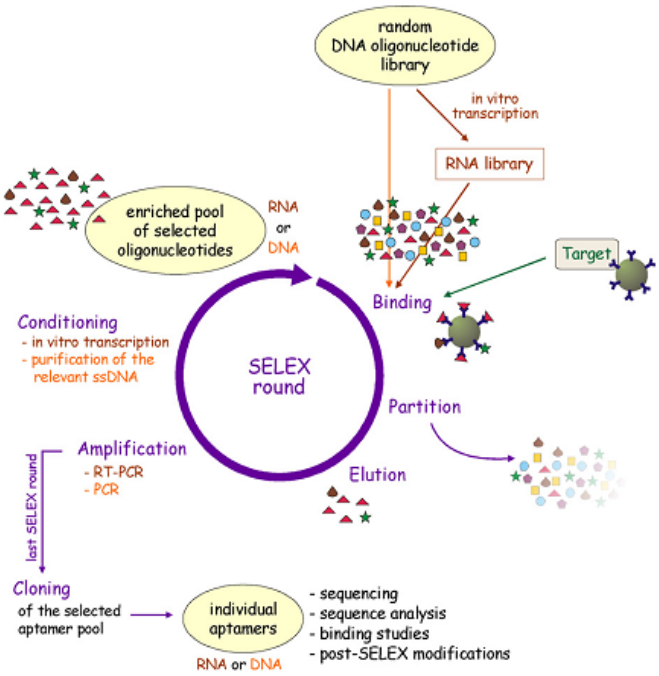


Figure 4-6 FluMag-SELEX schematic representing the steps of the process, adapted from Stoltenburg et al (2007),(Stoltenburg, Reinemann et al. 2007).

Despite the choice of the FluMag-SELEX, the structure-switch was found very appealing to this project because instead of working with derivatives it would allow to work directly with the LMC targets, Figure 7-1. It was considered to be used as a second aptamer generation method if time and resources would allow it. The structure-switch was not the principal generation method because it was considered less established.

The chosen aptamer generation method required the immobilisation of the LMC target derivative into the surface of Dynabeads®. The chosen LMC targets for aptamer generation were alanine and coprostane.

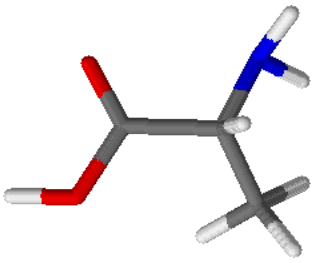


Figure 4-7 L- Alanine isomer (MW 89.09 g/mol).

Alanine, Figure 4-7, was chosen because of the interest in generating aptamers against an amino acid, and its simplicity would allow the aptamer to detect the amino acid backbone and it that way be generic to the amino acids family. The same principle was applied for coprostane, it would allow the generation of an aptamer able to recognise stearanes, Figure 4-8.

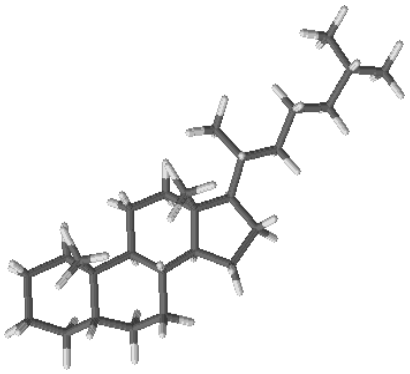


Figure 4-8 Coprostane (MW 330.59 g/mol).

The targets were chosen because of the desire to detect amino acids as simple units of organic compounds with nitrogen and carbon, and the stearanes were considered as a difficult target to antibody generation. Aptamers generation was tested against two very simple LMC targets. This simplicity made them very difficult targets due to their lack of charges, functional groups and in the case of coprostane, hydrophobicity.

After choosing the targets to test aptamer generation it was necessary to decide on derivatives and immobilisation chemistry.

4.5.2. Choice of initial targets derivatives, spacer chemistry and beads for testing immobilisation

The initial LMC target derivatives chosen to be immobilised onto the surface of Dynabeads® were L-cysteine (MW 121.16), and 5β-cholanic acid, (MW 360.57). L-cysteine was the L-alanine derivative and the L- form was naturally the most abundant. L-alanine presented a reactive thiol group at one end and the generic amino and carboxyl groups.

The chosen spacer was N-[ε-maleimidocaproyloxy] succinimide ester (EMCS), which allowed the coupling between an amino group and a thiol group and would give a length of 9.4 Ă, (Pierce 2013). L-cysteine was used with Dynabeads® surface modified with primary amino groups. EMCS was immobilised on the amino Dynabeads® surface and then reacted with L-cysteine via thiol, Figure 4-9. L-cysteine solubility was high in aqueous buffers which also made it a preferable target derivative to work with.

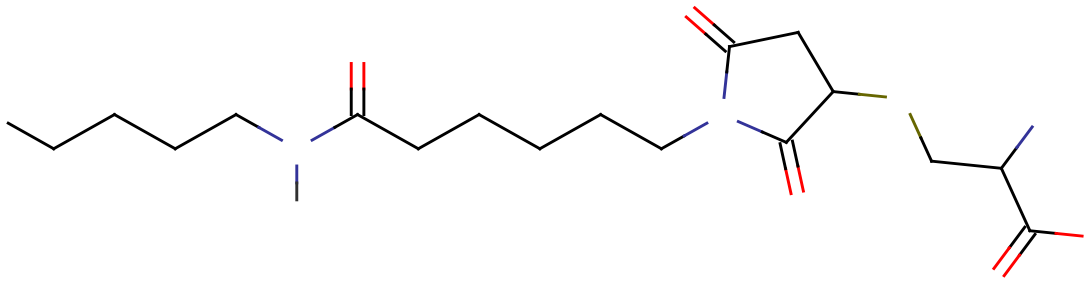


Figure 4-9 Amino modified Dynabeads® immobilised with EMCS spacer and L-cysteine.

The derivative for coprostane was 5β-cholanic acid. This presented a carboxylic group which could react with primary amines, so the di-amine spacer cadaverine was used to link the target derivative and carboxyl Dynabeads®, Figure 4-10. Cadaverine would give a length of five carbons after immobilisation.

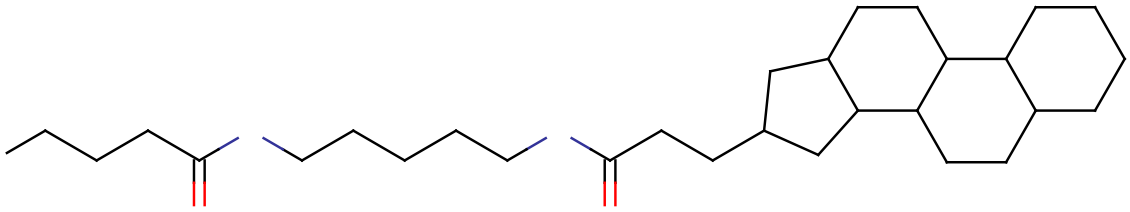


Figure 4-10 Carboxyl modified Dynabeads® immobilised with spacer cadaverine and 5β-cholanic acid

After choosing the targets and spacers the Dynabeads® immobilisation was performed.

4.6. Immobilisation of LMC target derivatives to magnetic beads and confirmation of the target immobilisation

To generate aptamers, using the chosen method FluMag-SELEX, it was required the immobilisation of LMC target derivatives on Dynabeads® surface.

This lead to the need of confirmation how efficient the immobilisation was. This was because of the nature of the derivatives, (low molecular weight and some hydrophobic), to prevent the use of resources in vain, and because it was found that it was not common to verify covalent immobilisation in aptamer generation using this method.

It was decided to search for a technique that could be used as a routine side-technique to confirm Dynabeads® immobilisation. The technique (s) was required to be compatible with the chemical nature of the derivatives and the Dynabeads® that were used a solid support, as well as to any of the immobilisation chemistry used.

The first step was the Dynabeads® immobilisation with spacers and derivatives.

4.6.1. Initial immobilisation of LMC target derivatives to magnetic beads and the confirmation of the target immobilisation

The LMC targets derivatives were, as seen before, the targets L-cysteine and 5β-cholanic acid. Which were immobilised via the spacers EMCS and cadaverine, Figure 4-9 and Figure 4-10.

5β-cholanic acid was also directly immobilised onto the surface of amino Dynabeads® but just to aid in the confirmation of immobilisation in some tests. The Dynabeads® used were surface modified with primary amino groups or surface modified with carboxyl groups. The immobilisation was done with 1-Erhyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and N-hydroxysuccinimide, (EDC/NHS) activation in the case of binding carboxylic acids to primary amines, or EMCS with reactive maleimide and ester groups in the case of thiols and amino groups.

Compatibility of Dynabeads® and cadaverine spacer with working buffers

It was found several problems with target derivatives solubility and Dynabeads® solvents compatibility, so buffers composition, chemicals and Dynabeads® were first tested.

a) Materials and Methods

i. Materials and chemicals

Carboxyl M-270 Dynabeads® from Invitrogen (14305D), phosphate buffered saline (PBS) pH 7.3; dimethyl sulfoxide (DMSO, CAS 67-68-5; D8418), methanol (CAS 67-56-1; 34860), 1-Erhyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, CAS 25952-53-8; 22980), all from Sigma, cadaverine (CAS 462-94-2; 33211) from Fluka,1.5 ml tubes rotator, 1.5 ml tubes from Eppendorff.

ii. Methods to study Dynabeads® compatibility with working buffers and LMC target derivatives solvents

To study the impact of working with different buffers, three buffers were tested at different percentages. The solvents were methanol, PBS and DMSO. These were used to solubilise derivatives and spacers. The buffers were tested on: i) carboxyl Dynabeads® with cadaverine activated with EDC, ii) carboxyl Dynabeads® with cadaverine and no activation, and iii) carboxyl Dynabeads®. It was only used carboxyl Dynabeads® because the standard composition of Dynabeads® was the same, (Invitrogen 2013).

b) Experimental and Results

i. Dynabeads® compatibility with working buffers and LMC targets derivatives solvents

The test consisted in washing Dynabeads® with different percentages of buffers to see if agglomeration (A), flocculation (F) or precipitation (P) occurred, Table 4-1.

Table 4-1 Dynabeads® compatibility with working buffers during immobilisation steps - agglomeration (A), flocculation (F), precipitation (P) or in solution (S).

	DB + EDC + cadaverine	DB + cadaverine	DB
50 % PBS : 50 % DMSO	P	P	P
50 % PBS : 50 % methanol	F	A	S
70 % PBS : 30 % DMSO	S	S	S
70 % PBS : 30 % methanol	S	S	S
30 % PBS : 70 % DMSO	P	P	P
30 % PBS : 70 % methanol	F	A	P
100 % DMSO	S	S	S

It was observed precipitation when organic buffers were at equal or higher part with aqueous buffers. Agglomeration in clusters was obtained of Dynabeads® when methanol was presented at higher percentage than 30%, and flocculation when EDC and cadaverine were added to those agglomerates. No problems were registered in aqueous solutions with low percentages of organic solvents.

c) Discussion

It was tested different percentages of the three solvents used to solubilise LMC target derivatives and spacers; this was relevant because of the need of immobilisation of a spacer and a target derivative sometimes with different polarity.

Precipitation was seen when organic solvents were present at 70% and PBS at 30%. On the other hand when PBS was 70% and the organic solvents were 30% Dynabeads® did not precipitated, flocculated, or aggregated. These were considered the best buffers to use in the need of solubilise an organic compound in an aqueous buffer.

Also the carboxyl Dynabeads® in 50 % DMSO and methanol changed their colour into a darker brown but no precipitation occurred. This changed when the spacer cadaverine was added. Moreover it was seen that using a solution of 100% cadaverine and then adding DMSO promoted Dynabeads® precipitation, which did not happen if cadaverine was diluted in DMSO first. No explanation was found for these situations, but it was decided to dilute cadaverine in DMSO and to use 100 % DMSO in the cases were spacers and targets were both hydrophobic, and to use 70 % PBS with 30 % organic solvent in the cases where the spacer was hydrophobic and the derivative hydrophilic. In all the other situations an aqueous buffer was used.

Once the Dynabeads® compatibility with working buffers was tested, it was proceeded to surface immobilised the chosen spacers and LMC target derivatives.

Immobilisation of spacers and target derivatives to Dynabeads®

Amino Dynabeads® were used to immobilise L-cysteine via EMCS and to immobilise directly 5β-cholanic acid. The L-cysteine immobilisation chemistry used was the reaction between the Dynabeads® primary amines and the EMCS ester, and then the maleimide reaction with thiol groups. The 5β-cholanic acid immobilisation was via EDC/NHS forming a covalent bond between the amines and the carboxylic groups.

a) Materials and Methods

i. Materials and chemicals

Amino M-270 Dynabeads® (14307D) and carboxyl M-270 Dynabeads® from Invitrogen, 5β-cholanic acid (CAS 546-18-9; C7628), from Sigma (appropriated dissolved), EMCS (CAS 55750-63-5; 22308) from Pierce (appropriated dissolved), PBS pH7.3; DMSO, methanol, L-cysteine (CAS 52-90-4; C7352), all from Sigma, NHS (CAS 6066-82-6; 130672), cadaverine from Fluka, and EDC from Thermo scientific, magnetic support, rotator, 1.5 ml tubes DNAse free from Eppendorff.

ii. Method for L-cysteine immobilisation to amino Dynabeads® using EMCS activation

100 µl of Dynabeads® were removed from the stock solution and the buffer removed by magnetic separation. The Dynabeads® were washed three times with 500 µl PBS. Each wash consisted in the addition of buffer, strong agitation with vortex and separation of liquid by magnetic separation. It was added to the beads 450 µl of PBS and 50 µl of EMCS (previously dissolved in DMSO at 10 mM). The mixture was incubated at RT for 1 hour with agitation. The liquid was removed using magnetic separation and the Dynabeads® were washed four times with 500 µl of PBS. It was added to the Dynabeads® 500 µl of L-cysteine at 10 mg/ml in PBS and incubated at RT for 30 min with agitation. The Dynabeads® were separated from the liquid and washed four times with 500 µl of PBS. All the wash-liquid was collected for further analysis. The Dynabeads® were re-suspended in 100 µl PBS and kept at 4ºC until further use.

iii. Method for 5β-cholanic acid immobilisation to carboxyl Dynabeads® via cadaverine and EDC/NHS

100 µl of Dynabeads® were removed from the stock solution and the buffer removed by magnetic separation. The Dynabeads® were washed three times with 200 µl DMSO (wash-steps as previously explained). Cadaverine was added to the Dynabeads® (100

µl stock solution in 400 µl DMSO) and 100 µl of EDC at 5 mg/ml in cold DMSO and 100 µl of NHS at 5 mg/ml in cold DMSO were also added. The Dynabeads® were mixed at RT with agitation for 2 hours. The liquid was collected and the Dynabeads® washed three times with 200µl DMSO, all the wash-liquid was collected and kept for further analysis. Afterwards it was added to the beads a solution of 5β-cholanic acid (5 mg/ml) and fresh solutions of EDC/NHS as explained. The mixture was treated as before and after the last washing step, the Dynabeads® were re-suspended and kept in the cold room to avoid DMSO freezing.

iv. Method for 5β-cholanic acid direct immobilisation to amino Dynabeads® via EDC/NHS

Same method as explained in the previous point, but without the immobilisation of the spacer. This direct immobilisation was tested only for confirming covalent chemistry reaction.

b) Experimental and Results

L-cysteine immobilisation to amino Dynabeads® via EMCS spacer and 5β-cholanic acid immobilisation to carboxyl Dynabeads® via cadaverine spacer and directly to amino Dynabeads® occurred as expected as no obvious problems were found. The subsequent analysis to confirm the successful immobilisation was explained in this chapter following sections.

c) Discussion

The surface immobilisation occurred as expected and suffered a few changes from the manufacturer´s recommended protocol. The working volumes were performed at a higher volume than recommended to ensure a visual mix during the washes and incubation, and the incubations were made for longer than recommended to ensure that the reactions had time to occur.

After obtaining three sets of immobilised Dynabeads®, it was necessary to confirm how efficient that immobilisation was before starting aptamer selection. This was, as explained before, necessary to do because of the targets derivatives nature, to reduce the risk of generating aptamers against unmodified Dynabeads® and because it was not found in literature a routine side-technique that would allow a direct, fast and reliable confirmation.

Test of trinitrobenzenesulfonic acid (TNBS) reagent for primary amine detection to confirm immobilisation Dynabeads®

The Dynabeads® immobilised sets for aptamer generation were L-cysteine via EMCS and 5β-cholanic acid via cadaverine. A third set was 5β-cholanic acid directly immobilised without any spacer. This set was used only to confirm surface immobilisation.

The carboxyl Dynabeads® were first immobilised with cadaverine and then with the 5β-cholanic acid. In order to confirm cadaverine immobilisation it was tested the presence of primary amino groups. This was done with a standard method, using the TNBS reagent.
TNBS reacted with primary amino groups and cadaverine as a di-amine presented two reactive primary amines. Cadaverine, if efficiently immobilised to carboxyl beads, would present one reactive primary amino group. TNBS was reacted with carboxyl Dynabeads® with immobilised cadaverine to confirm the efficiency of the covalent chemistry. The absorbance intensity would be proportional to the amount of available primary amine groups and in that way the presence of immobilised cadaverine could be confirmed.

a) Materials and Methods

i. Materials and chemicals

TNBS (CAS 2508-19-2; 28997) from Pierce, borate buffer pH 9.0, 4 % sodium bicarbonate, all from Sigma, carboxyl M-270 Dynabeads® from Invitrogen reacted with cadaverine via EDC/NHS reaction, IKA KS 4000 control incubator, 96 well Nunc microtiter plate, 1.5 ml Eppendorf tubes and Thermo Scientific Varioskan Flash Plate Reader.

ii. Method for TNBS reaction with primary amine groups

A solution of 0.1% w/v TNBS, was prepared by diluting 1:50 TNBS 5 % (w/v) solution in 18 MΩ.cm water. The samples and the positive controls at different concentrations were prepared in borate buffer. 100 µl of sample or positive control was pipetted to 1.5ml tubes and in another tube 100 µl of borate buffer was used as blank. To each tube was added 100 µl of 4 % sodium bicarbonate buffer and mixed. Afterwards, to each tube, was added 100 µl of 0.1 % TNBS and mixed. All the tubes were incubated for 1 hour at 37 ºC. After it was transferred from each tube 200 µl into the wells of a 96

well microplate and the absorbance was read between 300 to 700 nm. The results were plotted graphically.

b) Experimental and Results

The best absorbance wavelength was determinate by reading the absorbance from 300 to 700 nm with ranges of 10 nm. Primary amines reacted with TNBS were reported to have an absorbance peak at 340-350 nm or at 410-420 nm, (Goodwin, Choi 1970, Staden, MacCormack 1998).

i. Detection of cadaverine primary amino groups in solution

TNBS was run with cadaverine in solution at concentrations that could mimic the presence of cadaverine on the Dynabeads® surface if immobilised efficiently. This was done to study the assay in solution without the Dynabeads® presence. Cadaverine was diluted 1:4 with a higher concentration of 1 mg/ml ($5.7x10^{-9}$ M) and the lower of 0.001 mg/ml ($5.7x10^{-12}$ M). The assay worked well but the higher concentration of cadaverine showed to be too high and lower concentrations were used.

At that stage it was noticed and confirmed in literature that TNBS spontaneously hydrolysed to picric acid affecting the reaction efficiency. Picric acid was also detectable at 340-350 nm. The same author reported that higher pH values promoted the secondary product reaction with primary amine groups. To improve the reaction the assay was run at pH 7.5 in PBS buffer instead of borate buffer at pH 9.0. The concentrations of cadaverine were kept, (Surovtsev, Fjodorov et al. 2001).

The obtained results were similar to the ones using borate buffer at pH 9.0, except for the higher concentration of cadaverine where it was possible to detect also the presence of the reaction product at 410 nm, Figure 4-11.

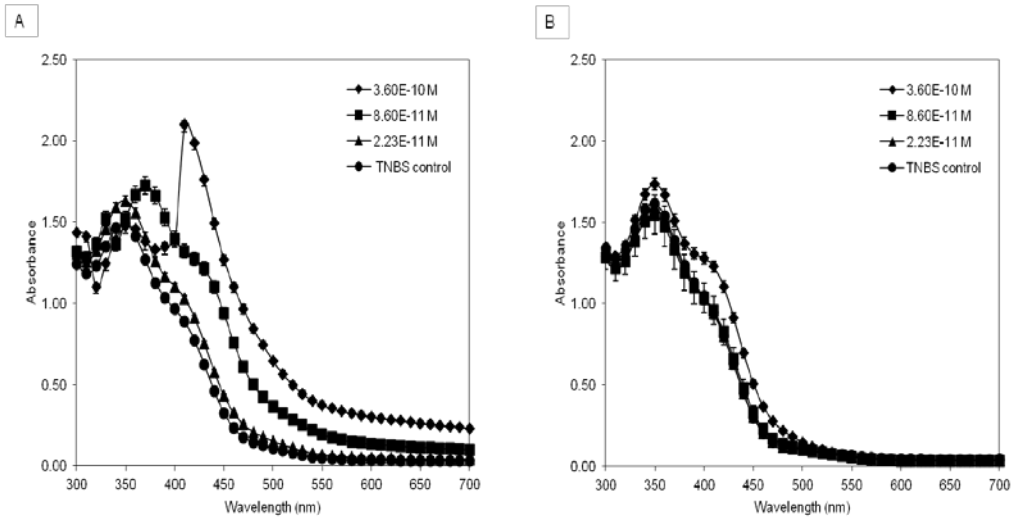


Figure 4-11 Reaction of TNBS with cadaverine at different concentrations and reagent buffer at A) pH 9.0 and B) pH 7.5.

There was a decrease in signal obtained between 420 and 450 nm and a shift of the obtained peak from 420 to 350 nm. The results became more defined at lower pH and there was an increase in signal at 350 nm, but at the same time it was not possible to distinguish between the different lower concentrations of cadaverine. The obtained results showed the two absorbing peaks for the highest concentration of cadaverine tested, 3.6 $x10^{-10}$ M. At higher pH it was possible to detect also two peaks with cadaverine at 8.6 $x10^{-10}$ M, but at 370 and 400 nm. The chosen conditions were to perform the assay at pH 7.5 because it gave a clear detection peak at 350 nm and a second peak at 400 at the highest cadaverine concentration. It was then tested the detection of surface immobilised cadaverine.

ii. Detection and quantification of immobilised cadaverine onto the surface of Dynabeads®

When absorbance was read after reacting TNBS with $1x10^{8}$ (~100 µl) Dynabeads® immobilised cadaverine, the obtained results were very high due to light scattering. It was then tested lower numbers of Dynabeads® with immobilised cadaverine, but in all, included the unmodified carboxylic acid Dynabeads® used for controls, it was verified light scattering.

c) Discussion

The obtained results of cadaverine reaction in solution were similar to the ones found in literature, and the assay performed at lower pH reduced the existence of secondary products and allowed the detection at 350 nm. This was supported by the work of

Cayot et al. (1997) that observed similar results, (Cayot, Tainturier 1997). When TNBS was tested with cadaverine immobilised Dynabeads®, no differences were recorded between modified and unmodified carboxyl Dynabeads®, showing that absorbance detection was not an adequate technique or that no immobilisation occurred.

It was decided to pursue another detection method for primary amino groups.

Summary and Conclusion

It was immobilised 5β-cholanic acid using the spacer cadaverine into carboxyl Dynabeads®, and L-cysteine into amine Dynabeads® using the EMCS spacer. The immobilisations were made as expected and analytical techniques were implemented to verify the cadaverine immobilisation. The tested analytical techniques were found to be unsuitable to confirm immobilisation of Dynabeads®. This was because the Dynabeads® caused light scattering, making impossible to detect differences between immobilised Dynabeads® and unmodified with direct optical detection.

There was the need to test other techniques that were compatible with the Dynabeads® or where the detected product would be in solution instead of surface immobilised.

4.6.2. Use of alternative analytical technique to confirm target immobilisation to address sensitivity and background issues

The fact that the reacted product with the primary amino groups was covalently attached to Dynabeads®, interfered with direct optical detection. A different approach was taken and it was opted to search for a method that would allow the difference of concentration in target derivative before and after covalent chemistry to be tested. This separation was possible because of Dynabeads® magnetic properties. This allowed the separation of the unreacted target derivative from the covalently attached to Dynabeads®. This detection would be in solution and without the presence of Dynabeads®.

Test of Ellman's reagent for thiol groups detection present in L-cysteine to confirm Dynabeads® immobilisation

Research into a technique that would allow the detection in solution of unreacted target derivative was conducted. At this stage no method was found to test primary amines in solution that would not require surface immobilisation, so the immobilisation of L-cysteine was considered. A new method was found, the Ellman´s reagent. The

Ellman's reagent reacted with free L-cysteine and the product remained in solution. By depletion it was possible to assume if immobilisation occurred or not and extrapolate its efficiency from a standard calibration curve.

a) Materials and Methods

i. Materials and Chemicals

Sodium phosphate pH 8.0 from Sigma, EDTA from Fisher, L-cysteine, from Sigma, in house immobilised amine M-270 Dynabeads®[3] from Invitrogen, Ellman's reagent (CAS 69-78-3; 22582) from Thermo scientific, 1cm^3 polystyrene cuvettes from Fisher, Beckman Coulter DU series 700 spectrophotometer.

ii. Method for Ellman's reaction with thiol groups

A reaction solution of 0.1 M sodium phosphate with 1 mM EDTA was prepared. Several dilutions of known concentrations of L-cysteine were made using the reaction solution as solvent. The same was done for the unknown solutions (immobilised amine Dynabeads®). A solution of Ellman's reagent was made adding 4 mg of reagent into 1 ml of reaction solution. New tubes were prepared to which it was add 50 µl of Ellman's reagent in 2.5 ml of reaction solution and 250 µl of standard or sample. The mixture reacted at RT for 15 min and 1 ml of solution was transferred to a 1cm^3 polystyrene cuvette and absorbance was read at 412nm. The values of absorbance obtained for the L-cysteine standards were plotted and the unknown sample concentrations extrapolated from the calibration curve. The calculations of thiol groups concentration presented in each sample were made based on L-cysteine molar absorptivity, the absorbance values obtained and the light path length taken in the measurement. That relation was described in Equation 4-1.

$$\varepsilon = \frac{A}{bc}$$

Equation 4-1 Formula to calculate concentration - c - (M) of thiol groups based on the absorbance obtained - A- , light path length - b - (cm), and TNB product molar absorptivity (E = 14.150 $M^{-1}cm^{-1}$), (Pierce 2010).

b) Experimental and Results

i. Detection and quantification by depletion of thiol groups present in immobilised L-cysteine

[3] The immobilisation of amino Dynabeads was made as explained in section 4.5.1(Chapter 4) but with L-cysteine solution of 1 mg/ml instead of 10 mg/ml, and five washing steps instead of four.

Solutions of known L-cysteine concentration were made and quantified with the Ellman´s reagent. The obtained calibration curve of L-cysteine was performed with r^2 of 0.997, Figure 4-12.

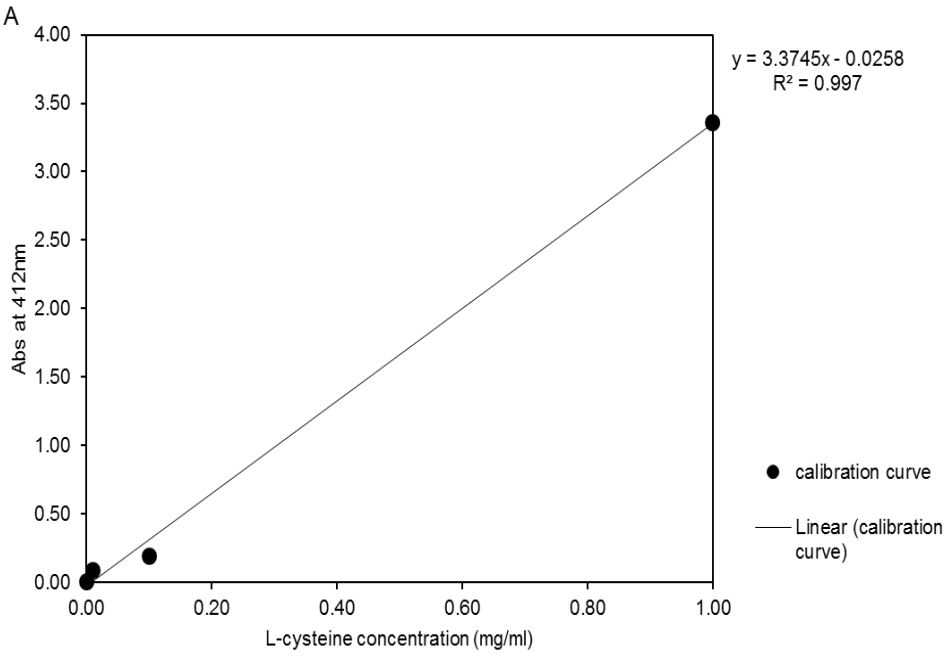


Figure 4-12 Content extrapolated of L-cysteine (mg/ml) in wash-liquid obtained after immobilisation, A) graph including all parameters, B) graph with lower scale showing values under 0.01 mg/ml.

The calibration curve presented concentration values wide spaced because of the need to cover the initial amount of L-cyteine added to the Dynabeads® (1mg/ml) and the lower values expected to cover the small difference of immobilised target. The content of thiol groups present on the standard solutions were calculated based on Equation 4-1, and represented on Table 4-2.

Table 4-2 Content of thiols in L-cysteine calibration curve.

Calibration curve L-cysteine (mg/ml)	Abs values	Thiol content in reading volume (M)	Thiol content in sample (mol)
1.000	3.36	2.37E-01	6.65E-04
0.100	0.191	1.35E-02	3.78E-05
0.010	0.087	6.15E-03	1.72E-05
0.001	0.008	5.65E-04	1.58E-06

Afterwards the Dynabeads® were immobilised with EMCS and L-cysteine, and the wash-liquid solutions obtained after the immobilisation were collected and reacted with

Elman´s reagent. The concentration of L-cysteine was extrapolated from the calibration curve (Figure 4-12), and the thiol groups calculated (Table 4-3) using the Equation 4-1.

Table 4-3 Quantity of thiol present in wash-liquid after L-cysteine immobilisation in sample and in control solutions.

		Sample	
	Abs values	Thiol content in reading volume (M)	Thiol content in sample (mol)
Wash1	3.364	2.38E-01	6.66E-04
Wash2	0.089	6.29E-03	1.76E-05
Wash3	0.007	4.95E-04	1.39E-06
Wash4	0.003	2.12E-04	5.94E-07
Wash5	-0.004	-2.83E-04	-7.92E-07
		Control	
	Abs values	Thiol content in reading volume (M)	Thiol content in sample (mol)
Wash1	3.306	2.34E-01	6.54E-04
Wash2	0.034	2.40E-03	6.73E-06
Wash3	0.006	4.24E-04	1.19E-06
Wash4	0.009	6.36E-04	1.78E-06
Wash5	0.002	1.41E-04	3.96E-07

The thiol sum obtained in the sample was 6.84E-04 mol and in the control 6.64E-04 mol, which was similar to the amount added initially to both (6.65E-04). Based on this and on the extrapolated concentrations, a significant difference between initial and final added L-cysteine was not achieved.

It was decided to calculate the expected variation in thiols content based on the Dynabeads® theoretical capacity of binding. Based on the manufacturer datasheet, the Dynabeads® binding ability of 100 µl was of 0.45 µmol, (Invitrogen 2013). In the L-cysteine, (MW 121.16), 1 mg/ml solution it was present 8.25 µmol. It was added 500 µl of that solution which was equal to 4.125 µmol. If all of the added solution bound, the 100 µl of Dynabeads® (0.45 µmol) would represent around 11% of the initial value. It was used that percentage to be the expected decrease in the thiols groups' concentration before and after immobilisation. It was expected to obtain a value in the same order of magnitude but 11% lower and maybe that small difference was not possible to detect using the Elman's reagent.

c) Discussion

The detection by depletion was tested for L-cysteine, using the Ellman's reagent as a thiol reactive chemical. No major differences between what was added for immobilisation and what was collected were found. These results were inconclusive because there were no studies made regarding the technique resolution to understand if it would be possible to detect variations of 11%. Also it was found the need to have a positive control that would confirm immobilisation under the same conditions. Because of these it was not possible to understand if there was or not immobilisation.

It was necessary to find another way to verify immobilisation.

Summary and Conclusion

The tested analytical technique was found to be unsuitable to confirm immobilisation of L-cysteine to Dynabeads®.

The obtained data was inconclusive because it was not understood if there was no change in thiol groups' concentration and no immobilisation or if the technique did not allow the detection of a small change in the thiol content (around 11%). Also it was necessary to have a positive control that could be used as an indicative for immobilisation and technique resolution.

It was necessary to test additional techniques that would overcome the targets derivatives small size and were a positive control would be used.

4.6.3. Additional use of an analytical technique to confirm targets and spacers immobilisation to address targets small size

Considering the need for detecting the variation of target derivative concentration before and after immobilisation (depletion), and maintaining the detection method free of the Dynabeads® presence, HPLC was tested.

HPLC was chosen because it was a common separation method and was readily available. Also HPLC was the technique used by Kim et al (2010), (Kim, Hyun et al. 2010) to confirm immobilisation of ibuprofen. Ibuprofen was used as a positive control to validate the technique and the EDC/NHS immobilisation chemistry.

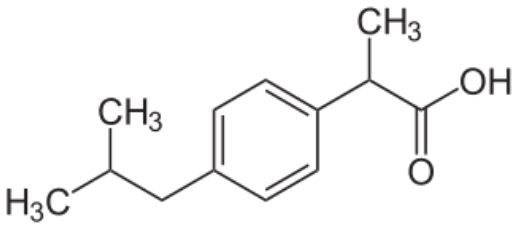


Figure 4-13 Ibuprofen.

The use of HPLC as a side-technique for Dynabeads® surface immobilisation, allowed testing a new target derivative, the phytanic acid (MW 312.53). It was tested with the condition if proved immobilised it could be used in aptamer generation.

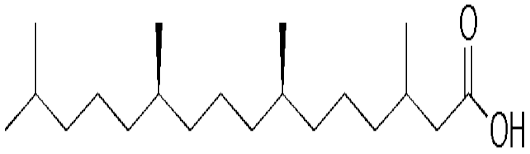


Figure 4-14 Phytanic acid.

Moreover, it was also tested the use of N-BOC-cadaverine instead of cadaverine for immobilisation. This compound presented one of the primary amines protected with N-tert-Butoxycarbonyl (BOC) and in that way it was thought to allow better detection of immobilisation. In that case the aim was to immobilise and confirm immobilisation of protected cadaverine and afterwards de-protect the second primary amine and react it with the derivative.

Immobilisation to Dynabeads® of new target derivative, a modified spacer and a control target

Carboxyl Dynabeads® were used to immobilise N-BOC-cadaverine and phytanic acid, via cadaverine spacer. Amino Dynabeads® were used to immobilise ibuprofen (MW 206.28). Ibuprofen was used as the positive control.

a) Materials and Methods

i. Materials and chemicals

Carboxyl M-270 Dynabeads® and amino M-270 Dynabeads®, from Invitrogen, phytanic acid (CAS 14721-66-5; P4060), ibuprofen (CAS 15687-27-1; I4883) (appropriated dissolved), cadaverine, and N-BOC-cadaverine (CAS 51644-96-3; 15406), (appropriated dissolved), sodium hydroxide (NaOH), acetonitrile HPLC grade (CAS 75-05-8), methanol HPLC grade and water HPLC grade from Sigma, 85%

phosphoric acid, PBS pH 7.3; 25 mM 2-(N-morpholino) ethanesulfonic acid (MES), DMSO, from Sigma, 18 MΩ.cm water magnetic support, rotator, tubes DNAse free from Eppendorff.

ii. Method for carboxyl Dynabeads® immobilisation of cadaverine and phytanic acid

Cadaverine and phytanic acid were immobilised following the same protocol used for the immobilisation of cadaverine and 5β-cholanic acid, described in section 4.5.1. The difference was that methanol was the solvent used instead of DMSO.

iii. Method for carboxyl Dynabeads® immobilisation of N-BOC-cadaverine

The N-BOC-cadaverine immobilisation protocol was the same used for the immobilisation of cadaverine in section 4.5.1., but instead of DMSO, it was used 25mM MES because this version of cadaverine was water soluble and MES was the buffer recommended by the manufacture for Dynabeads® immobilisation, (Invitrogen 2013).

After immobilisation it was necessary to de-protect N-BOC-cadaverine. MES buffer was removed from the Dynabeads® and 100 µl of acetonitrile was added, and then 50 µl of 85% solution of phosphoric acid was added drop wise. It was left three hours at RT with agitation. The solution was removed from the Dynabeads® and 50 µl of NaOH was added and cooled on ice, once cooled 50 µl of water was added. The mixture was agitated on ice for 5 min and this procedure was repeated three times. Afterwards the Dynabeads® were separated from the solution and re-suspended in 100 µl of MES.

iv. Method for amino Dynabeads® immobilisation of ibuprofen

The ibuprofen immobilisation was performed in the same way as 5β-cholanic acid, described in section 4.5.1., but in MES instead of DMSO.

b) Experimental and Results

i. Immobilisation of phytanic acid via cadaverine spacer, of N-BOC-cadaverine and ibuprofen

The immobilisations were made as explained and occurred as expected. The evaluation of the success of those immobilisations was made later on this section and chapter.

c) Discussion

The immobilisation of phytanic acid and cadaverine were made in methanol to avoid changes of working buffers. The immobilisations of ibuprofen and N-BOC-cadaverine were performed in MES buffer as recommended by the Dynabeads® manufacturer. Similar behaviour was observed during immobilisation as seen for L-cysteine and 5β-cholanic acid.

Test of High Performance Liquid Chromatography for detection and quantification of LMC targets and spacers to confirm Dynabeads® immobilisation by depletion

After Dynabeads® immobilisation, HPLC was tested as a technique to quantify unbound spacers and target derivatives. The simple structures was a problem because there were not easily detectable under UV, and there was no knowledge about best running buffers, retention time or appropriated detection wavelength. Therefore the tested conditions were based on published work, when found, and in personal communications. Only initial tests were made for the targets that did not show retention times under the initial tested conditions to avoid extensive waste of time and resources.

a) Materials and Methods

i. Materials and Chemicals

HPLC vials from Chromacol, HPLC machine and software from Shimadzu, C18 150 x 4.6 mm column from Phenomenex, UV detectors SPD-20A, HPLC grade acetone (CAS 67-64-1; 270725), acetonitrile, methanol, and water, from Sigma

Samples and standards were solubilised in the chosen running buffer and standards dilutions were made for a calibration curve. All the samples were transferred into appropriated HPLC vials. Before the run, the C18 column was cleaned with a run of HPLC grade acetone for 2 min. Afterwards the running buffer run for at least 20 min or until a stable baseline signal was seen. The samples were then disposed correctly on the injection tray, they were injected through the column and the retention time required for each sample was visualised by the presence of a peak in the baseline. The peak area was directly correlated to target concentration versus the known standard injected solutions retention time. The samples were quantified by extrapolation of the given peak area in the calibration curve.

ii. Method for detection of ibuprofen

Ibuprofen was used as a positive control and its direct immobilisation into Dynabeads® was confirmed by depletion using HPLC. Following Kim et al (2010),(Kim, Hyun et al. 2010).

iii. Method for detection of phytanic acid and 5β-cholanic acid

The first attempt for detection was a gradient test with Water:Acetronitrile (34:66), pH 3.4, with 20µl injection volume, a run of 20 min and the UV detectors at 214 and 280nm. The targets were at 0.1, 0.01 and 0.001 mg/ml and solubilised in methanol (phytanic acid), and DMSO (5β-cholanic acid). The same conditions were tested but with the running buffer at pH 6.5.

A different running buffer, Water:Methanol (34:66) was tested under the same two pH values (3.4 and 6.5) and running conditions.

A repetition of the Water:Methanol (34:66) pH 3.4 run was performed only to 5β-cholanic acid to see if it would be possible to make a calibration curve.

iv. Method for detection of L-cysteine and N-BOC-cadaverine

L-cysteine and N-BOC-cadaverine experimental detection tests were the same conditions described in the previous point.

b) Experimental and Results

i. Detection and quantification by depletion of ibuprofen

HPLC was used as a technique to verify if target derivatives depletion occurred caused by Dynabeads® immobilisation. Ibuprofen was used as a positive control because it was physiochemically similar to the LMC derivatives and it was used in published aptamer work, where Dynabeads® immobilisation was accessed with HPLC, (Kim, Hyun et al. 2010).

Ibuprofen was detected at 7 min retention time with a running buffer of Water:Acetronitrile (34:66), pH 3.4. It was possible to run a calibration curve with known concentration solutions and assume its direct immobilisation onto the surface of amino Dynabeads® by extrapolating the ibuprofen concentration present in the wash-liquid solution, Figure 4-15.

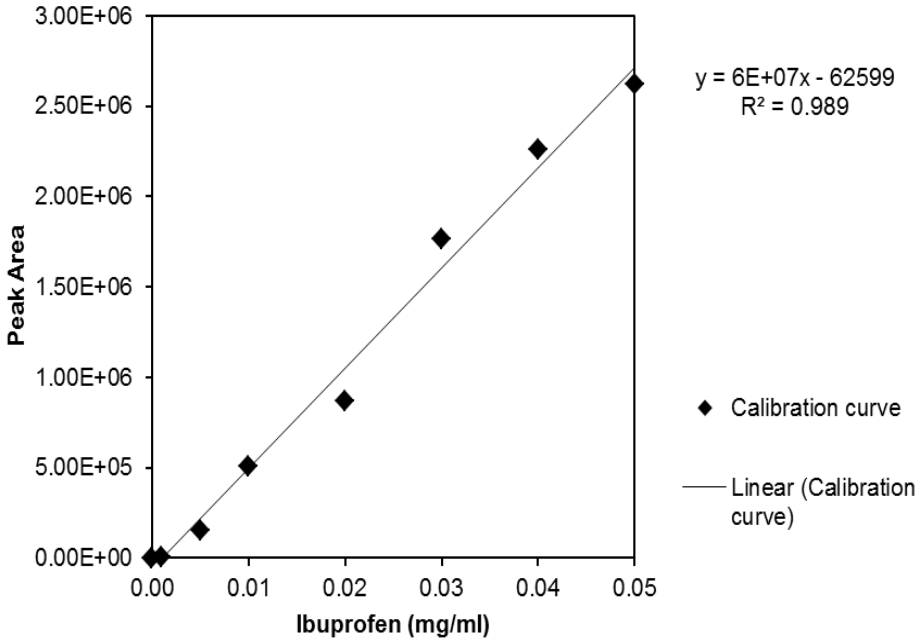


Figure 4-15 Ibuprofen detection by HPLC, calibration curve and extrapolation of Ibuprofen present in wash-liquid of activated Dynabeads® versus controls.

Table 4-4 Ibuprofen values obtained in the wash-liquid after immobilisation and on controls.

	Wash1	
	Peak area	Ibuprofen (mg/ml)
Dynabeads® activated with EDC and reacted with ibuprofen	430988.6	0.008226
Dynabeads® with ibuprofen (control)	604647.4	0.011121
Dynabeads® (control)	0	0.001043
	Wash2	
	Peak area	Ibuprofen (mg/ml)
Dynabeads® activated with EDC and reacted with ibuprofen	266903.1	0.005492
Dynabeads® with ibuprofen (control)	235104.9	0.004962
Dynabeads® (control)	0	0.001043

From verifying that the depleted concentrations in the wash-liquid solutions were lower than the obtained in the controls (unmodified Dynabeads® and unspecific binding of ibuprofen); it was assumed that the difference was caused by surface immobilisation. This assumption also validated the EDC/NHS coupling protocol used, because it showed differences between the immobilised surfaces activated with EDC/NHS and surfaces with unbound ibuprofen (unspecific binding).

ii. Detection of phytanic acid and 5β-cholanic acid

Several initial running conditions (three running buffers and two pH values) were tested to detect phytanic acid and 5β-cholanic acid retention times. In both cases no detection was seen with the running conditions tested. For that reason the detection was abandoned.

iii. Detection of L-cysteine

The initial detection of L-cysteine was successful and was detected at 1.45 min retention time at pH 3.4 of the running buffer Water: Acetonitrile (34:66). The peak area values changed because were proportional to the analyte concentration. It was run a calibration curve with known concentration solutions, Figure 4-16.

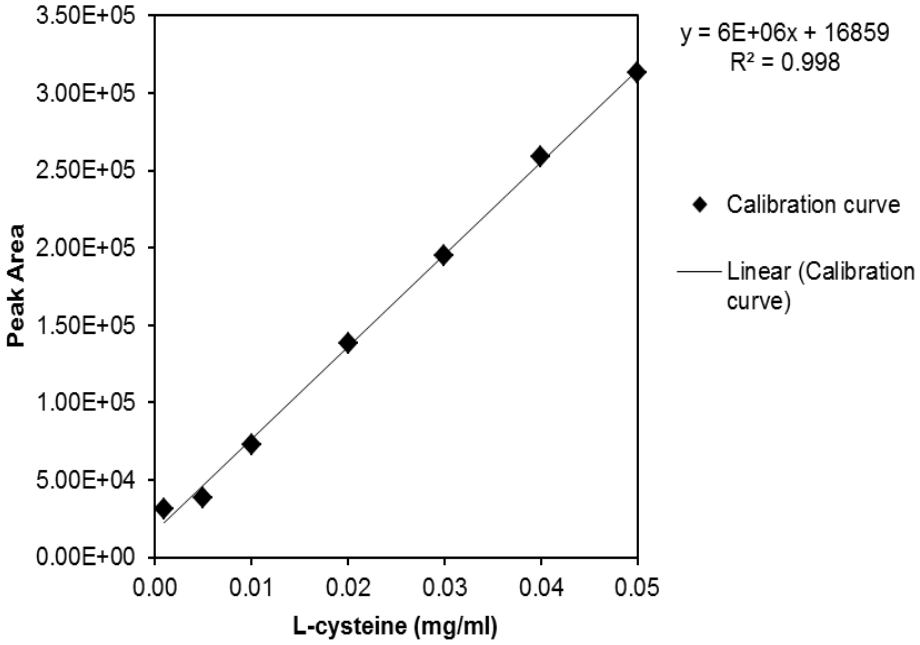


Figure 4-16 L-cysteine calibration curve based on the obtained peak area with HPLC analysis.

When the L-cysteine wash-liquid solutions were analysed after surface immobilisation it was seen a shift in the initial retention time. This was seen initially as a contaminant but in some situations there was no evidence of L-cysteine at the expected retention time of 1.45 min, when it was known to have L-cysteine in solution. After reading the absorbance at 210 nm of one unique solution of L-cysteine, during a period of time, it was seen a decrease of concentration of L-cysteine. Later was concluded that L-cysteine oxidised into cystine making the UV detection unreliable, Figure 4-17.

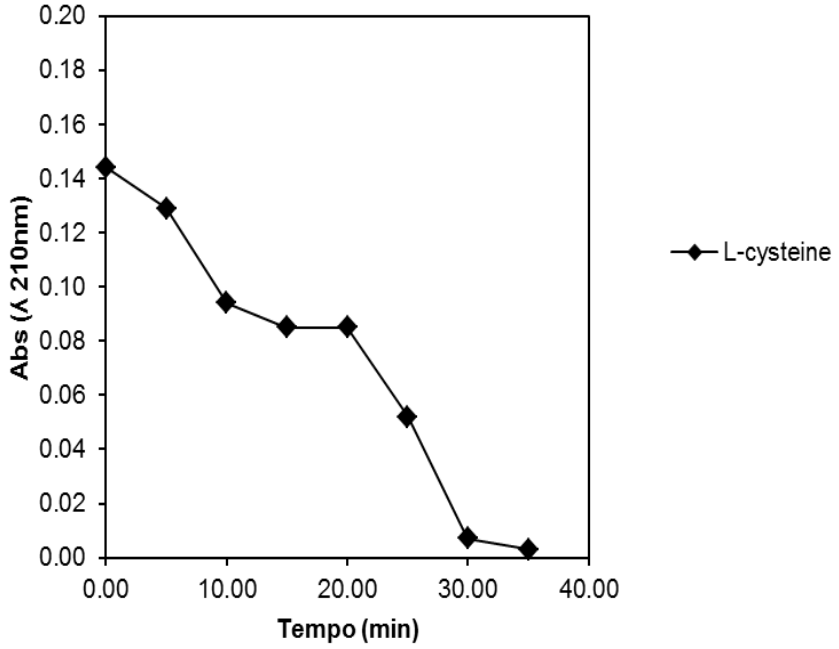


Figure 4-17 Detection of L-cysteine oxidation in PBS during 35 min.

In the previous graph it was possible to verify experimentally almost the full oxidation of L-cysteine in less than 40 min. This showed that the detection of L-cysteine obtained in the wash-liquid solutions was not reliable because the unbound L-cysteine would oxidise between the collection and analysis of the samples turning the detection ineffective.

iv. Detection of N-BOC-cadaverine

The initial attempt to use HPLC to detect N-BOC-cadaverine did not show a clear peak, easy to confirm that it was the target. Because of lack of comparison to peak identification it was not possible to proceed with N-BOC-cadaverine detection in HPLC.

c) Discussion

There was no information about the required conditions to detect the target derivatives, such as mobile phase, detection wavelength, if isocratic or gradient running buffer and retention time. It was necessary to look at literature but no clear examples were found to the more simple targets, so a few conditions were tested based in *in house* discussion. The running buffers chemicals composition chosen was considered transparent at the low wavelengths tested; in order to try to capture as much as possible.

The phytanic acid proved to be very difficult to detect because it had not a functional group besides the –COOH which presented low detection under UV. Also the 5β-cholanic acid did not present a peak under the tested conditions.

The L-cysteine was detected easily in the first assay but afterwards it was not possible to detect it again because it was noticed the formation of disulphide bonds (cystine) as a peak shift was observed in the analysis of wash-liquid solutions from Dynabeads® immobilisation. L-cysteine was found to convert easily in solution to cystine. It was found later that the addition of EDTA to solution would prevent the oxidation, but it was not applied by then, (Thermo Scientific Pierce 2010).

Because HPLC as a side technique was time consuming due to the lack of information about the LMC target derivatives detection conditions, it was chosen to test other techniques.

Summary and Conclusion

It was detected and assumed surface immobilisation of ibuprofen using HPLC to quantify the obtain depletion between initial and final solutions. Ibuprofen worked as a validation control for the immobilisation EDC/NHS chemistry used because it was assumed that the difference in concentrations obtained was caused by efficient immobilisation chemistry.

The target derivatives with their simplistic chemical structure were not detected by the tested conditions with the exception of L-cysteine, which was found to easily oxidise and become cystine in solution. Because of this phenomenon it was inconclusive if L-cysteine bound to the Dynabeads® or not. L-cysteine was the only LMC target derivative that was possible to quantify by the tested HPLC conditions. Unfortunately it was verified that oxidised in solution making the detection inadequate because of the time between sample collection and analysis.

After testing HPLC as an additional technique, it was obvious that the nature of the LMC derivatives in conjugation with Dynabeads® were complex for detection using the standard or well-known analytical techniques. It was necessary a different approach. It was decided to test additional techniques focused on surface analysis.

4.6.4. Supplementary use of analytical techniques to confirm target immobilisation and address targets small size and beads physical characteristics

The LMC derivatives of interest were L-cysteine and 5β-cholanic acid, immobilised on the surface of amino and carboxyl Dynabeads® via a spacer. Complementary to this it was used 5β-cholanic acid directly immobilised into the surface of amino Dynabeads®. Phytanic acid was discarded due to its simplicity, and another derivative was used to test immobilisation. The derivative was lysine but in the protected version to prevent unwanted chemistry. N-BOC-lysine was water-soluble and also an amino-acid that could be used instead of L-cysteine in aptamer generation.

It was reviewed techniques used for physical surface analysis and the approached changed from detecting specifically functional groups by direct or indirect detection to compare immobilised surfaces from unmodified.

Five new techniques were tested to confirm immobilisation: Infra-Red (IR) and Fourier Transform Infra-Red (FTIR), elemental analysis, another method to detect primary amino groups (ninhydrin reagent) and X-ray photoelectron spectroscopy (XPS).

Dynabeads® immobilisation with new target derivative - Amino Dynabeads® immobilisation of N-BOC-lysine

A new target derivative was added to the previous tested derivatives (L-cysteine, 5β-cholanic acid and phytanic acid). The derivative was used to study surface immobilisation in one of the tested techniques and was the N-BOC-lysine, an amino acid with one end protected with a BOC structure. It was surface immobilised the new derivative and then de-protected. In both situations it was followed the same protocols as in previous points.

a) Materials and Methods

i. Materials and chemicals

N-BOC-lysine was immobilised on amino Dynabeads® as explained in section 4.5.1 via EDC/NHS and to de-protect lysine, it was followed the same protocol to de-protect N-BOC-cadaverine, explained in detail in section 4.5.3.

b) Experimental and Results

The N-BOC-lysine immobilisation on the surface of amino Dynabeads® occurred as expected as well as the de-protection. Both actions were accessed later on this chapter.

c) Discussion

The immobilisation and de-protection was made as previously. The assessment of both steps was only confirmed once a suitable technique was found to confirm surface immobilisation.

Test of ninhydrin reagent for primary amine detection to confirm Dynabeads® immobilisation

Ninhydrin was a colorimetric detection reagent that reacted with primary amines. It was used to test the immobilisation both on the surface of amine and carboxyl Dynabeads®, to which a primary amine would be present after immobilisation.

a) Materials and Methods

i. Materials and Chemicals

Ninhydrin reagent (CAS 485-47-2; N7285), ethanolamine (CAS 141-43-5; E9508), 0.05 % glacial acetic acid, from Sigma, 95 % ethanol, 6 ml glass vials, 1 cm^3 polystyrene cuvettes from Fisher, Beckman Coulter DU series 700 spectrophotometer, boiling water bath, immobilised M-270 Dynabeads® with L-cysteine via EMCS and directly with 5β-cholanic acid.

ii. Method for ninhydrin reaction with primary amines

Standard solutions were made from a starting solution of 50 µM ethanolamine, in 0.05 % glacial acetic acid. The standards were diluted with 18 MΩ.cm water in a total volume of 2 ml in 6 ml glass vials. The blank was 18 MΩ.cm water. To the standards, blank, and samples, it was added 1 ml of ninhydrin reagent, mixed gently and immersed into a bubbling boiling water bath for exactly 10 min. After, the vials were removed from the bath and allowed to cool down to RT. To each solution, 5 ml of 95 % ethanol was added. From each solution, 1 ml was transferred into 1 cm^3 polystyrene cuvettes and absorbance was read at 570 nm. In the case of absorbance values higher than one, dilutions were made in 95 % ethanol. A calibration curve was obtained and the samples at unknown concentrations of amine groups were extrapolated.

b) Experimental and Results

i. Use of ethanolamine as standard for calibration curve

The confirmation of immobilisation was made by comparison between immobilised Dynabeads and controls, so it was necessary initially to run a calibration curve for extrapolation of concentrations. Ethanolamine was used as standard for a calibration curve. Dilutions from 6.25 µM to 43.75 µM in 18 MΩ.cm water were made and reacted with ninhydrin.

ii. Detection and quantification by depletion of immobilised L-cysteine via EMCS spacer with ninhydrin

Amino Dynabeads® and Amino Dynabeads® with unspecific adsorbed L-Cysteine were reacted with ninhydrin to be used as comparison of immobilisation. Because ninhydrin provided a colorimetric detection method it was possible to visually confirm the presence of primary amino groups, Figure 4-18.

Amino Dynabeads® immobilised only with EMCS and amino Dynabeads® immobilised with EMCS and L-cysteine reacted with ninhydrin and the concentration values were extrapolated from the obtained absorbance, Figure 4-19.

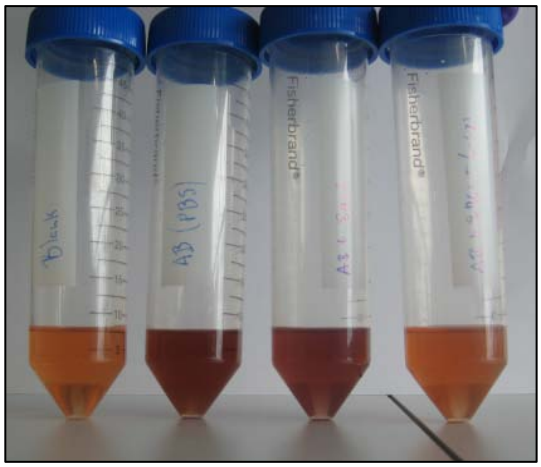


Figure 4-18 Colour change in the presence of primary amines. (Left to right: Blank solution, unmodified amino Dynabeads®, amino Dynabeads® with EMCS and amino Dynabeads® with EMCS and L-cysteine.)

It was possible to visualise a colour change in comparison to the blank solution, which occurred according to the amino content. As expected the unmodified amino Dynabeads® presented the darkest colour. It was expected to have a similar result in the amino Dynabeads immobilised with EMCS and L-cysteine. This would happen

because of the increase of amino content present in L-cysteine. Such increase was not seen and the solution that followed in colour gradient was the amino Dynabeads® immobilised with EMCS spacer. This was perhaps caused by poor EMCS immobilisation. In the case of the addition of L-cysteine after EMCS immobilisation it was seen a decrease in colour. This could have been because of efficient immobilisation but no reaction with the amino group or by unspecific coated of the Dynabeads® surface, preventing the reaction with the surface amines.

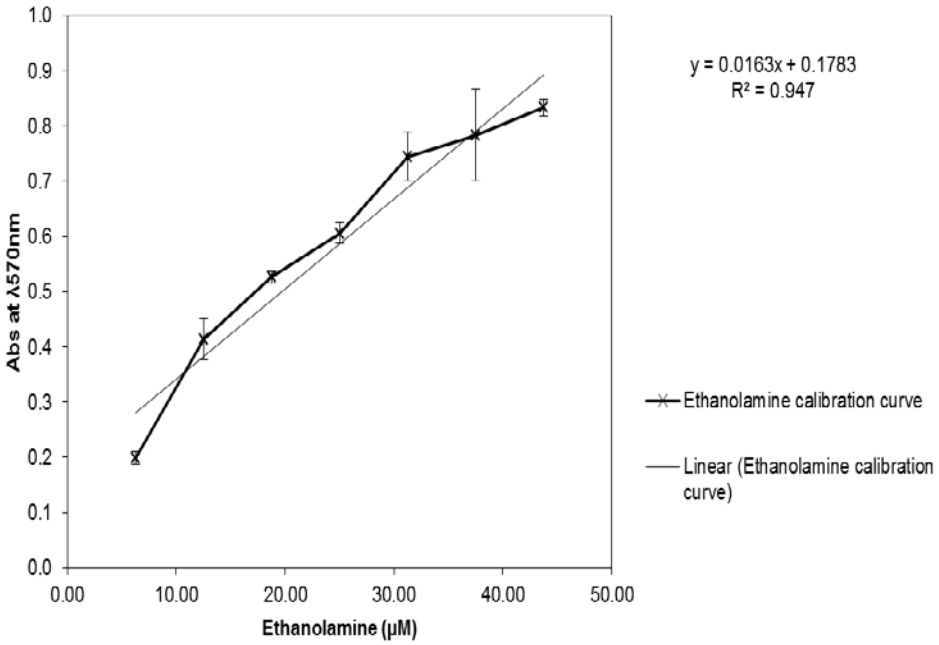


Figure 4-19 Calibration curve and extrapolated concentrations of primary amino groups present at different stages of L-cysteine immobilisation.

Table 4-5 Extrapoleted values of amines present at each L-cysteine immobilisation stage.

	Abs	SD	Amino groups (µM)
Dynabeads® with EMCS and reacted with L-cysteine	0.184	0.030	0.370
Dynabeads® with EMCS (control)	0.460	0.030	17.282
Dynabeads® with unspecific L-cysteine (control)	0.819	0.141	39.286
Dynabeads® (control)	0.740	0.021	34.481

The extrapolated concentration values from the calibration curve showed clearly the lower signal obtained in the L-cysteine immobilised Dynabeads®, Figure 4 15.

iii. Detection and quantification by depletion of immobilised 5β-cholanic acid with ninhydrin

Similar procedure was done with amino Dynabeads® either unmodified for comparison or direct immobilised with 5β-cholanic acid, Figure 4-20 and Table 4-6.

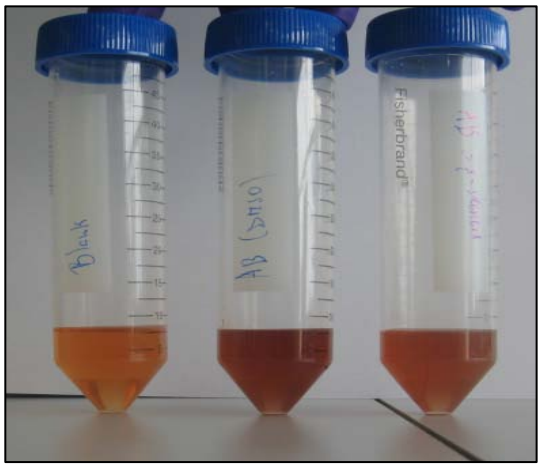


Figure 4-20 Colour change in the presence of primary amines. (Left to right: Blank solution, unmodified amino Dynabeads®, amino Dynabeads® with 5β-cholanic acid)

In this case it was seen a decrease in colour when compared unmodified Dynabeads® with modified ones. This was the desired result that confirmed surface immobilisation.

Table 4-6 Extrapolation of amino groups present on Dynabeads® surface after immobilisation and in controls

	Abs	SD	Amino groups (μM)
Dynabeads® with 5β-cholanic acid	0.430	0.026	15.442
Dynabeads® with unspecific 5β-cholanic acid (control)	0.907	0.035	44.726
Dynabeads® (control)	0.635	0.0097	27.998

It was seen reduction in reactive amine groups on the immobilised Dynabeads® in comparison with unmodified Dynabeads®. That was assumed to be caused by the previous amines reaction with 5β-cholanic acid. It was considered that direct surface immobilisation occurred efficiently.

c) Discussion

Ninhydrin detection was based on the presence of reactive primary amino groups. The amino Dynabeads® reacted well with ninhydrin and it was possible to determine if they were or not immobilised.

It should have been seen an increase of amino groups due to L-cysteine so the solution should had been darker than the immobilised EMCS, than in turn should had

been lighter than the unmodified Dynabeads®. This might show that there was no immobilisation of L-cysteine or that unspecific cover of the Dynabeads® preventing the primary amines reaction with ninhydrin.
In the case of the direct immobilisation it was possible to assume immobilisation of 5β-cholanic acid by decrease of available amino groups. Again it was possible to assume the validation of the EDC/NHS chemistry and protocol.

There were several hypotheses for ninhydrin non-reactivity after the second step immobilisation, but it was not possible to confirm neither. For that reason it was seen as inappropriate to use ninhydrin reaction as side-technique for the Dynabeads® immobilisation when using a spacer and target, which was exactly what was decided to have for aptamer generation.

It was necessary to pursue a technique able to confirm immobilisation when more than one molecule was surface immobilised.

Test of X-ray Photoelectron Spectroscopy for detection of functional groups to confirm Dynabeads® immobilisation

XPS allowed surface analysis by the detection of atomic elements, the percentage of each, and the neighbourhood interaction between elements. XPS was used to access how efficient LMC target derivatives were immobilised onto the surface of Dynabeads®.

a) Materials and Methods

i. Materials and Chemicals

In house immobilised Dynabeads®, Swallon LTE scientific oven, and X-ray spectrophotometer (Kratos AXIS ULTRA (1486.6 eV) with fixed analyser transmission and with 12 kV anode potential).

ii. Samples preparation

In house immobilised Dynabeads® and controls were dried at 37 °C, until no visible liquid was observed, labelled and sent for analysis. From each sample three replicates were taken and measured.

iii. XPS analysis

The XPS analysis was done at the XPS laboratory of Nottingham University, and the costs were supported by EPSRC.

The procedure was reported as being the samples were put into a sample track and exposed to mono-chromated Al kα X-ray after being introduced overnight into the instrument ultra-high vacuum chamber. The emission current was of 10 mA. The scans were high resolution with 20 eV pass energy and a take-off angle of 90°.

b) Experimental and Results

i. L-cysteine immobilisation

- L-cysteine immobilised via EMCS spacer

Considering the chemical bonds of L-cysteine immobilisation on the surface of amino Dynabeads®, via the spacer EMCS (Figure 4-9), it was expected to see an increase or new appearance of the following bound elements:

carbonyl (R(C=O)R´), carboxamide (R-(C=O)NR´), sulphydril (RC-SH) and carboxylic (R(C=O)OH).

Also a shift in elements position due to new neighbourhood was expected and the atomic percentage of elements should have varied and the full width at half maximum between elements (FWHM) should also have changed.

The easiest detectable element for immobilisation validation was sulphur as it was the only new introduced element. Also it was expected a decrease of primary amino groups after EMCS spacer immobilisation and an increase after L-cysteine immobilisation.

After data comparison of amino Dynabeads®, amino Dynabeads® with immobilised EMCS and amino Dynabeads® with the target derivative and spacer immobilisation, it was clear that there was no evidence of immobilisation of L-cysteine. The immobilisation protocol was inefficient.

ii. 5β-cholanic acid immobilisation

5β-cholanic acid immobilisation was tested directly on the surface of amino Dynabeads® and via cadaverine spacer on carboxyl Dynabeads®.

- 5β-cholanic acid immobilisation via spacer cadaverine

When comparing the general elements positions after calculating the replicates average it was seen two elements peak shifts between the different stages of immobilisation. The N 1s and O 1s were different in the Dynabeads® immobilised with cadaverine and also with cadaverine and 5β-cholanic acid, Table 4-7, Figure A-1.

Table 4-7 Comparison of binding energy position average, standard deviation and coefficient of variation of C, N and O present in carboxylic Dynabeads® immobilised with 5β-cholanic acid via spacer cadaverine, and controls.

Element	CB (DMSO)			CB with immob. cadaverine		
	Position(eV)	SD	CV%	Position(eV)	SD	CV%
C1s	282.67	0.58	0.2	283	0	0
N1s	397	0	0	399	0	0
O1s	530	0	0	529.67	0.58	0.11
Element	CB with immob. cadaverine and 5β-cholanic acid			CB with 5β-cholanic acid		
	Position(eV)	SD	CV%	Position(eV)	SD	CV%
C1s	283.33	0.58	0.2	282.33	0.58	0.2
N1s	398.67	1.15	0.29	397.33	0.58	0.15
O1s	529.67	1.15	0.22	530	0	0

It was after studying the sub-peaks produced by each element that it was clear the influence of immobilisation in each element neighbourhood.

This was particularly visible in the full immobilised Dynabeads® , were the bond C=O appeared under the O 1s at the binding energy average of 531.06 eV with a standard deviation of 0.07 and a coefficient of variation of 0.01%. This binding energy presented an increase in concentration percentage inside the element binding energy range in comparison to the unmodified Dynabeads®, Figure A-2, Figure A-3 and Figure A-4.

The same was seen under the N 1s, were a new bond appeared at the average of 400.09 eV, with a standard deviation of 0.57 and a coefficient of variation of 0.14%, Figure A-5, Figure A-6 and Figure A-7.

Another way of confirming immobilisation was by studying the full width at half maximum average of each element at the different immobilisation step, Table 4-8.

Table 4-8 Comparison of full width at half maximum average, standard deviation and coefficient of variation of C, N and O present in carboxylic Dynabeads® immobilised with 5β-cholanic acid via spacer cadaverine, and controls.

Element	CB (DMSO)			CB with immob. cadaverine		
	FWHM	SD	CV%	FWHM	SD	CV%
C1s	2.85	0.09	3.33	2.72	0.14	5.20
N1s	2.56	0.19	7.37	3.05	0.12	3.80
O1s	3.22	0.04	1.35	3.28	0.14	4.24
Element	CB with immob. cadaverine and 5β-cholanic acid			CB with 5β-cholanic acid		
	FWHM	SD	CV%	FWHM	SD	CV%
C1s	3.60	0.84	23.31	2.71	0.13	4.62
N1s	3.41	0.30	8.70	3.35	0.14	4.06
O1s	3.59	0.11	3.02	3.09	0.03	0.99

There was clearly an increase of FWHM values for the three elements, in the full immobilised Dynabeads®. This was sign of the existence of more sub-peaks inside each element, which occurred due to immobilisation.

A last point of study for the obtained data was the atomic percentage of each element at the different immobilisation steps for the three elements, Table 4-9.

Table 4-9 Comparison of atomic percentage average, standard deviation and coefficient of variation of C, N and O present in carboxylic Dynabeads® immobilised with 5β-cholanic acid via spacer cadaverine, and controls.

Element	CB (DMSO)			CB with immob. cadaverine		
	average	SD	CV%	average	SD	CV%
C1s	66.28	1.62	2.45	67.35	0.095	0.14
N1s	1.90	0.30	15.81	5.58	0.248	4.34
O1s	25.32	0.83	3.26	21.95	0.34	1.54
Element	CB with immob. cadaverine and 5β-cholanic acid			CB with 5β-cholanic acid		
	average	SD	CV%	average	SD	CV%
C1s	67.77	1.56	2.30	67.32	0.73	1.09
N1s	6.74	0.14	2.08	2.20	0.26	11.87
O1s	22.96	1.44	6.26	25.43	0.44	1.74

It was seen a shift in percentage towards the N 1s and the O 1s in the immobilised Dynabeads® as expected.

- 5β-cholanic acid direct immobilisation

The direct 5β-cholanic acid immobilisation on the surface of amino Dynabeads® was confirmed by sub-peaks biding energy values. When looking at the general binding value for each element, it was not seen a significant change between immobilised and control Dynabeads®, Table 4-10, Figure A-8.

As in the 5β-cholanic acid immobilisation via a spacer, shifts in the internal positions after immobilisation were seen. At C 1s it was seen a new sub-peak binding energy with 287.91 eV average, with 0.07 of standard deviation and 0.02% of coefficient of variation, Figure A-9 and Figure A-10. The O 1s also presented a new binding position averaged at 530.93 eV, with standard deviation of 0.15 and 0.03% of coefficient of variation, Figure A-11 and Figure A-12. The N 1s presented a new internal peak 399.76 eV with standard deviation of 0.12 and a coefficient of variation of 0.03%, Figure A-13 and Figure A-14.

Table 4-10 Comparison of binding energy position average, standard deviation and coefficient of variation of C, N and O present in amino Dynabeads® immobilised with 5β-cholanic acid, and controls.

Element	AB (DMSO)			AB with immob. 5β-cholanic acid		
	Position(eV)	SD	CV%	Position(eV)	SD	CV%
C1s	283,33	0,58	0,20	284,00	0,00	0,00
N1s	397,00	0,00	0,00	397,33	0,58	0,15
O1s	530,33	0,58	0,11	530,67	0,58	0,11
Element	AB with 5β-cholanic acid			5β-cholanic acid		
	Position(eV)	SD	CV%	Position(eV)	SD	CV%
C1s	284,00	0,00	0,00	283,00	0,00	0,00
N1s	397,33	0,58	0,15	Not present	Not present	Not present
O1s	531,00	0,00	0,00	531,00	0,00	0,00

Analysing the FWHM it was observed that there was not a significant difference between the immobilised Dynabeads® and the unmodified. This was expected because of the low molecular of the 5β-cholanic acid, which did not added significant length to the molecule, Table 4-11.

Table 4-11 Comparison of full width at half maximum average, standard deviation and coefficient of variation of C, N and O present in amino Dynabeads® immobilised with 5β-cholanic acid, and controls.

Element	AB (DMSO)			AB with immob. 5β-cholanic acid		
	FWHM	SD	CV%	FWHM	SD	CV%
C1s	2.95	0.07	2.46	2.86	0.10	3.49
N1s	2.30	0.23	9.87	2.45	0.24	9.61
O1s	2.41	0.05	2.01	2.41	0.29	12.08
Element	AB with 5β-cholanic acid			5β-cholanic acid		
	FWHM	SD	CV%	FWHM	SD	CV%
C1s	2.93	0.41	14.04	2.04	0.13	6.48
N1s	2.43	0.34	13.97	Not present	Not present	Not present
O1s	2.54	0.14	5.60	3.07	0.18	5.81

Also the comparison of atomic percentages of negative controls to the immobilised samples, confirmed the direct immobilisation of 5β-cholanic acid to the surface of amino Dynabeads®. There was an increase in the percentage of N 1s and O 1s which caused a decrease in the value for C 1s,Table 4-12.

Table 4-12 Comparison of atomic percentage average, standard deviation and coefficient of variation of amino Dynabeads® direct immobilisation of 5β-cholanic acid and controls

Element	AB (DMSO)			AB with immob. 5β-cholanic acid		
	At.%	SD	CV%	At.%	SD	CV%
C1s	71.81	1.46	2.03	67.43	0.29	0.44
N1s	1.31	0.20	15.25	3.80	0.15	3.8
O1s	25.95	1.19	4.60	28.30	0.39	1.37
Element	AB with 5β-cholanic acid			5β-cholanic acid		
	At.%	SD	CV%	At.%	SD	CV%
C1s	70.71	3.98	5.63	86.35	7.58	8.77
N1s	1.25	0.76	60.35	Not present	Not present	Not present
O1s	27.34	3.61	13.22	12.34	6.42	52.03

iii. Lysine immobilisation

N-BOC-lysine was surface immobilised and de-protected afterwards. The correct immobilisation and de-protection would have the structure represented in Figure 4-21.

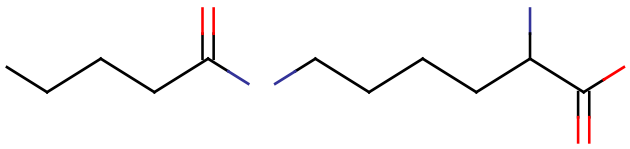


Figure 4-21 Surface immobilised lysine to amino Dynabeads®.

Lysine was directly immobilised on the surface of amino Dynabeads® using the protected version of the target, N-BOC-lysine. The immobilisation and de-protection were made and XPS was used to confirm both steps. The binding energy was compared between immobilisation and controls to confirm changes in the atomic bonds, Table 4-13 and Figure A-15.

Table 4-13 Comparison of binding energy position average, standard deviation and coefficient of variation of C, N and O present in carboxyl Dynabeads® immobilised with lysine, and controls.

Element	CB (PBS)			CB with immob. lysine		
	Position(eV)	SD	CV%	Position(eV)	SD	CV%
C1s	283,67	0,58	0,20	283,67	0,58	0,20
N1s	400,67	0,58	0,14	398,33	0,58	0,14
O1s	530,67	0,58	0,11	530,67	0,58	0,11
Element	CB with immob. BOC-lysine			CB with BOC-lysine		
	Position(eV)	SD	CV%	Position(eV)	SD	CV%
C1s	283,00	0,00	0,00	283,33	0,58	0,20
N1s	394,00	6,08	1,54	398,00	0,00	0,00
O1s	530,00	0,00	0,00	530,33	0,58	0,11

No position changes were observed for C 1s or O 1 s, but it was verified a change in the N 1s at each stage, Figure A-17, Figure A-19 and Figure A-21. This could confirm the changes of surface immobilisation and de-protection. It was necessary to look at other parameters.

Looking at the FWHM values, they did not show the presence of a long molecule, this was the same as in the case of the direct immobilisation of 5β-cholanic acid. The low molecule weight did not add significant length to the initial molecule to allow a significant increase in the FWHM, Table 4-14.

Table 4-14 Comparison of full width at half maximum average, standard deviation and coefficient of variation of C, N and O present in amino Dynabeads® immobilised with 5β-stearane, and controls.

Element	CB (PBS)			CB with immob. lysine		
	FWHM	SD	CV%	FWHM	SD	CV%
C1s	2,80	0,27	9,75	3,34	0,52	15,65
N1s	4,29	0,38	8,86	3,95	0,70	17,65
O1s	3,01	0,36	12,05	3,63	0,62	17,02
Element	CB with immob. BOC-lysine			CB with BOC-lysine		
	FWHM	SD	CV%	FWHM	SD	CV%
C1s	3,02	0,16	5,44	2,88	0,40	13,87
N1s	2,50	0,17	6,74	2,46	0,40	16,39
O1s	3,24	0,04	1,16	3,19	0,27	8,48

The atomic percentage was an indicator of immobilisation, so the percentage of C 1s, N 1s and O 1s was compared between the stages of immobilisation, Table 4-15.

Table 4-15 Comparison of atomic percentage average, standard deviation and coefficient of variation of carboxyl Dynabeads® direct immobilisation of lysine and controls

Element	CB (PBS)			CB with immob. lysine		
	At.%	SD	CV%	At.%	SD	CV%
C1s	71,06	0,58	0,81	68,88	0,47	0,68
N1s	2,65	0,53	20,09	5,17	0,20	3,84
O1s	24,74	0,15	0,59	24,38	0,04	0,14
Element	CB with immob. BOC-lysine			CB with BOC-lysine		
	At.%	SD	CV%	At.%	SD	CV%
C1s	63,08	1,23	1,95	62,96	1,49	2,37
N1s	4,86	0,13	2,77	1,38	0,18	13,02
O1s	25,81	0,35	1,37	28,84	0,32	1,09

It was seen an increase in N atomic percentage after surface immobilisation, showing the presence of two amino groups, from the Dynabeads® and from the target. The de-protection appeared to have been also efficient because there was reduction in O atoms and C from the protection to the de-protected target.

c) Discussion

By analysing binding energy values it was possible to detect the presence of specific molecular bonds. The increase of specific atomic percentages and FWHM allowed understanding of the changes made by efficient immobilisation.

It was found that L-cysteine did not appear to be immobilised because there were not found shifts in atomic neighbourhoods of C 1s, N 1s or O 1s, it was not found an increase of atomic percentage towards N 1s and C 1s as expected, and the presence of a new atomic element S 2s was not found as it should had in an efficient immobilisation.

5β-cholanic acid was found immobilised either via the spacer cadaverine or directly into the surface of Dynabeads®. It was found that the immobilisation method used, proved to be efficient for both cases. The several binding energy shifts were confirmed in literature to be caused by –R(C=O)NR´- covalent bonds,(Chen, Nikles 2001, Eralp, Shavorskiy et al. 2011).

The immobilisation of lysine via its protected version, BOC-lysine, was confirmed by the atomic percentage increase of amines and oxygen. Due to the atomic nature of compounds, there were no different elements and the fact that lysine and carbodiamines both have nitrogen and oxygen, increased the difficulty to confirm de-

protection. The only factor to support the efficient de-protection was the decrease in carbon and oxygen that was as expected after de-protection. Lysine was surface immobilised and assumed to be de-protected because it would be necessary to compare the obtained data with lysine.

Summary and conclusion

In summary, IR and FTIR were inadequate to confirm LMC target derivatives immobilisation. Ninhydrin reaction with primary amines showed to be compatible only with direct immobilisation of amino Dynabeads®, and XPS proved to be appropriated to be used to confirm Dynabeads® immobilisation in general.

XPS allowed confirmation of surface modifications. It proved to be adequate to be used with Dynabeads® and the low molecular size LMC target derivatives. Despite this, it was found that ideally the chemical nature of the target immobilised should be different from the Dynabeads® used. This would add new atomic species making the detection easier.

The fact that it was concluded that L-cysteine immobilisation was inefficient lead to the understanding that previous techniques used to detect the presence of L-cysteine, in special the Ellman's reagent technique, did not present positive results, probably not because it was not adequate for Dynabeads® immobilisation detection, but because there was no immobilisation. Because XPS was used in other immobilised sets it was possible to understand that the technique itself worked and that it was the immobilisation the problem. This did not happen in previous techniques because they were too specific for target detection and there were no positive controls available for technique validation.

4.7. Initial testing of aptamer generation against small molecules relevant for the LMC, using the FluMag Systematic Evolution of Ligands by Exponential Amplication (SELEX) method

The initial objective of testing if it would be possible to generate aptamers within the project time frame and with the available resources was affected by the obstacles found in section 4.5. For this reason it was necessary to modified the objective and contextualise it with the task of confirming the derivatives surface immobilisation. It was decided to focus on highlighting problems found in the chosen SELEX method prior to

attempt aptamer generation against the LMC immobilised target derivatives. This allowed the test of FluMag-SELEX while confirming Dynabeads® immobilisation. It was decided to study two major FluMag-SELEX steps: the amplification of eluted strands and the separation of the positive strand after amplification. The new objectives were to study the use of real time PCR, which was introduced as a novel technique and to compare the standard use of a denaturing gel and the less common use of streptavidin coated Dynabeads® for ssDNA separation.

The LMC target derivatives chosen to test aptamer generation were L-cysteine and 5β-cholanic acid and immobilisation was assumed successful until confirmation in order to allow the study of the FluMag-SELEX during the project time frame. This was contrary to the reason why immobilisation wanted to be confirmed, but in order to proceed with the feasible part of the aptamer generation work it was necessary to use assumed immobilised Dynabeads®.

Due to the fact that the confirmation of immobilisation was more complex than expected and that the studied steps in the FluMag-SELEX showed sensitive points in the method, it was not possible to complete a cycle of aptamers generation in the allocated time frame and resources.

Nevertheless the learned lessons allowed the input of suggestions and knowledge for future work.

4.7.1. Establishment and optimisation of iterative steps in preparation for applying FluMag SELEX method to LMC targets

As explained in section 4.4, the chosen method for aptamer generation was the FluMag-SELEX. This method used Dynabeads® as solid surface for target immobilisation and modification of the positive strand with fluorescein during the amplification step, (Stoltenburg, Reinemann,C., Strehlitz,B. 2005).

The chosen target derivatives for aptamer generation were L-cysteine and 5β-cholanic acid. In order to improve the knowledge of the FluMag-SELEX it was assumed, until section 4.5 completion, that the target derivatives had been immobilised.

The FluMag-SELEX was an established method but in order to implement it in house and to introduce the real time PCR (qPCR) instead of the traditional PCR and to

choose from two different techniques for ssDNA separation, it was necessary to test the amplification step and the ssDNA separation step individually.

qPCR was not a technique used in aptamer generation, especially within the FluMag-SELEX. qPCR was used in literature with RNA aptamers. There was an example where selected strands that bound the target (E-coli surface proteins) were amplified by reverse transcriptase qPCR, (L, Kim et al. 2009).

qPCR was a technique that allowed following the amplification in real time. Moreover based on a calibration curve it was possible to quantify the eluted material obtained in each iteration, introduce wanted modifications to the strands via the primers, and discuss the DNA variability of the eluted strands based on their temperature melting point. qPCR has three major cycle steps: denaturation of existing dsDNA, annealing of primers, and amplification by taq polymerase using each strand as template. Usually those cycles are repeated at least 25 to 30 times and afterwards a melting curve analysis is performed. The amplification is divided in two parts, the exponential and the plateau. The amplification is detected by using SYBR green dye. In the presence of dsDNA the dye SYBR green intercalates it and its fluorescence is registered. Once the taq polymerase amplified each ssDNA into a dsDNA, the SYBR green binds to it and there is a raise of fluorescence, which is proportional to the presence of dsDNA, When analysing the data the obtained Ct (threshold cycle) values are considered as the level of fluorescence above the defined baseline, at which is considered a sign of DNA amplification, and used for quantification. (BioRad 2006).

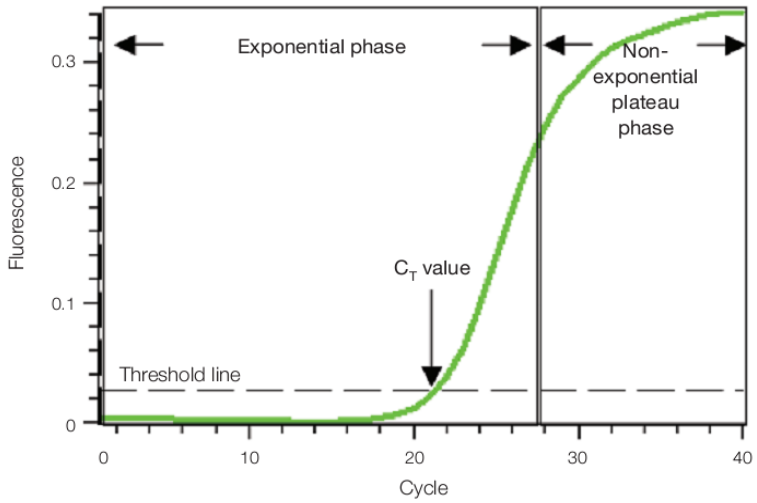


Figure 4-22 qPCR amplification graph showing the exponential and plateau phases and the Ct value, adapted from (BioRad 2006).

The separation of the positive strand was tested using either the standard method with a denaturing gel or a less common method where streptavidin coated Dynabeads® were used.

The initial step tested was the amplification by qPCR implementation.

Amplification of selected ssDNA by real time Polymerase Chain reaction (qPCR)

qPCR allowed the amplification of the eluted strands obtained after selection of the ssDNA pool with the immobilised target. As qPCR was implemented it was necessary to test the chosen primers, to decide on the best running conditions and to obtain a calibration curve that would allow the posterior quantification of the eluted strands.

a) Materials and Methods

i. Materials and chemicals

ssDNA random library from Invitrogen, primers from Sigma and Invitrogen, SyBr Green mix with taq polymerase from PrimerDesign (Precision Mastermix), DNAse and RNAse free water (CAS 7732-18-5; W4502) from Sigma, 96 qPCR transparent or white plates (MSP-9601) and transparent optical sealing tape (223-9444), CFX 96 thermal cycler from BioRad, Picodrop spectrophotometer and UV tips from Picodrop, UV light cabinet, 70 % isopropanol (IPA), ice.

ii. Method for qPCR

The material and DNase free water were set under the UV light for at least 18 min after the chamber surface had been wiped with 70 % IPA. The primers, SyBr Green mix and sample were kept on ice until use. A mixture of SyBr Green mix, primers and either sample or water was prepared freshly at every run inside the working chamber. To avoid contamination the sample was the last to be added. The plate was sealed and introduced into the qPCR machine and the run started. While the materials were UV treated the software had been set according to the run conditions. The final adopted conditions were a cycle of two steps (95 ºC for 10 min, 95 ºC for 15 sec, 60 ºC for 1 min), 50 µl of final volume, primers at 200 nM and in the first FluMag-SELEX iteration 50 cycles and 40 in all others.

iii. Method for qPCR libraries and primers

Two ssDNA libraries were tested. Each had specific fixed ends to allow primer annealing and a random sequence between the flanked regions. The first library was adapted from Nutiu et al (2005), (Nutiu, Li 2005). This was chosen because the

structure-switch was considered an interesting method, as explained previously. The second library was adapted from the original description of FluMag-SELEX, Table 4-16.

Table 4-16 Tested random ssDNA, adapted from published work.

Adapted from Nutiu et al (2005), (Nutiu, Li 2005)	5´-CCTGCCACGCTCCGCAAGCTT-(40)-TAAGCTTGGCACCCGCATCGT-3´
Adapted from Stoltenberg (2005), (Stoltenburg, Reinemann,C., Strehlitz,B. 2005)	5´-ATACCAGCTTATTCAATT-N(40)-AGATAGTAAGTGCAATCT -3´

The primers tested were the published ones and their modified versions. The modified versions were re-designed and analysed with free software from IDT technologies, (IDT 2013).

Table 4-17 Primer sets tested for random ssDNA libraries.

Forward primer	Reverse primer
5´-GCGGAGCGTGGCAGG -3´ 5´-FAM-ACGGAGTGTGGC -3´ 5´-FAM-AAGCTTGCGGAGCGTGGCAG G-3´	5´-ACGATGCGGGTGCCAAGCTTAr-3´ 5´-Poly A(20)-ACGATGCGTGTGTCAA -3´ 5´-Biotin-ACGATGCGTGTGTGCAAA- 3´ 5´-ACGATGCGGGTGCCAAGCTTA-3´
5´-FAM-ATACCAGCTTATTCAATT-3´	5´-PolyA(20)-HEGL-AGATTGCACTT ACTATCT -3´ 5´-Biotin-AGATTGCACTTACTATCT-3´

iv. Method for qPCR runs

Several running conditions were tested with the aim to obtain a working running assay. The qPCR chosen detection dye was SyBrGreen which was fluorescent at 524 nm, allowing the visualisation of dsDNA by intercalating it.

Table 4-18 Tested qPCR running conditions with random ssDNA libraries.

Performed assays	ssDNA library (adapted from Nutiu et al (2005), (Nutiu, Li 2005)			ssDNA library (adapted from Stoltenburg et al (2005))
	Primer set 1	Primer set 2	Primer set 3	Primers set I
Initial run mimicking references	✓	✓	✓	✓
Primers at different concentrations	✓	✓	✓	✓
Different Annealing temperature	✓	✓		✓
Calibration curve	✓	✓		✓
Agarose 2% gel	✓	✓		
Analyse of melting temperature products		✓		
Run at two or three steps		✓		

v. Methods for qPCR melting curve and products quality control

The calibration curve was only performed after having a running assay. The stock library was diluted and triplicates of each concentration were made. The melting curves were also run and analysed to detect the presence of primers dimers and variability of the sample. The melting curve was performed after the run and consisted in an increase of temperature from 65°C to 95°C in a short period of time (0.05 min).

b) Experimental and Results

i. Run of qPCR primers and chosen libraries

The libraries tested, Table 4-16, were random sequences with fixed sequences at their ends for primer annealing. The library chosen was adapted from Nutiu et al (2005) and the chosen primers set were the second pair. It was then tested the best annealing temperature. The temperature gradient was tested with the annealing temperature ranging from 59° to 69°C. The primers were at 200nM and the ssDNA library at 40 ng/µl. The melting curve presented products with melting temperatures of 77.0°C or 77.5°C, Figure 4-23 and Table 4-19.

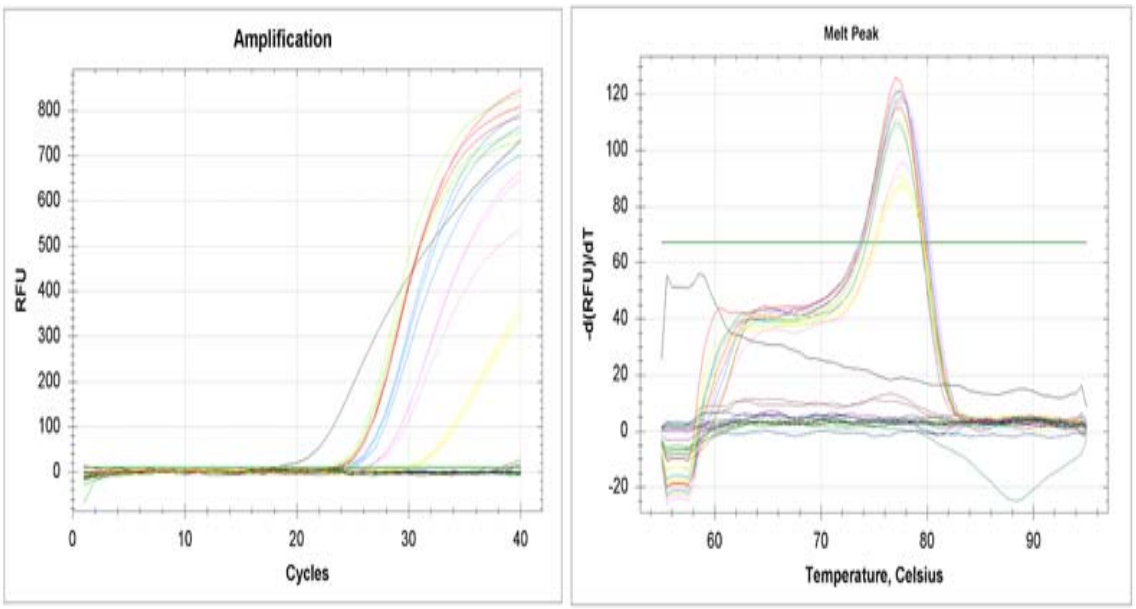


Figure 4-23 Temperature gradient, from 59°C to 69°C, for best primer annealing temperature.

The amplification curve showed amplification in all the samples with the exception of the negative controls and Ct values according to the initial ssDNA concentration. The melting curve confirmed that the nature of samples was the same and no primer dimers or other false positives were amplified.

Table 4-19 Average of Threshold cycles obtained at each annealing temperature.

Temperature	Ct Average	SD	CV%
69.0	n.a.		
68.5	n.a.		
67.4	38.78	0.84	2.17
65.4	29.86	0.48	1.60
63.0	26.27	0.09	0.35
61.0	24.50	0.50	2.03
59.7	23.59	0.20	0.87
59.0	23.25	0.26	1.12

The Ct obtained at the different temperatures allowed to choose the best annealing temperature versus the initial amplification starting point. It was chosen to use the standard 60 °C, even if it was not tested directly on that temperature range, it was seen that the obtained Ct at 59.7 °C was 23.59 which was accepted as desired.

ii. qPCR running conditions and calibration curve

The best running conditions were a hot start of 95ºC for 10 min, a denaturation step at 95ºC for 15 sec, the annealing and extension steps were made at 60ºC for 1 min, and the melting curve run from 55 to 95°C in 0.05 min. Afterwards a calibration curve was obtained. The calculation of reaction efficiency (E%) was made using the formula based on curve slop, Equation 4-2.

$$E\ (\%) = \left[10^{\left(\frac{-1}{m}\right)} - 1\right] \times 100$$

Equation 4-2 The calculation of qPCR amplification efficiency was depended on the obtained calibration curve slop.

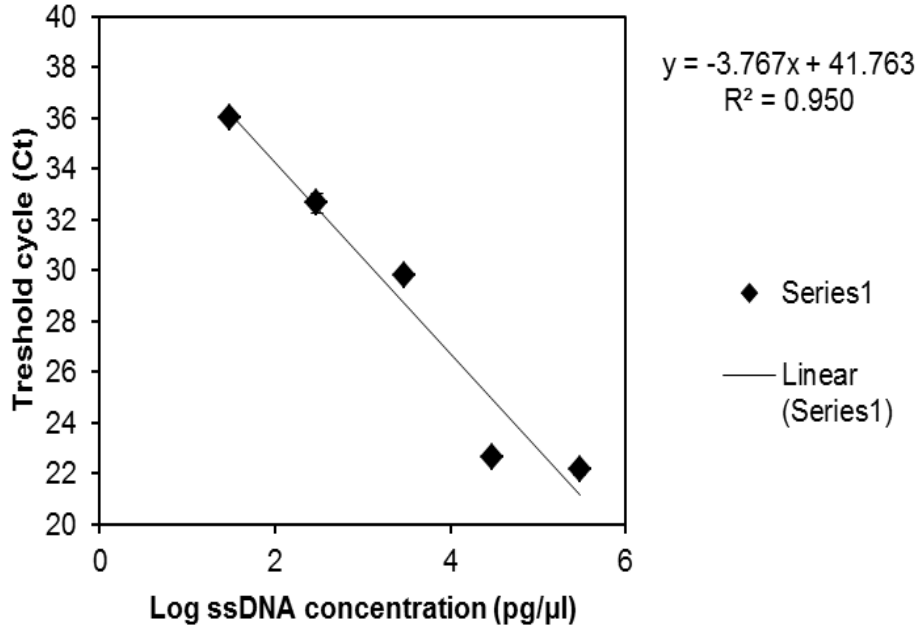


Figure 4-24 Calibration curve of ssDNA library diluted 1:10 using FAM-Fw primer and Poly A tail- Rw primer.

The obtained reaction efficiency was of 84.3%. That was not high as desired and required for the standard MIQE guidelines (above 90%), so a better calibration curve had to be obtained (Bustin, Benes et al. 2009).

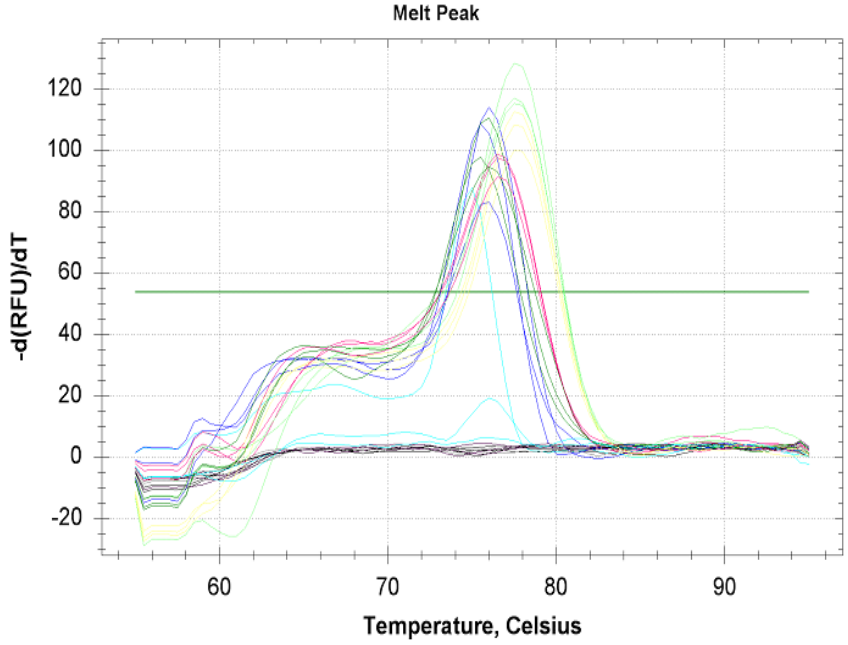


Figure 4-25 Melting curve obtained for calibration curve of ssDNA library diluted 1:10.

The obtained melting curve did not show any primer dimers as desired and confirmed the specificity of the amplified material.

A new calibration curve was made with a lower interval between concentrations, Figure 4-26.

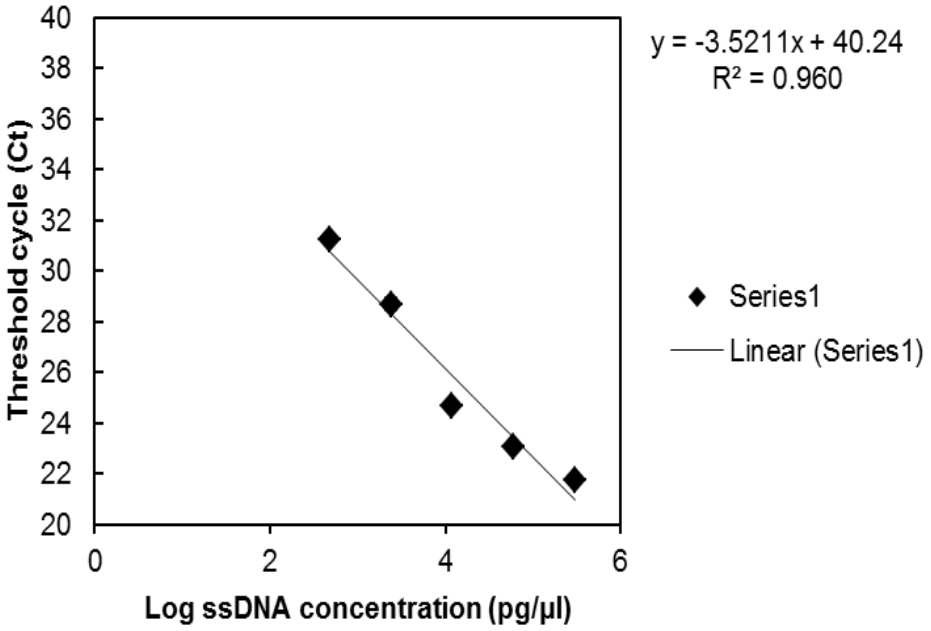


Figure 4-26 ssDNA library calibration curve diluted 1:5 using FAM-Fw primer and Poly A tail- Rw primer.

When testing a calibration curve using smaller dilution factors it was possible to obtain a reaction efficiency of 92.3%. This was under the required standards for qPCR work that required at least three orders of magnitude but did not satisfied the recommendation of representing at least 5 $\log_{10}$ concentrations, (Bustin, Benes et al. 2009). Nevertheless it was expected to cover the eluted working concentrations of ssDNA (obtained after target and library pool mixture).

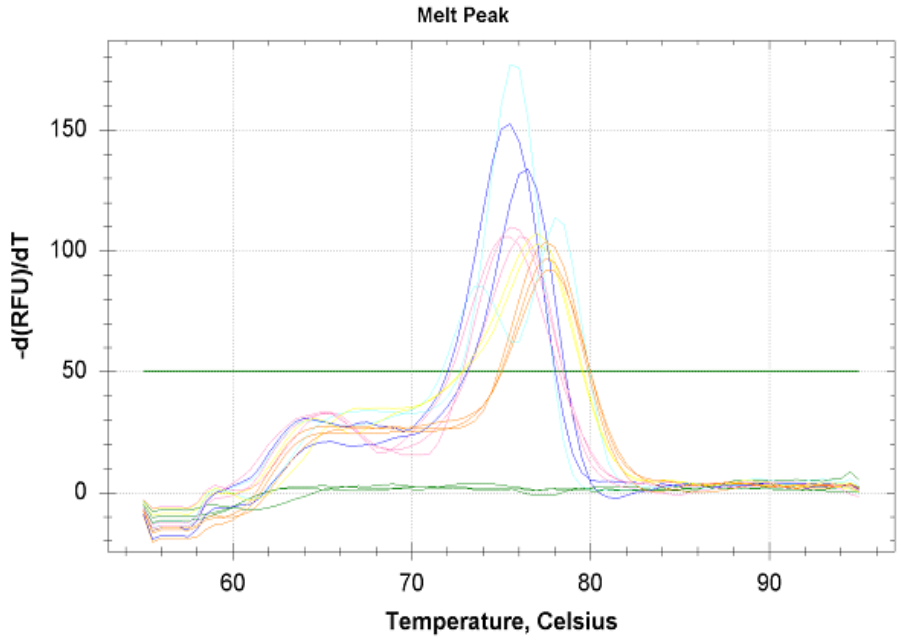


Figure 4-27 Melting curve obtained for the ssDNA calibration curve diluted 1:5.

Once more the obtained melting curve showed products specificity, but at lower degree than before showing that a higher number of different sequences were amplified. This was expected because the ssDNA library was rich in variability. It was not seen any primer dimers which was positive and confirmed that only the library was amplified.

c) Discussion

After testing two different libraries and primers sets, running conditions and finding the best annealing temperature for the primers set, it was obtained the best qPCR running conditions. Based on that, a calibration curve was obtained with ssDNA stock dilutions of 1:5 with a reaction efficiency of 92.3%.

Separation of positive ssDNA after qPCR amplification into dsDNA

The separation of the positive ssDNA after qPCR was made either using a denaturing gel were strands were separated by size, or the use of streptavidin coated Dynabeads® that captured the biotin modified strands.

a) Materials and Methods

i. Materials and chemicals

Streptavidin coated Dynabeads from Invitrogen, binding buffer (100mM NaCl, 20mM Tris-HCl, 2mM $MgCl_2$, 5mM KCl, 1mM CaCl2, 100mM NaOH, 5M NaCl, magnetic support from Invitrogen, 6% TBE-urea pre-cast acrylamide gels from Invitrogen

(EC68655BOX), TBE-urea loading buffer, running gel tank from Invitrogen, TBE running buffer from Invitrogen (155581-028), SyBr Green Safer view (G108) from abm, ethanol, 3 M sodium acetate.

ii. Method to use streptavidin coated magnetic beads (SA) for DNA strand separation

The protocol was adapted from Wochner et al (2007), (Wochner, Cech et al. 2007). It was noticed that there was no information about the volume to add of dsDNA to SA Dynabeads®, which were washed three times with 500 µl of Binding buffer. To 180 µl of qPCR product was added 45 µl of 5 M NaCl and mixed for 30 min at RT. The Dynabeads® were magnetically separated from the solution which was discarded. Three washes of 3 min each with agitation with 1 ml of Binding buffer were made. After the last wash and discarding the liquid, 200 µl of 100 mM NaOH was added, the Dynabeads® were agitated at different times and the liquid separated from the Dynabeads® and collected. To this 760µl of Binding buffer was added, and precipitation with ethanol was done. After it was re-suspended in 10 µl DNAse free and loaded into a 2% agarose gel or quantified with picodrop for method validation.

iii. Method of denaturing polyacrylamide gel (PAGE) for DNA strand separation

It was added to 5 µl of sample (negative strand was modified with HEGL-poly A tail in qPCR), 5 µl of TBE-urea loading buffer. The samples were denatured and loaded into the gel. It was tested as denaturing agents i) NaOH at 50 mM, temperature was tested for 3min at ii)70°C, and iii) 90°C and at iv) 95°C for 10 min. The gel was run at 180V. Afterwards was immersed in a SYBR green safe view staining bath and revealed under UV.

b) Experimental and Results

i. Use of streptavidin coated magnetic beads (SA)

The way to verify the separation efficiency was to use either an agarose gel or UV reading with picodrop. The picodrop was chosen because it was more sensitive and required less material than the gel, (Picodrop 2011). The addition of dsDNA to SA Dynabeads® was tested twice. The negative strand was modified with biotin via qPCR. It was necessary to test two times for NaOH actuation because it was not found information on the reference article, Table 4-20.

Table 4-20 Amount of dsDNA added to SA Dynabeads® and recovered ssDNA.

	Added dsDNA (ng/µl)			Recovered ssDNA (ng/µl)			
NaOH actuation time (min)	Average	SD	CV%		Average	SD	CV%
1.00	481.87	1.27	0.26	Samp. 1	n.a.	n.a.	n.a.
1.00	452.57	1.17	0.26	Samp. 1	2.57	0.42	16.22
				Samp. 2	n.a.	n.a.	n.a.
				Samp. 3	n.a.	n.a.	n.a.
5.00	427.93	1.92	0.45	Samp. 1	5.53	0.74	13.32
				Samp. 2	1.70	0.56	32.75

n.a.- not available

The recovered ssDNA was very low compared with the added concentration. This could have been caused by poor immobilisation, denaturation or recovery. It was repeated the assay but similar results were obtained. The technique was not performing as desired.

ii. Use of denaturing polyacrylamide gel conditions (PAGE)

It was tested a PAGE gel to obtain the positive strand of dsDNA after an efficient denaturation. The PAGE gel was run at 180V and the samples were denatured either by the action of NaOH or by temperature. The dsDNA was obtained from qPCR and the positive control from stock library. The results were exposed in Figure 4-28.

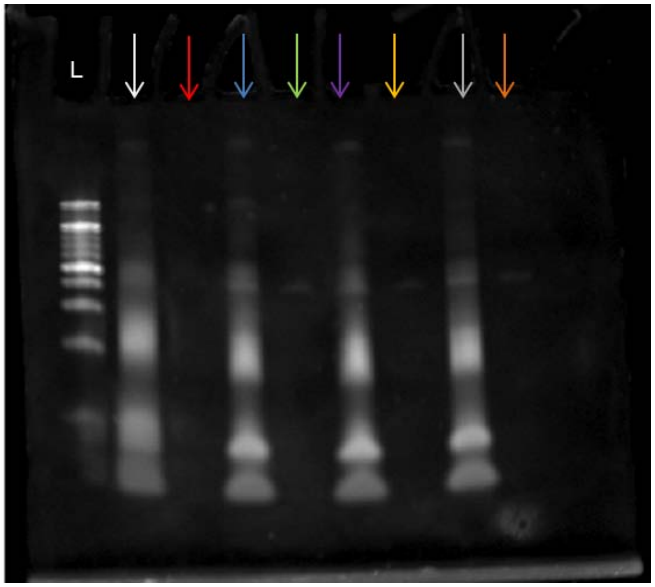


Figure 4-28 PAGE gel to test the best denaturing conditions of dsDNA, from left to right: ladder, dsDNA treated with 50 mM NaOH and positive control (white and red), dsDNA heated at 70 °C for 3 min and positive control (blue and green), dsDNA heated at 90 °C for 3 min and positive control (violet and yellow), dsDNA heated at 95°C for 10 min and positive control (grey and orange), negative control (not seen).

The PAGE gel showed first of all an overload of wells with excessive concentration of dsDNA and primers. Also it was seen sample degradation when treated with NaOH. The temperature treatments were gentler but only the higher temperature showed a sufficient separation between strands. Those conditions were adopted to denature the dsDNA before PAGE loading.

c) Discussion

The SA separation method was tested first by following the manufacturer protocol (not shown) and after being mentioned in several articles in aptamer generation, the protocol published by Wochner et al (2007) was tested. There were two major points that required more detail. The first was the concentration of dsDNA used, as the article only referred the volume added and the binding capacity of 100 pmol per 1.5 mg Dynabeads®, (Wochner, Menger et al. 2008, Wochner, Cech et al. 2007). The second point was the low detailed information about the use of the denaturing agent, sodium hydroxide, because no time of contact was mentioned. Also sodium acetate 3 M was used instead of sodium phosphate to neutralise and precipitate the ssDNA.

The inefficiency of SA separation might have been due to low time of denaturing NaOH incubation, inefficient modification during qPCR, or a mixture of both.

The PAGE gel presented degradation and also a high content of primers, as well as wells overloaded. Despite this it was chosen to adapt as separation process the use of PAGE with heating at 95 °C for 10 min as denaturing step. This was because it was the option where the separation and run looked neater as well as it was the better establish method. It was also decided to reduce the running voltage to 130 V (better separation) and to load less concentration of sample.

Detection and quantification of ssDNA to access quality during the iterative selection steps

The modification of the positive strand with fluorescein during qPCR allowed a visualisation in the PAGE, but also the quantification by fluorescence reading. This was considered important because it would allow quantifying the amount of ssDNA pool before each selection after iteration 1.

a) Materials and Methods

i. Materials and chemicals

Fluorescein salt, 18Ωcm water, eppendorf tubes, FAM modified forward primer, black microplate

ii. Method to detect ssDNA modified with fluorescein

A stock solution of fluorescein was made with water and from there several dilutions. Those were used to obtain a calibration curve. Three different concentrations of fluorescein modified primer were also diluted in water. 200 µl of each of the solutions was transferred to a black microplate and fluorescence was read at excitation wavelength of 485 nm and emission wavelength 535 nm.

b) Experimental and Results

i. Fluorescent detection of modified ssDNA smaller than 100bp

It was run a calibration curve with known concentration solutions of fluorescein and three solutions of FAM modified primer were tested and their concentrations extrapolated from the calibration curve. The assay showed possible to detect small oligonucleotides as primers and at concentrations as low as 4.8×10^{-3} µg/ml, Figure 4-29, Table 4-21.

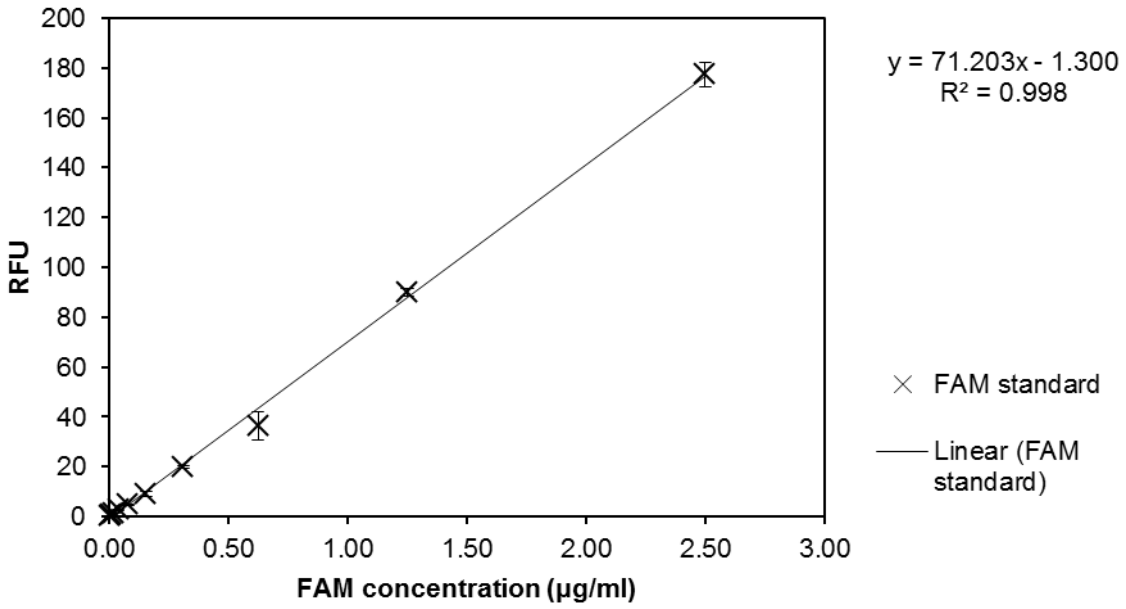


Figure 4-29 Fluorescein calibration curve for positive strand quantification.

Table 4-21 Extrapolation of unknown FAM primer concentrations obtained with qPCR.

	Abs	FAM primer concentration (µg/ml)
Solution 1	0.097004	5.607
Solution 2	0.332851	22.4
Solution 3	0.805724	56.07

c) Discussion

The calibration curve made from fluorescein salt was compatible to the quantification of modified strands of low molecular weight. The concentration of modified forward primer, used in the qPCR amplification, was obtained based on the made calibration curve extrapolation. It would be possible to use this curve to quantify before a new iteration the amount of positive strands present in the pool.

Summary and Conclusion

The FluMag-SELEX was an iterative method where each step needed to be tested and optimised. The amplification and separation steps were tested and optimised and the detection of modified fluorescein primers was assessed.

Here the amplification was modified into qPCR, which was introduced, as far as known, for the first time in the amplification step and proved to be a good alternative to

standard PCR, allowing eluted strands quantification and real-time quality control of amplified strands.

Two separation methods of ssDNA after qPCR were tested and PAGE was chosen because it was the most well-known method, and due to time constrains it was not possible to expand the test on SA Dynabeads® separation. This was supported after the initial tests made did not present an efficient separation using SA Dynabeads®.

Fluorescein shown that could be used as a quantifying agent because it was possible to verify the presence of fluorescein modified primers at different concentrations.

4.7.2. Initial Aptamer development using the FluMag SELEX method

Aptamer generation was attempted, due to the problems found in implementing the method it was not possible to generate aptamers within the LMC timeframe. It was possible to understand and study in detail each step of the FluMag-SELEX, to introduce novelty in the amplification step and to test a couple of iterations for both the LMC target derivatives.

As the confirmation of target derivatives immobilisation took longer than expected, and in order to test the method, it was run two iterations of FluMag-SELEX against L-cysteine. The target was assumed immobilised and the iterations implemented. Those two iterations showed a few fragile points of the method. After the implementation of XPS it was possible to confirm that L-cysteine was not surface immobilised so the aptamer generation was abandoned and new iterations against 5β-cholanic acid were made. Again problems with the method were found which lead to the impossibility of generating aptamers within the time frame.

FluMag SELEX iterative rounds

a) Materials and Methods

i. Materials and chemicals

Binding buffer, Extraction buffer (500 mM ammonium acetate, 0.1 % SDS, 0.1 mM EDTA), DNAse free water, qPCR material and chemicals, heating plate, rotor, magnetic support, pre-cast PAGE gels 6 % urea from Invitrogen, gel cast from Invitrogen, TBE running buffer from Invitrogen and TBE-urea loading buffer, in house immobilised Dynabeads®, ssDNA library from Invitrogen, low-binding Eppendorff 1.5 ml tubes.

ii. Method of aptamer generation with FluMag-SELEX

Iterative steps: The immobilised Dynabeads® were washed three times with 500 µl Binding buffer, mixed with the prepared ssDNA and incubated at RT for 30 min with gentle agitation. 3 nmol (~$1x10^{15}$) of ssDNA was prepared by heat at 90 ºC for 10 min and cooled on ice, kept at 4 ºC for 15 min and at RT for 8 min (Stoltenburg, Reinemann et al. 2007) After the Dynabeads® were separated from the liquid that was discarded and washed five times with Binding buffer (500 µl), afterwards were re-suspended in DNAse-free water (100 µl).

Elution: The Dynabeads® in DNAse-free water were heated at 80ºC for 10 min, being agitated a couple of times during that time. After were placed on the magnetic support and the liquid collected into a low-binding eppendorf tube. 100 µl of DNAse free water was added to the Dynabeads® and the procedure was repeated. The total of elution steps were three.

Amplification by qPCR: The final volume of each elution was 100 µl, which was divided in five parallel qPCR reactions to a final volume of 50 µl. The qPCR run for 50 cycles in the first iteration and 40 in the rest in order to prevent material loss. The conditions were detailed elsewhere in this document.

Precipitation of dsDNA: To each qPCR product solution was added two times its volume in ethanol, 40 µl of sodium acetate and 5 µl of linear acrylamide. Kept at -20ºC for 1hour and centrifuged at the highest G force (either 21.100 or 15.200 rpm, depending of the centrifuge). The liquid was discarded, let air dry for a couple of minutes and the pellet was re-suspend in 5 µl of DNAse-free water. Or instead of precipitation the qPCR products were clean with mini-elute kit (28004) from Qiagen.

ssDNA separation by PAGE gel: To each tube from the previous step 5 µl of TBE-urea loading buffer was added. The samples were heated at 95 ºC, in the two initial rounds for 10 min and later for 15 min, as it showed necessary to maintain the denaturation, and were kept immediately on ice. Each sample was loaded on the gel as well as a DNA ladder. The gel was run at 130 V. After the gel was stained with SYBR green bath for 30 min, and rinsed with DI water. The gel was visualised under short wave UV light (λ 280 nm) and also under blue light filter in order to visualise specifically the fluorescein modified positive strands. The pictures of the gel were saved and the gel wrapped with transparent film and set under the horizontal UV lamp. The bands with the fluorescein were marked and excised from the gel. To each gel extracted band it

was add 100 µl of Elution buffer, the gel was crushed and left overnight at RT with agitation (140 rpm).

Recovery of the selected ssDNA: The liquid was removed from the crushed gel and precipitation of the ssDNA was done as described previously. Each ssDNA eluted band was re-suspended in 10 µl of DNAse-free water. One sample was kept at -20 ºC and the others used for the next iterative round.

b) Experimental and Results

i. Initial L-cysteine aptamer generation

Previous to confirmation of Dynabeads® immobilisation, it was assumed the immobilisation of L-cysteine to test the FluMag-SELEX. The amplification of the selected and eluted strands with qPCR was made for iteration 1. From the obtained Ct values it was extrapolated the amount of ssDNA present in each elution solution. Only Ct values from 30 to 36 were considered for calculations (Ct values below 30 were not seen), and the calibration curve with efficiency of 92.3 % was used, see Figure 4-26 and Table 4-22.

Table 4-22 Extrapolation of eluted ssDNA of aptamer generation against L-cysteine, iteration 1.

	Ct average (y)	SD	CV%	y = -3.5211x +40.24	Eluted ssDNA (pg/µl)
Elution 1	34.89	0.41	1.16	1.52	$3.31E^{+01}$
Elution 2	34.88	0.72	2.06	1.52	$3.33E^{+01}$
Elution 3	35.14	1.18	3.36	1.45	$2.81E^{+01}$

The qPCR melting curve showed primer-dimers production in the latest cycles; also the positive control presented a Ct average of 23.5 which was equivalent to ~57.0 ng/µl. The considered eluted ssDNA melting temperatures ranged from 77.0 °C to 78.0 °C.

Afterwards the samples were precipitated and run in a PAGE gel. The presence of primer-dimers and some contamination of the negative control were visible. Also the loading of the positive control did not occurred as expected and it was lost in the run. Despite this the ssDNA separation occurred and instead of two bands it was seen three. This indicated a poor denaturation, also the gel wells were overload, Figure 4-30.

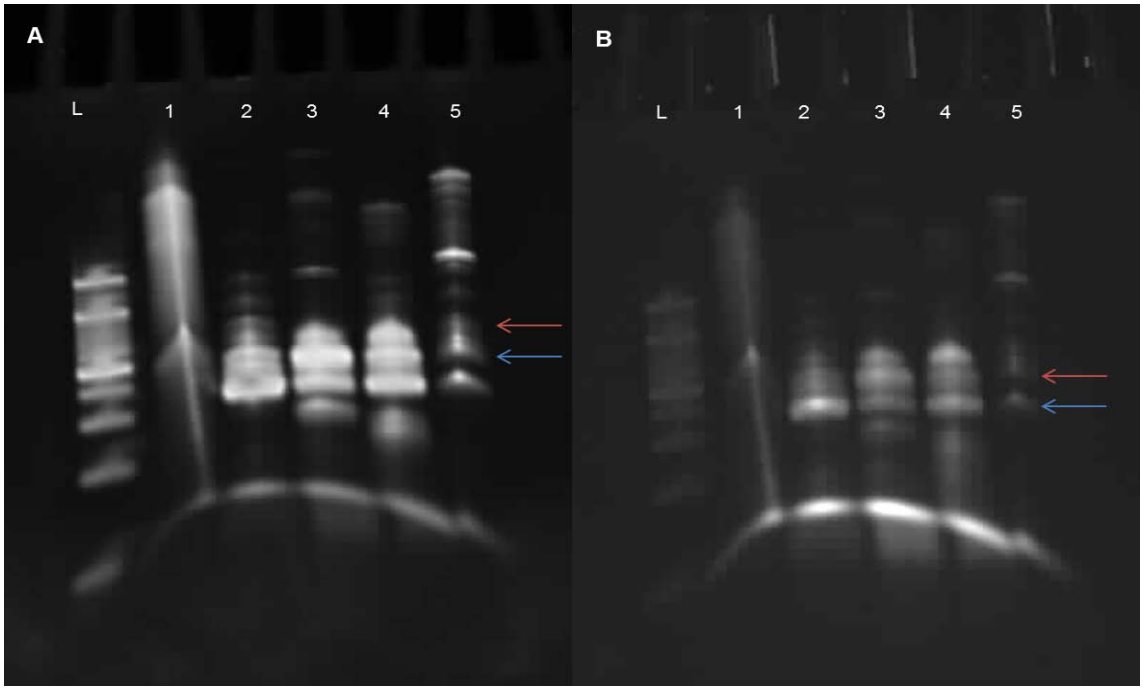


Figure 4-30 PAGE gel for separation of ssDNA after FluMag-SELEX round 1 for L-cysteine, from left to right: ladder, positive control, elution 1, elution 2, elution 3, negative control. A) PAGE gel under UV short wavelength and B) Gel under blue filter for FAM visualisation.

It was possible to visualise the presence of the positive strand via the fluorescein visual detection. That showed that the positive strand was present in three bands, one that should have been the double strand, the other with the majority of the band being the modified Rw primer and the third the band of interest with only positive strands. That strand was recovered and the rest of the material lost. The eluted material was taken into the second iteration.

The second iteration was performed with the obtained material from the previous round. Selection, elution, precipitation and amplification was made, the amplified material was calculated as before, Table 4-23.

Table 4-23 Extrapolation of eluted ssDNA of aptamer generation against L-cysteine, iteration 2.

	Ct average (y)	SD	CV%	y = -3.5211x +40.24	Eluted ssDNA (pg/µl)
Elution 1	17.21	3.54	20.55	6.54	$3.48E^{+06}$
Elution 2	22.05	8.59	38.96	5.17	$1.47E^{+05}$
Elution 3	11.96	2.00	16.73	8.03	$1.08E^{+08}$

It was seen a decrease in ssDNA according to the elution number and varied from $3.48E^{+06}$ to $1.08E^{+08}$ pg/µl.

The qPCR melting curve did not show primer-dimers and the positive control presented a Ct average of 25.6 which was equivalent to ~15.0 ng/µl. The considered eluted ssDNA melting temperatures ranged from 78.0 °C to 78.5 °C.

The PAGE gel presented contamination of the negative control but also showed a better separation between strands, being possible to observe also three bands, confirmed by gel visualisation under a blue light filter (not shown), Figure 4-31.

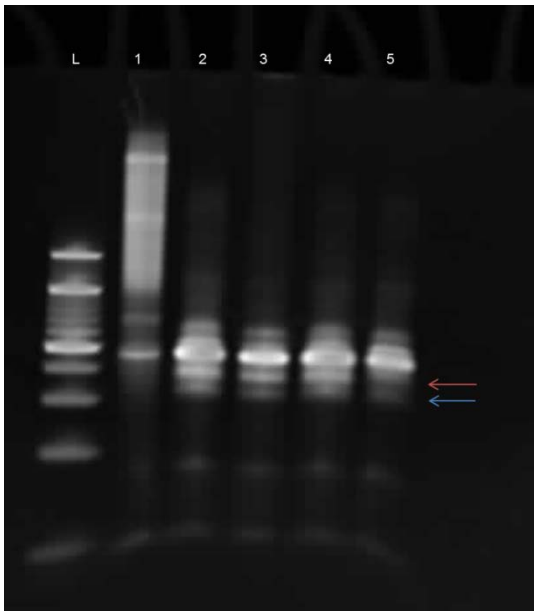


Figure 4-31 PAGE gel for separation of ssDNA after FluMag-SELEX round 2 for L-cysteine, from left to right: ladder, positive control, elution 1, elution 2, elution 3, negative control. PAGE gel seen under UV short wavelength.

The positive control confirmed that the initial band (band with higher content) was the double strand DNA again the denaturation was not as efficient as necessary. The three eluted and amplified samples presented two more bands, which were the negative band and the positive band. The situation in this case was that the negative control

presented library contamination and because of that it was not possible to continue the iterations. This was because if library contamination was present in the negative control, it meant that either it was from the qPCR samples evaporation and condensation due to poor plate sealing, or one of the chemicals was contaminated with library and it was added initial library to the refined material. In any case the generation was stopped.

ii. Initial 5β-cholanic acid aptamer generation

After confirmation of 5β-cholanic acid surface immobilisation, the aptamer selection was attempted. The initial concentration of ssDNA added to the Dynabeads® had an average of 393.77 ng/µl, a standard deviation of 2.75, and a coefficient of variation of 0.70 %.

The obtained material after selection, elution and amplification was described in Table 4-24.

Table 4-24 Extrapolation of eluted ssDNA of aptamer generation against 5β-cholanic acid, iteration 1.

	Ct average (y)	SD	CV%	y = -3.5211x +40.24	Eluted ssDNA (pg/µl)
Elution 1	22.96	1.43	6.21	4.91	$8.07E^{+04}$
Elution 2	20.07	10.99	54.74	5.73	$5.36E^{+05}$
Elution 3	30.09	1.25	4.16	2.88	$7.65E^{+02}$

The eluted material was quantified from $8.07E^{+04}$ to $7.65E^{+02}$ pg/µl. It was seen a decrease in eluted material according to the elution number.

The qPCR melting curve presented a small presence of primer-dimers. The positive control had a Ct average of 21.8 which was equivalent to ~15.0 ng/µl. The considered eluted ssDNA melting temperatures ranged from 78.0 °C to 78.5 °C. The dsDNA precipitation added a very high salt content which lead to the samples lost. Two new initial rounds were tested and similar problems were found. That time the PAGE extraction step added a high content of salts that were not completely removed which lead also to loss of sample from iteration to iteration. It was decided to use every time precipitation that had to be done to clean the DNA from salt content, a di-salting membrane, a mini-elute kit (28004).

After all the samples lost because of salt precipitation, new iteration was tested, Table 4-25.

Table 4-25 Extrapolation of eluted ssDNA of aptamer generation against 5β-cholanic acid, iteration 1.

	Ct average (y)	SD	CV%	y = -3.5211x +40.24	Eluted ssDNA (pg/µl)
Elution 1	30.68	0.84	2.73	2.72	$5.19E^{+02}$
Elution 2	26.59	0.99	3.74	3.88	$7.55E^{+03}$
Elution 3	30.12	2.22	7.36	2.88	$7.51E^{+02}$

The obtained values of ssDNA were lower than before, with the highest concentration of $7.55E^{+03}$ pg/µl. The qPCR melting curve presented two samples with primer-dimers but no melting peak between 70 °C and 80 °C. The considered eluted ssDNA melting temperatures ranged from 75.5 °C to 78.5 °C. The positive control presented a Ct average of 20.1 which was equivalent to ~535.0 ng/µl.

The PAGE gel presented several bands both on the eluted samples and on the negative control. It did not show a clear band or a better separation between strands of interest. The positive control was not very clear. One of the three eluted samples presented a similar band to the positive control, Figure 4-32.

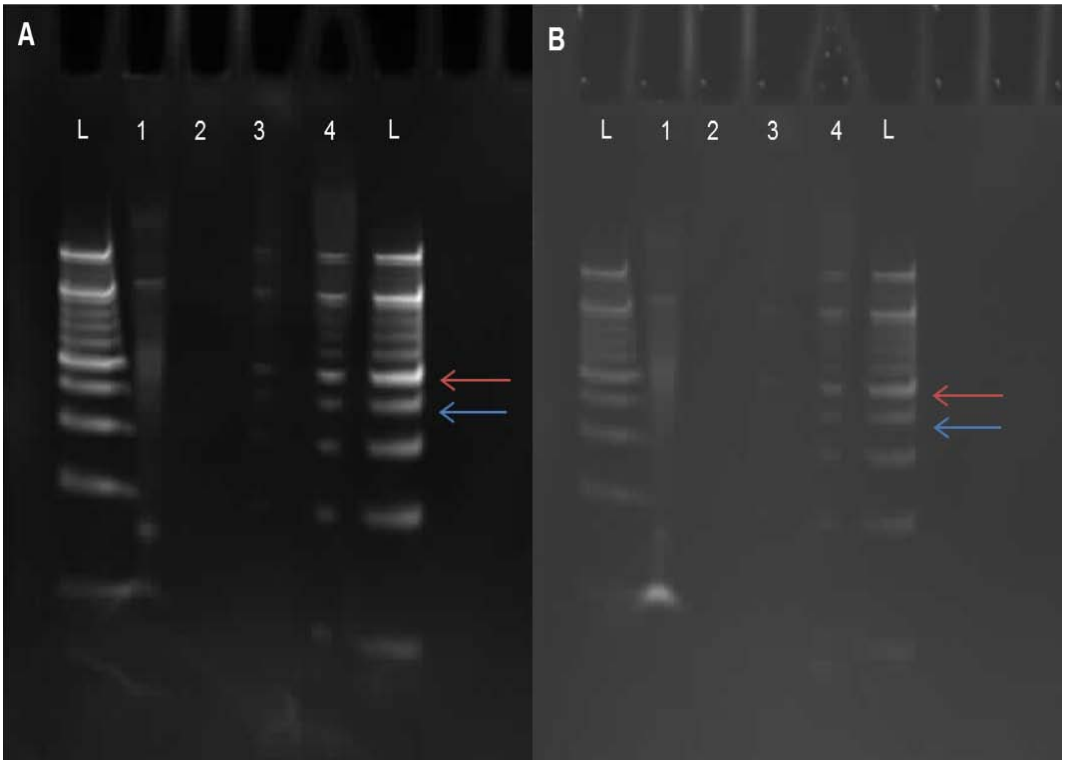


Figure 4-32 PAGE gel for separation of ssDNA after FluMag-SELEX round 1 for 5β-cholanic acid, from left to right: ladder, positive control, elution 1, elution 2, elution 3, negative control and ladder. A) PAGE gel under UV short wavelength and B) Gel under blue filter for FAM visualisation.

Another three more iterations were made from the obtained material from PAGE but it was clear that because of this PAGE gel low quality separation the following iterations did not work and only artefacts were amplified.

In a last attempt to generate aptamers using the FluMag-SELEX, ibuprofen was used as a positive control, and the target 5β-cholanic acid Table 4-26.

Table 4-26 Extrapolation of eluted ssDNA of aptamer generation against 5β-cholanic acid and Ibuprofen as positive control, iteration 1.

5β-cholanic acid	Ct average (y)	SD	CV%	y = -3.5211x +40.24	Eluted ssDNA (pg/µl)
Elution 1	24.90	0.30	1.22	4.36	$2.28E^{+04}$
Elution 2	25.10	0.12	0.47	4.30	$1.99E^{+04}$
Elution 3	25.08	0.53	2.12	4.30	$2.02E^{+04}$
Ibuprofen	Ct average (y)	SD	CV%	y = -3.5211x +40.24	Eluted ssDNA (pg/µl)
Elution 1	25.83	0.09	0.36	4.09	$1.24E^{+04}$
Elution 2	25.78	0.15	0.59	4.11	$1.28E^{+04}$
Elution 3	26.73	2.28	8.53	3.84	$6.86E^{+03}$

The highest concentration eluted for the 5β-cholanic acid was $2.28E^{+04}$ pg/µl, which was higher than in the previous attempt. The highest concentration for ibuprofen was $6.86E^{+03}$ pg/µl. It was not understood if this showed an initial better affinity to ibuprofen than to 5β-cholanic acid because there were no more iterations with the two. The obtained qPCR melting curve presented primer-dimers in all samples with the exception of the positive control. The positive control presented a Ct average of 22.7 which was equivalent to ~96.4 ng/µl.

The PAGE gel did not show a band at the expected length so it was discarded and the iteration abandoned.

c) Discussion

The initial application of FluMag-SELEX was made assuming that L-cysteine was efficiently surface immobilised. After two iterations there was qPCR contamination and aptamer generation was abandoned after confirmation that the target was not immobilised. It was pursued the aptamer generation against 5β-cholanic acid because it was confirmed surface immobilisation and was the other chosen LMC target derivative to test aptamer generation against. The implementation of the method was very changeling and was impossible to perform a full generation cycle. This occurred because of problems as salts accumulation, sample degradation, contamination and

ssDNA loss after PAGE. That lead to the understanding of the critical stages of FluMag-SELEX that prevented the full cycle of aptamer generation.

Summary and Conclusion

The FluMag-SELEX was tested initially against L-cysteine. It was assumed target immobilisation because of time constrains. It was possible to perform two iterations before qPCR contamination. The method was abandon and once target immobilisation was confirmed the generation was tested against 5β-cholanic acid. Several iterations were attempted but in each were found problems, either increase of salt concentration, sample loss in PAGE elution or qPCR contamination. Due to these setbacks it was concluded that an aptamer generation cycle against the chosen targets was not possible to perform under the LMC project timeline.

4.8. Discussion to identify if it was possible to generate Aptamers against the LMC small targets and to identify adequate technique (s) to confirm beads immobilisation of the LMC targets

The LMC targets derivatives and spacers were, with the exception of L-cysteine, hydrophobic. The use of Dynabeads® magnetic properties allowed to change buffer solutions from hydrophobic to aqueous and vice-versa. The best conditions for 5β-cholanic acid immobilisation were using a solution of DMSO and for the L-cysteine immobilisation a mixture of DMSO and PBS. The chosen chemistry (EDC/NHS) to form a carbodiimide between 5β-cholanic acid carboxylic acid and an amine proved to work and promote surface immobilisation. The same was not found for the L-cysteine immobilisation via a maleimide and an ester. Unfortunately there was no time to search for a reason, but because it was pH dependent it might have not been efficiently stable or the oxidation of L-cysteine prevented the coupling.

The confirmation of immobilisation was a critical step because of the simple nature of the derivatives. Also to avoid the risk of generating aptamers against Dynabeads® and because only one example was found in literature where immobilisation was confirmed before aptamer generation using the FluMag-SELEX, (Kim, Hyun et al. 2010).

It was found that immobilisation confirmation was complex and that traditional analytical chemistry techniques were not suitable to confirm immobilisation either because of the target derivative or the surface characteristics.

Also the decision of not using chemical derivatisation, as that would go against the wanted desire of finding a routine technique which would be ideally simple and fast to perform, made even more difficult the detection of simple molecules as the LMC derivatives. This was particularly important for the use of HPLC as it was found that derivatisation was a common way of quantifying fatty acids and simple molecules, (Corradini, Philips 2011), also it was found that liquid and gas chromatography were used with mass spectroscopy (LC/MS and GC/MS), instead of HPLC, for detection of carboxylic acids, (Johnson 2005, Fallon, Booth et al. 1987).

To add to this, the fact that Dynabeads® had a core of iron for their magnetic properties and that were present in solution despite the fact of being the solid phase, made direct detection impossible using spectrophotometry due to light scattering. This was a problem especially in the case where the colorimetric or florescent product was attached to the bead as with TNBS, Fluorescamine and OPA.

Also easy contamination with detergents primary amines, in the case of fluorescent detection with OPA and fluorescamine was another obstacle that made them not suitable for side-detection techniques.

Hinterwirth et al (2010) presented a review on techniques used for primary amines detection onto surfaces. It mentioned three of the tested chemicals, TNBS, OPA, and Ninhydrin. The authors presented similar results for TNBS which was also considered not as sensitive as required. For the OPA test, the detection after the reaction was different, and instead of reading the fluorescence in solution, the authors observed the immobilised glass beads under a fluorescent microscope. This could be possibly one of the reasons why the detection did not occur in this case. The same authors mentioned that ninhydrin was not appropriated for quantification assays. They claimed that the reagent was soluble and in that way could react with primary amines present in solution, (Hinterwirth, Strobl et al. 2010). But in this case the Dynabeads® were washed previously, and as seen with HPLC in the case of ibuprofen, they seemed to be sufficiently efficient to reduce the primary amines presence. Nevertheless there could have been some effect that could only have been studied in detail if L-cysteine had been immobilised or a positive control had been present for data comparison.

Another considered point was that the proportions of the Dynabead itself compared with the thin layer of immobilised biomolecules onto its surface could have obstructed detection techniques as IR and FTIR. Despite this hypothesis it was not expected because it was found previous work were surface modification was accessed with FTIR, (Panek, Pietrow et al. 2012). A similar result was seen in the Dynabeads® elemental analysis.

Later was also noticed that due to resources constrains it was analysed by IR, FIRT and elemental analysis only L-cysteine immobilisation. It was found with XPS analysis that the immobilisation was not efficient in that case, which lead to the discussion that, in special these techniques, could work but due to the lack of a positive control it was not possible to understand if the inadequacy was of the technique or the sample.

The XPS analysis was a direct detection by surface atomic analysis and in that way it was more reliable and easy to use than the previous colorimetric methods; however there were also some problems with XPS. The sample analysis was sensitive to oxidation, so it was important to minimise the time from immobilisation to analysis (Fairley 2009). Also as the samples needed to be dry and grinded it was difficult to understand the impact of the material from which Dynabeads® were made of in the analysis. Again it was trusted that the washes performed after immobilisation were sufficient to remove the majority of unbound compounds. XPS allowed quantification of each element compound in the sample but it was difficult to take elations about the concentration immobilised due to the fact that unmodified Dynabeads® versus modified presented the same compounds but at different percentages. XPS proved to be the most adequate side-technique from all the tested ones to verify Dynabeads® immobilisation for the FluMag-SELEX.

Despite the fact that Dynabeads® interfered in some of the detection methods tested, their use allowed the change between matrix buffers required by the target derivatives and spacers that were either hydrophobic or hydrophilic. Also by being in solution, while working as a surface, was considered best for interaction between immobilised target and ssDNA library, (Baumann 2010).

The application of the FluMag-SELEX proved to required optimisation and familiarisation with each iterative step prior to aptamer generation. In that way it was possible to understand the critical steps and the need of quantification or at least confirmation of the presence of the selected ssDNA at the several steps. Also it was

found that the separation of the positive strand after amplification was critical and lead to loss and possible degradation of ssDNA. Another issue was the need to clean efficiently the ssDNA between rounds to avoid the build-up of salts that interfered with the conformation and amplification, (BioRad 2006, Baumann 2010).

It was found that qPCR was a good way of verifying the amount of ssDNA selected at each round and also to confirm the presence of contaminants or integrity of the sample. The best reaction efficiency obtained was 92.3 %, it was considered for quality control and quantification of selected ssDNA of each iteration.

The FluMag-SELEX, once adapted to the targets, with optimised iterative steps, and probably with a well-known library seemed to be a reliable method. Because of time and resources limitations it was not possible to verify if aptamers against the LMC derivatives would be generated using this method.

4.9. Summary and Conclusions

The chosen method to test aptamer generation was the FluMag-SELEX because of the use of Dynabeads® allowed target-ssDNA interaction in solution and magnetic partition.

This method required the target surface immobilisation into Dynabeads®. Because of the simple chemical structure of the chosen LMC targets to test aptamer generation, L-alanine and coprostane, it was necessary to use their derivatives L-cysteine and 5β-cholanic acid. In order to prevent or minimise steric hindrance it was added a spacer between the Dynabead surface and the target derivative. 5β-cholanic acid was reacted with carboxyl Dynabeads® via the spacer cadaverine. The reaction chemistry was EDC/NHS based. The L-cysteine was reacted to amino Dynabeads® via the spacer EMCS using pH changes.

It was found that there was a need to confirm Dynabeads® immobilisation via a reliable side-technique. Several analytical techniques were tested in order to confirm immobilisation, but in the majority of the cases the nature of the LMC target derivatives and the composition of Dynabeads® proved incompatible with the detection methods. It was found that for a direct immobilisation and in the presence of primary amines, ninhydrin was appropriated for a simple diagnose. It was found that the appropriated technique to determine the surface immobilisation of Dynabeads® to the LMC targets derivatives was XPS. This technique allowed confirming the presence of new atomic

compounds, to quantify them and to understand their neighbourhood arrangement. The several analytical techniques tested were resumed in Figure 4-33.

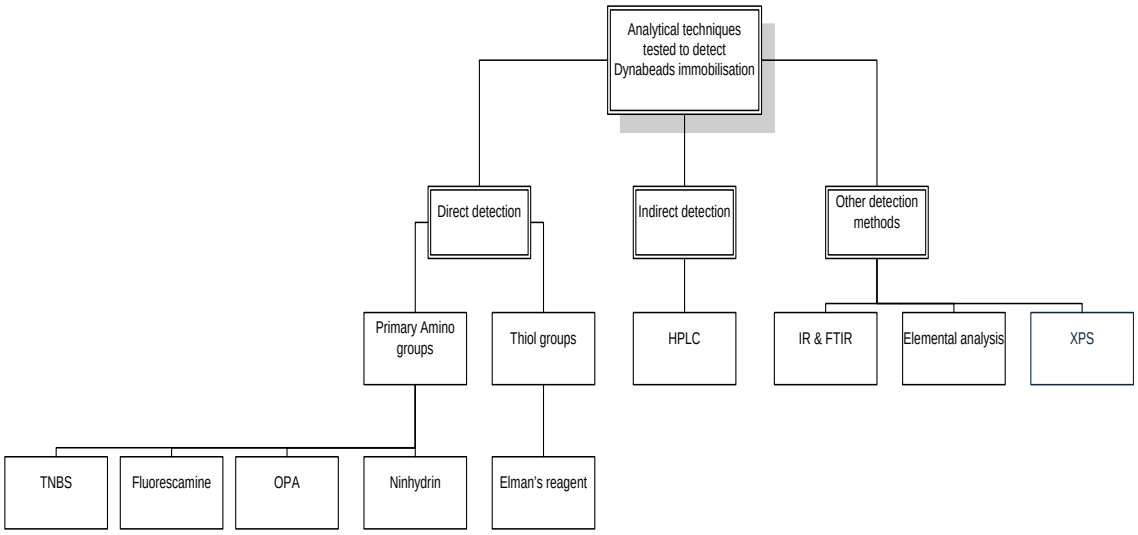


Figure 4-33 Diagram with all the analytical techniques tested to determinate Dynabeads® immobilisation of L-cysteine or 5β-cholanic acid.

Two major steps of the iterative aptamer generation method were studied and one of them modified. The amplification step was performed with qPCR instead of the traditional PCR to allow real time monitoring, quantification of eluted material in each round and an idea of the sample variability. It was achieved a calibration curve with an efficiency of 92.3 %. qPCR was assumed a better technique then the standard PCR because it gave the possibility to quantify the amount of eluted material in each round and to access its quality without the use of agarose gels, preventing material loss.

The second step tested was the positive ssDNA separation procedure in which the standard method, denaturing gel, was tested versus a less used method, the streptavidin coated Dynabeads®. It was found that both the techniques had negative points and that the denaturing gel was the less time consuming to optimise because it was better known.

The FluMag-SELEX showed to have specific problems in its iterative steps. The risk of salts accumulation and loss of material due to de-salt washes and purification at the end of each round was a problem. Another point was the need of ssDNA correct folding before interacting with the target. This was influenced by ion content in the binding solution and their strength, and also how the ssDNA reacted to the denaturing

conditions applied in the previous round. This was impossible to monitor and it was considered that it was part of the selection process. In summary Figure 4-34 represents the tested steps in the FluMag-SELEX.

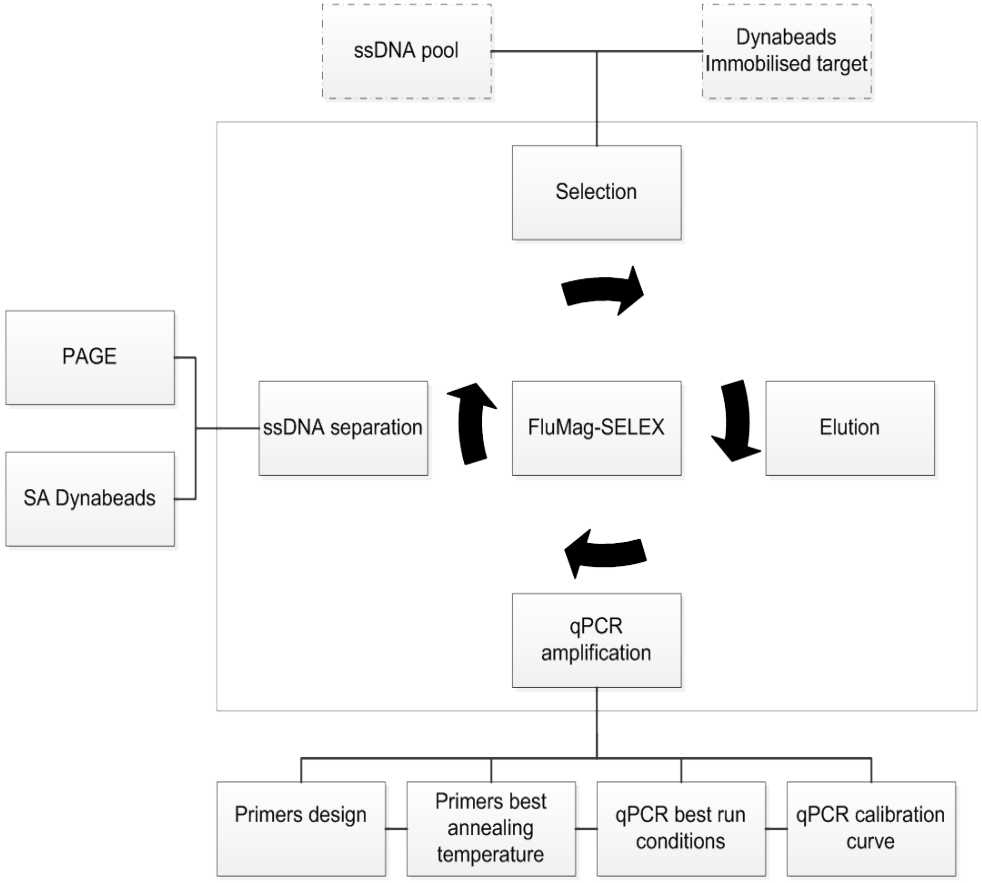


Figure 4-34 FluMag-SELEX diagram with tested steps.

In conclusion it was found that the route forward to follow regarding aptamer generation against LMC target derivatives applying the FluMag-SELEX would require the use of XPS to confirm target immobilisation. The molecular size, simplicity and hydrophobicity made LMC target derivatives difficult to solubilise, very un-reactive and undetectable using simple analytical standard methods. qPCR was an useful tool and should be used for quality control and quantification of eluted strands in each iteration. The separation of the positive ssDNA after amplification was a critical step and a source of material loss. Ideally the best method would be one that would allow a clear separation with the less strands damage and loss possible. Also the use of fluorescein and its quantification might be used in the future for ssDNA characterisation as it was not possible to go further than the detection of primer concentrations.

Considering the time frame and resources available to test the aptamer generation against LMC target derivatives, it was not achieved a complete cycle of FluMag-SELEX and for this reason it was not possible to test the aptamer generation as initially aimed, but all the steps of the iteration were optimised and problems within the method pointed out for future work.

Chapter 5. Can existing aptamers against small molecules be integrated into LMC type assays?

5.1. Chapter Introduction

The LMC assay, as explained in detail in section 2.4.1, was an inhibition ELISA assay type. The target derivatives were surface immobilised and the detection was made via optical detection of labelled bio-receptors.

For simplification during the development of the LMC, an inhibition ELISA assay was used with antibodies as bio-receptors and a colorimetric enzymatic detection system. This work aimed to test aptamers as bio-receptors for the LMC as alternatives to antibodies[4]. Aptamers were tested in simple models that could evolve to the final assay type if results and resources would allow.

Aptamers were tested in systems with surface immobilisation as required for the LMC, and in both labelled or label free detection systems. The label free system was used only because it allowed real time monitoring which gave a step-by-step understanding of the assay and an understanding of the association/ dissociation constants between the target and the aptamer.

Ideally these tests would have been made with aptamers against LMC targets, but as seen in the previous chapter (Chapter 4) LMC aptamers generation was not achieved. In order to overcome that problem, literature search for already existing aptamers against small molecules that could be studied under the LMC assay conditions was conducted. It was anticipated that any demonstration would be applicable to the future availability of LMC target specific aptamers.

The work developed in this chapter consisted of testing three different published small molecule targets and their aptamers into the LMC assay and similar assay formats. Based on this, it was impossible to have a full working LMC type assay against small molecules that could mimic LMC targets using aptamers due to the existence of several parameters that were found to need further research. In order to understand better the LMC assay format compatibility with aptamers, a protein target and its

[4] When using aptamers instead of antibodies the assay was called enzyme linked aptamer assay (ELAA).

aptamers were tested in LMC similar assay formats. Based on the work developed conclusions and recommendations about aptamer implementation in the LMC assay were made.

5.2. Literature review of existing aptamers against small molecules and their use in LMC relevant assays

5.2.1. Review of aptamers against small molecules

Aptamers against small molecules were reviewed in detail in section 2.5.2 (Chapter 2). There were aptamers against a wide range of small molecules, which were mainly hydrophilic, and presented biochemical properties as aromatic rings or specific functional groups or elements. For this work it was chosen to work with DNA aptamers because they were less sensitive to enzymatic degradation than RNA aptamers.

Several reviews were made about aptamers against small molecules, but those included RNA and DNA aptamers and all types of low molecular weight targets. When restricting the search to DNA aptamers that recognised and bound molecules with similar physiochemical properties to the LMC targets, only ten examples were found. Those targets were ibuprofen, (Kim, Hyun et al. 2010), estradiol, (Kim, Jung et al. 2007), ochratoxin A, (Prieto-Simon, Campas et al. 2008), cholic acid, (Kato, Takemura et al. 2000), ethanolamine,(Mann, Reinemann et al. 2005), acetamiprid, (He, Liu et al. 2011), anthracyclines (daunomycin and doxorubicin),(Wochner, Menger et al. 2008), several amino-acids, as L-tryptophan,(Yang, Bing et al. 2011), diclofenac (Joeng, Niazi et al. 2009), and bisphenol A, (Jo, Ahn et al. 2011).

5.2.2. Impact of pH, ions content, chemical modification and surface immobilisation on aptamer conformation and small target binding

The interaction between aptamers and small molecules at the molecular level was not well known and only a few studied examples were found. The aptamers and targets interactions were studied with X-ray crystallography or nuclear magnetic resonance (NMR).

Hermann and Patel (2000) reviewed the studied aptamer interaction with their targets. The authors demonstrated that aptamers usually adopted a specific binding folding

only in the presence of their target, and that in the cases of higher affinity, the target was involved in the aptamer structure by complementing it. It was also stated that the type of interactions observed between aptamers and targets that could occur besides structure complementarity were hydrogen bonds, electrostatic interactions, and π-stacking, (Hermann, Patel 2000).

Based on this, polarity or molecule charge changes could affect the target and aptamer interactions. This could be caused by pH or ionic content of buffers. Palecek et al (2012) reviewed the electrochemistry of DNA and the impact in its structure when surface immobilised in electrochemical electrodes and in polypropylene tubes, and reported that the DNA suffered conformation changes as well as a small denaturation that lead to surface adsorption. It was also mentioned that DNA stability when surface immobilised was depended on the surface electrostatic nature, the ionic nature of the buffer solution and the distance between DNA and the surface. The same authors explained that so far it was not possible to obtain detailed information on how the aptamer´s conformation was affected by surface immobilisation (Paleček, Bartošík 2012).

Another form of interference in the aptamer-target complex was referred by Meir et al (2007) who mentioned that when adding a fluorophore into the aptamer-target complex, the binding signal would decrease because it would change the aptamer conformation, (Meir, Marks et al. 2007).

DNA is known to adopt three forms: A. B and Z, according to bases electrical charges, (Calladine, Drew et al. 2004). Examples of DNA sequences G-rich (at least four guanine bases) were also found in nature, which adopted a square planar structure stabilised by a monovalent ion; these were nominated G-quadruplexes. There were several examples were aptamers instead of being depended on the target to adopt a binding folding structure, they presented a G-quadruplex structure that was stable on its own. These G-quadruplexes were special examples that required a metal ion to interact with the oxygen present in position six of each guanine, and the formation of hydrogen bonds between the bases, (Baumann 2010). Besides the example of G-quadruplexes that required metal ions for structure stabilisation, standard DNA also required the presence of metal ions but in a more versatile way.

Sigel et al (2012) described the interaction between nucleic acids and metal ions in general. They classified and characterised the monovalent and divalent ions such as

Mg^{2+} and Na^{+} or K^{+} that played a role in nucleic acids folding and structure stabilisation. Mg^{2+} bound especially to negative oxygens that did not make hydrogen bonds present either in the DNA backbone or in the different bases that allowed better stabilisation and smaller distances between negative elements, leading to a better folding and less solvation. Monovalent ions as Na^{+} or K^{+} bound preferentially to the nitrogens present in the bases, and was speculated that also balanced the negatively DNA molecule as a whole, (Sigel, Sigel et al. 2012).

Therefore several parameters seemed to play an important role in the interaction between aptamers and targets, being important to monitor and control them whenever possible (e.g. testing different pH and ionic buffer concentrations), in order to maximise the aptamer implementation into an LMC assay format.

5.2.3. Consideration of thrombin aptamers (and why were used)

The aim of this work was initially to implement already published aptamers against small targets into the LMC assay format. Because it was not straightforward as expected, it was decided to test aptamers against a protein. The chosen target was thrombin because thrombin aptamers were intensively studied and used in several assay formats and also in biosensors, (Bock, Griffin et al. 1992, Wang, Yang et al. 2011, Ni, Castanares et al. 2011, Aviñó, Fàbrega et al. 2012, Qi, Shangguan et al. 2013). Those included similar LMC assay formats and studies of the molecular interaction between aptamer and target, (PDB 2005, Russo Krauss, Merlino et al. 2012). Thrombin aptamers were also known to adopt the G-quadruplex which presented a more stable structure in solution, (Macaya, Schultze et al. 1993).

5.2.4. LMC relevant analytical assays

As reviewed in section 2.4.1 (Chapter 2), the LMC assay had several requirements. Because it was an inhibition assay, a target derivative was surface immobilised and the bioreceptor was labelled for optical detection. In order to develop a final assay, a standard inhibition ELISA as a working assay within the LMC group was used. Aptamers were tested under the LMC assay conditions, but also tested under other circumstances, such as aptamer surface immobilisation instead of target derivative and in a label free system. This was done because the implementation of the standard LMC assay did not show a straightforward response and alternatives were tested.

Different surfaces were also tested in order to reduce unspecific signal. The label system for optical detection of the colorimetric enzymatic product, when kept, was tested either connected to the aptamer or the target.

In all assays the aptamer or the target was modified either for surface immobilisation or for labelling. In some situations both interventionists were modified.

5.3. Chapter Aims and Objectives

The work presented in this chapter aimed to answer the question whether aptamers could be used in an LMC assay format and more specifically, whether aptamers against small molecules could be used in an LMC assay format.

The objectives of this study were:

- To identify existing / published aptamers that were against small molecules similar to the LMC targets and identify the ones used in analytical methods that mimicked LMC analytical assay formats.
- To test, in a structured approach, chosen aptamers in relevant LMC assay formats.

5.4. Chapter experimental rationale

The application of aptamers against small targets into the LMC assay format was tested. Other assays formats were also tested in an attempt to understand the impact on the detection of the target surface immobilisation.

Because it was not possible to have aptamers generated against LMC targets it was found in literature examples that could be used. The first experimental part of this chapter described the test of ibuprofen target and its aptamers using SPR in several assay formats. Either target surface immobilised and addition of free aptamer, or aptamer surface immobilised and addition of free target. It was seen that SPR was not appropriated for the targets of interest because of their low molecular weight, and it was then tested the LMC assay format and variants in microplates using enzymatic colorimetric reactions for optical detection. The targets OTA and estradiol were used in those assays and only OTA presented positive results in the LMC assay format. Although it was possible to verify aptamer binding to surface immobilised OTA, the assay shown to be very unstable and difficult to reproduce. Based on that,

Dynabeads® were used to see if the interaction between targets and aptamers would improve in comparison to the microplates, which did not occurred. It was then taken a new assay approach which gave positive results. It was seen that the assay improved if the aptamers and target interacted first in solution and were then surface immobilised, instead of one of the molecules being initially surface immobilised. Because it was not possible to achieve a stable working assay similar to the LMC format, it was tested a different target. In the last part of this chapter it was tested a protein as target instead of a low molecular target to verify if the LMC assay format was more compatible with large targets than smaller and to understand its impact on the interaction between low molecular weight targets and aptamers. It was possible to run a direct binding curve but when a competitive assay was tested, it was seen a higher affinity to the target in solution which gave a lower binding signal to the immobilised target.

This chapter improved the understanding of the impact of surface immobilisation of small targets in the aptamers interaction, and the differences obtained in assay formats based on the targets nature.

5.5. Choice of existing aptamers against small molecule targets and analytical implementations relevant to LMC assay formats

Considering the reviewed DNA aptamers against low molecular targets similar to the LMC mentioned in section 5.2.1 (Chapter 5), three targets were chosen to be tested in this work. The low molecular targets were ibuprofen, Figure 4-13, β-estradiol and ochratoxin A (OTA), Figure 5-1 and Figure 5-2.

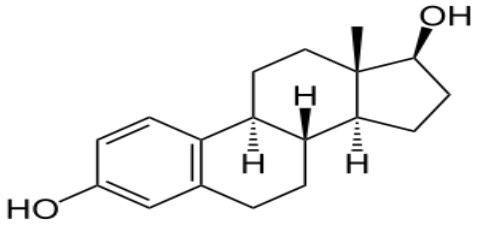


Figure 5-1 β-Estradiol.

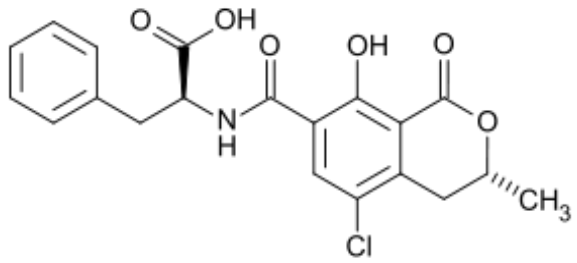


Figure 5-2 Ochratoxin A.

In the previous chapter (Chapter 4, section 4.6.2), an aptamer generation was attempted and Ibuprofen was used as the positive control. Because it was ready available, it had similar physiochemical properties to the LMC targets and had published aptamers against it, it was decided to be tested in an assay.

When searching in literature for published examples of DNA aptamers against small molecules (with similar physiochemical properties to LMC targets) and being used in an LMC assay type, only two examples were found: the OTA and the β-estradiol.

OTA aptamers had many examples of assay application in literature and versatility was shown. It was referred to be used in a competitive ELAA where both the target and the aptamer were surface immobilised and tested in wine samples with a detection limit of 1 ng/ml, (Barthelmebs, Jonca et al. 2011). In another example a competitive ELAA was also performed but this time on the surface of Dynabeads®, and the assay was used in wheat samples with a detection limit of 0.07 ng/ml, (Bonel, Vidal et al. 2011). Besides that the OTA aptamers were applied in several biosensors, (Castillo, Lamberti et al. 2012, Duan, Wu et al. 2012, Galarreta, Tabatabaei et al. 2013, Tong, Zhang et al. 2011, Wu, Chu et al. 2012). Aptamers or antibodies have also been used in existing biosensors to detect OTA (Hianik 2012).

There was one published work that reported an indirect competitive assay with a β-estradiol aptamer. β-estradiol-BSA was surface immobilised and the competition assay was made with β-estradiol and fluorescently labelled aptamer in solution. The fluorescent intensity of bound labelled aptamer to β-estradiol-BSA, was detected at a detection limit of 2.1 nM, using an evanescent wave all-fiber biosensor (Yildirim, Long et al. 2012). In the second example found, the aptamer was covalently attached to glass beads and the β-estradiol was captured in solution. The authors quantified the bound β-estradiol after aptamer denaturation with HPLC. The authors obtained a binding constant of 35.19 μM, (Huy, Jin et al. 2011). A third example referred to the β-

estradiol aptamer to be bound directly to the target that was electrochemically detected at 0.1 nM, (Kim, Jung et al. 2007).

In this chapter the chosen aptamers and targets were tested in standard microplate assays, in Dynabeads® assays, and a label free system as surface plasmon resonance (SPR) was used in order to monitor the interaction between the aptamer and the target in real-time. The microplate was tested because it was the surface used in the working LMC assay. The Dynabeads® were tested when appropriated based on published work and to verify if they were better surfaces than the microplates for assay development. The SPR was used because it allowed real time observation of the assay via the mass index change. It also allowed studying the aptamer and target binding constant through the experimental association and dissociation constants.

5.6. Common Assay Methodology

5.6.1. General assay format considerations

The implementation of the LMC assay format with small molecule targets and aptamers was tested in four different related assay format varieties. The use of a protein target and aptamers were also tested into one of the four assay formats.

Three of the tested formats were labelled assays while one of them was label free.

In the LMC assay format the surface immobilisation was made via a target derivative. The assay was performed with an aptamer in solution that was modified with biotin.

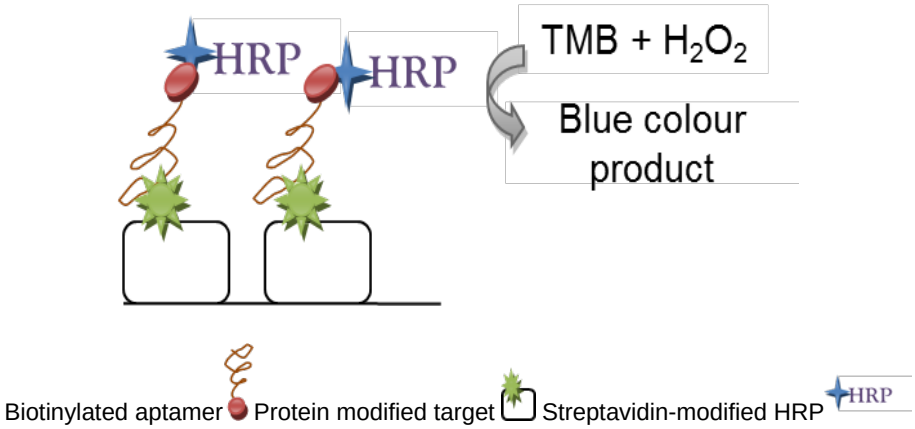


Figure 5-3 LMC assay format consisted in three steps; i) the target derivative was surface immobilised via protein adsorption, ii) the biotinylated aptamer was added in solution, and iii) a streptavidin-modified enzyme was added. The successful assay would generate an enzymatic blue product that was quantified with optical detection.

The second format was the inversed assay of the LMC assay, where the biotinylated aptamer was surface immobilised and the biotinylated target was added.

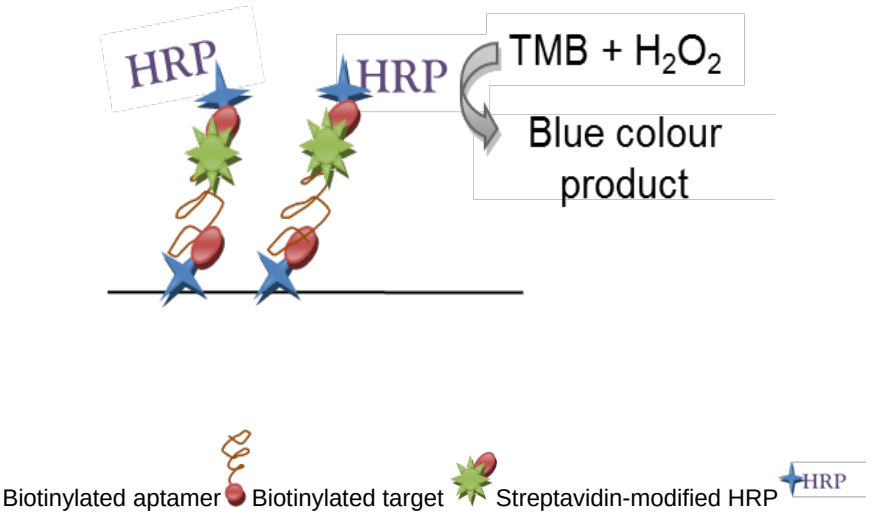


Figure 5-4 The second assay format tested consisted in three steps: i) the biotinylated aptamer was surface immobilised via streptavidin, ii) the biotinylated target was added in solution, and iii) a streptavidin-modified enzyme was added. The successful assay would generate an enzymatic blue product that was quantified with optical detection.

The third format was tested with aptamer and target derivative incubated in solution and then surface adsorbed.

All the previous assays required a label for quantification of the formed complexes, while in the fourth example there was no label. The binding quantification was made in

real time. The assays tested were similar to the LMC assay and its inversed assay but without the final addition of the enzymatic label.

It was this assay format that was the initial test performed to study aptamers against small molecules.

5.6.2. Surface Plasmon Resonance - based assays

One of the objectives of this work was to test already published aptamers against small molecules under the LMC assay format.

A way to verify in real time the interaction between an aptamer and its target was by using SPR. SPR was an instrument with optical detection that indicated any changes made into a chip surface. This made possible to verify the surface immobilisation and the molecular binding between the immobilised molecule and the molecule in solution, (Sadana, Sadana 2011).

The LMC assay required the target derivative immobilisation and the competition in solution of the labelled bioreceptor with the unmodified target. The use of SPR allowed studying the possibilities of either chip immobilisation of the target derivative or the biotinylated aptamer. SPR was the first method to be used as a detection system because it allowed real time observation and it was ready available. In the aptamer generation work (chapter 4), ibuprofen was used as a positive control, consequently it was chosen as the first target to be tested under the LMC assay format. In the published work three aptamers showed best affinity to ibuprofen, so they were all tested here (Kim, Hyun et al. 2010).

The SPR optical detection, or the change in the surface refractive index, was made in resonance units (RU). The variation obtained in the RU was caused by the concentration of the molecule surface immobilisation, which was also affected by its molecular weight. SPR was sensitive to mass changes occurred on the chip surface, the bigger the molecule the higher the change in the refractive index. Variations in the concentration or size of molecules attached to the chip surface changed the refractive index and that was visualised on a sensorgram in real time, Figure 5-5, (GE Healthcare 2010).

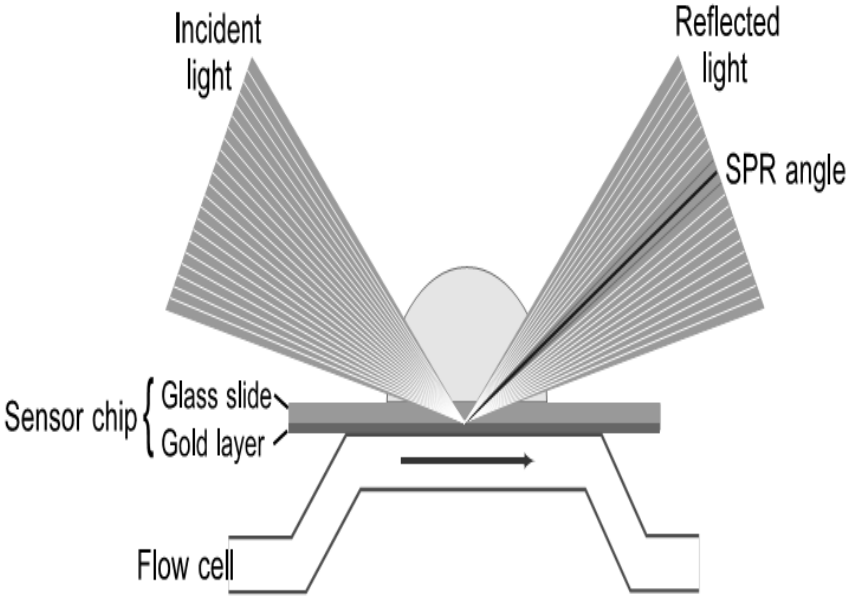


Figure 5-5 SPR detection system based on the change of the surface refractive index which was measured as resonance units visualised on a sensorgram, adapted from (GE Healthcare 2010).

a) Materials and Methods

i. Materials and Chemicals

Surface plasmon resonance (SPR) Biacore 3000, sensor chip CM-dextran with immobilised streptavidin, (SA chip with four surface channels, BR-1000-32), ibuprofen, 5β-cholanic acid, aptamers (see experimental section), streptavidin Horse radish peroxidase (SA-HRP, 43-4323) from Zymed, HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20, BR-1001-88), from GE healthcare life sciences, DMSO, HPLC grade methanol, NaOH from Sigma and in house synthesised target modified versions, 0.22 µm pore diameter size syringe filter (SF-J301322) from Jaytee, 18 MΩ.cm water, Binding buffer (see section 4.6.1).

ii. Methods for samples, reagents, SPR preparation and run conditions

The running buffer, aptamers and target (dissolved in appropriated aqueous buffer and minimal percentage of an organic buffer) were filtered and degassed. 100 µl of HPLC grade methanol was added and then 900 µl of running buffer, for total solubilisation. The unmodified aptamers and biotinylated aptamers were pre-heated before injection

at 90 °C for 1 min and kept on ice for 10 min as made by Polonschii et al (2010), (Polonschii, David et al. 2010).

The SA chip was put inside SPR and the running buffer was set to run at a desired flow, and let to do so until a stable baseline was visualised in the sensorgram.

iii. Method for immobilisation on the streptavidin modified chip surface of biotinylated modified molecules - Phase 1

According to the assay type it was surface immobilised the biotinylated aptamers or the biotinylated ibuprofen. In both cases, once a stable baseline was achieved with the running buffer, the biotinylated molecule was automatically injected at a certain flow rate and period of time. After the injection, it was waited until the running buffer baseline stabilised again. The difference in resonance units in the running buffer baseline before and after injection indicated immobilisation onto the surface of the SA chip. For unspecific binding control purposes, in some cases, it was used in parallel biochemically similar molecules and unmodified surface channels.

iv. Method for immobilisation of molecule in solution - Phase 2

According to the assay type the molecule in solution was the ibuprofen or the aptamer, both unmodified. After the biotinylated molecule surface immobilisation (in previous point), and stabilisation of the running buffer baseline, the molecule in solution was injected with a certain flow rate and for a certain period of time (10 µl for 1-5 min). Again the difference in resonance units obtained after a new baseline stabilisation indicated the interaction between the surface immobilised molecule and the one in solution. Also, in some cases, it was used in parallel to the assay surface channel, biochemically similar molecules in solution to study unspecific interactions.

v. Method for SPR affinity and kinetics studies

Once both assay phases were made it was possible to study the aptamer affinity to ibuprofen. During the phase 2 injection a tool called “Kinject”, quantified the association and dissociation time occurred between the immobilised molecule and one in solution. Based on the association/dissociation times occurred between the aptamer and ibuprofen and the obtained RU, it was possible to calculate the complex equilibrium kinetics. That was made using the 1:1 Langmuir fitting binding model, Equation 5-1. This model assumed that the immobilised molecule was homogenously bound on the chip surface and that the interaction between the immobilised molecule and the molecule in solution was one to one. (Nguyen, Tanious et al. 2007). This was the

simplest model that simulated the molecular interaction between each aptamer and ibuprofen molecule.

$$A + L \underset{Kd}{\overset{Ka}{\leftrightarrow}} AL, \quad K_A = Ka / Kd$$

Equation 5-1 Langmuir simple model version, where the free molecule binds to the surface immobilised molecule in the proportion of one to one, (Nguyen, Tanious et al. 2007).

5.6.3. Microplate - based assays

The LMC assay type was based on a inhibition ELISA and the developed working assay was a microplate assay.

a) Materials and Methods

i. Materials and Chemicals

Nunc 96-well microplate (442404), streptavidin (SA) coated Nunc 96 microplate (DIS-995-010A), adhesive seals for microplates (DIS-984-505J), aptamer and biotinylated aptamers from Invitrogen or Sigma, OTA (CAS 303-47-9; 32937) from Fluka; human biotinylated thrombin (69672-3) from Merk; OTA-HRP (P9949) from TrendPharmaTech, Centaur molecular products ; OTA-BSA (O3007), β-estradiol-BSA (β-estradiol 6-(O-carboxymethyl)oxime:BSA, E5630), human thrombin (CAS 9002-04-4; T7009), TMB tablets (3,3´, 5,5´- tetramethylbenzidene dihydrochloride; T3405), phosphate citrate with sodium perborate capsules (P4922), casein (CAS 9000-71-9; C3400), BSA (CAS 9048-46-8; A4503), 0.05 M carbonate buffer pH 9.6 (C3041), 10 mM HEPES pH 8.0, Tween 20 (CAS 9005-64-5; P1379) from Sigma; PBS pH 7.4, 1M sulphuric acid (J/8420/17) from Fisher; casein hammersten grade (101289) from MO-Biomedicals; β-estradiol selection buffer solution pH 8.0 (Kim, Jung et al. 2007, Yildirim, Long et al. 2012); Binding buffer 1 (Barthelmebs, Jonca et al. 2011); 18 MΩ.cm water; spectrophotometer Thermo Scientific Varioskan Flash Plate Reader; milk powder; temperature incubator.

ii. Method to test β-estradiol aptamers into the LMC context

100 µl per well of β-estradiol-BSA in PBS, was incubated overnight at 4 °C in a 96 microplate. The liquid was discarded and 200 µl of BSA (1 mg/ml in PBS) was added and incubated at RT for 1 hour. Afterwards 100 µl of biotinylated aptamer, in its

selection buffer, (Kim, Jung et al. 2007) was added and incubated at RT. A solution of 100 µl of SA-HRP (in selection buffer), was added for 30 min at RT and 50 µl of TMB was added until colour developed. 50µl of sulphuric acid was added and the plate read at 450 nm. Between each step the plate was washed three times with PBS (300 µl). All the incubations were incubated with agitation.

iii. Methods to test ochratoxin A aptamers into the LMC context

The protocols were adapted from Barthelmebs, J. et al (2011), (Barthelmebs, Jonca et al. 2011). The OTA aptamers were tested under three assay formats; i) where OTA was surface immobilised, ii) the aptamer was surface immobilised and iii) where the complex was made in solution and then surface adsorbed.

- Surface immobilisation of OTA

The microplate was overnight coated with 100 µl of OTA-BSA in 0.05 M carbonate buffer, at 4 °C. The microplate was blocked with a blocking solution for 1 hour at RT. 100 µl of OTA biotinylated aptamer was added in Binding buffer 1 and incubated for 1 hour at RT. A 1 µg/ml solution of SA-HRP was added and 50 µl of TMB was added until colour developed. 50 µl of sulphuric acid was added and the plate read at 450 nm. Between each step the plate was washed three times with PBS (300 µl). All the incubations were light protected and incubated with agitation.

- Surface immobilisation of OTA aptamer

A SA microplate was coated with biotinylated OTA aptamer (100 µl for 30 min). The microplate was blocked with 200 µl of 0.5 % casein solution in Binding buffer 1 for 45 min. 100 µl of OTA-HRP was added for 30 min. 100 µl of TMB was added and allowed to react and 50 µl of 1M sulphuric acid was added and the microplate absorbance read at 450 nm. All the steps were made at RT, light protected and with agitation. Between each step, three washes were made with 300 µl of Binding buffer 1.

- Surface immobilisation of OTA-aptamer complex formed in solution

Several concentrations of OTA-BSA were incubated in solution with the biotinylated aptamer and then it was transferred to a microplate (100 µl per well). The microplate was incubated for 20 min at 37 °C and then blocked with 2 % milk powder for another 30 min at RT. It was added the SA-HRP, the TMB and reacted as before. The obtained signal was read at 450 nm.

iv. Method to test thrombin aptamers into the LMC context

The protocol was adapted from Baldrich et al (2005), (Baldrich, Acero et al. 2005). The SA coated microplate was washed three times with 300 µl of 50 mM PBS, and then it was coated with 100 µl of biotinylated aptamer in PBS for 30 min at 37 °C. The SA microplate was blocked with 200 µl of biotin in PBS for 30 min at RT. 100 µl of biotinylated thrombin in 10 mM HEPES pH 8.0, was added and incubated for 1 hour at 4 °C. 1 µg/ml solution of SA-HRP was added (100 µl) and incubated for 10 min, and 100 µl of TMB was added until colour developed. 50 µl of sulphuric acid was added and the plate read at 450 nm. Between each step the plate was washed three times with PBS Tween 20 (300 µl).

In order to study an alternative to the standard microplate as a solid surface and because Dynabeads® allowed better interaction in solution between the immobilised molecule and the free molecule, they were also tested as solid surfaces.

5.6.4. Dynabeads® - based assays

Dynabeads® because of their size (µm) and better mass transfer between the molecule in solution to the one immobilised, were expected to allow better interaction between aptamers and targets. Also the magnetic partition was a vantage to separate the formed complexes from the unbound molecules. Dynabeads® were tested as alternative to the microplates.

a) Materials and Methods

i. Materials and Chemicals

Streptavidin Dynabeads® M-270, OTA, biotinylated aptamers from Sigma or Invitrogen, Nunc 96-well black microplate (437111) from Fisher, spectrophotometer, rotator, magnetic support, SA Dynabeads® buffer C, D and D´ (Centi, Messina et al. 2008), buffer OTA aptamer (Cruz-Aguado, Penner 2008).

ii. Method to test ochratoxin A aptamers into the LMC context

The protocol was based in Bonel et al. (2011) work, (Bonel, Vidal et al. 2011), 500 µl of biotinylated aptamer was added to 50 µl of SA Dynabeads® after being washed three times with buffer C (500 µl). The Dynabeads® and aptamer were incubated at 37 °C for 30 min with agitation. 200 µl of OTA or OTA-BSA, in buffer OTA aptamer, was added to the Dynabeads® and incubated at RT for 15 min. Three washing steps were made between each step with 500 µl of buffer. Afterwards the Dynabeads® were re-

suspended in 100 µl of OTA aptamer buffer and heated at 90 °C for 5 min and the fluorescence of the collected liquid was read in a spectrophotometer. The last step was repeated two more times.

iii. Method to test trombin aptamers into the LMC context

Protocol adapted from Centi et al (2008), (Centi, Messina et al. 2008). 50 µl of SA Dynabeads® were removed from the stock solution and washed three times with 500 µl of buffer C. it was added 150 µl of 1 µM biotinylated thrombin aptamer in buffer C, incubated for 15 min. A new wash set was made and 500 µl of 500 µM biotin solution was added for 30 min. After washing the Dynabeads® as before, 200 µl of biotinylated thrombin was added to 25 µl of Dynabeads® and incubated for 30 min. The Dynabeads were washed twice with buffer D and SA-HRP in buffer D´ was added.100 µl of TMB was added for 5min and then 50 µl of sulphuric acid was added and the liquid collected into a microplate and read at 450 nm. All the steps were made at RT with agitation.

5.7. Experimental testing of chosen aptamers in LMC relevant assays

One of the objectives of this work was to test existing aptamers against small molecules into relevant LMC assay formats. This involved in some cases, the surface immobilisation of a target derivative and the detection of binding via the enzymatic labelled aptamer. The enzyme would oxidise a substrate into a colorimetric product. Or in another cases it was surface immobilised the aptamer and added the target. In the situations where the detection method required a label, the target was modified in order to have the enzymatic label. In the cases where the detection system was label free it was tested the unmodified target. In another cases the fluorescent properties of the target also allowed the direct detection. After testing the existent aptamers against small molecule into the LMC assays and similar formats, it was tested the thrombin aptamer and target. This was tested with the same enzymatic detection method but only with aptamer surface immobilisation.

5.7.1. Initial testing of aptamers in LMC relevant assays: aptamers against ibuprofen

As mentioned before, ibuprofen was used in the previous chapter as positive control in aptamer generation, and because it was physiochemically similar to the LMC targets and had already existing aptamers against it, it was used as the first target to study the aptamers implementation into LMC relevant assays.

The initial choice of instrumentation was the SPR. Despite the fact that SPR was a label free system, while LMC assay required labels, it was used because it allowed a real time analysis of aptamer-target interaction, which was fundamental to a working assay and has been mentioned in literature in aptamer related studies, (Gopinath 2010).

Ibuprofen aptamers tested into the LMC context – SPR assay

It was not found examples of binding assays similar to the LMC assay format using the ibuprofen and its aptamers. It was here attempted the development of a binding assay using SPR in which it would be required the surface immobilising of one of the molecules and the addition in solution of the other.

a) Experimental and Results

i. Selection of the ibuprofen aptamers to be used

The tested aptamers were described by Kim et al (2010), and are reproduced in Table 5-1, (Kim, Hyun et al. 2010). In summary three aptamers against ibuprofen were used and one control sequence consisting of the complementary sequence to ibuprofen aptamer sequence 1. For surface immobilisation, the aptamers where synthesised with biotin-C6-spacer added onto the 5’ end of their sequences, Table 5-1.

Table 5-1 Ibuprofen tested sequences and control random sequence, adapted from (Kim, Hyun et al. 2010).

Ibuprofen aptamer sequence 1	5´- ATACCAGCTTATTCAATTACAGTAGTGAGGGGTC CGTCGTGGGGTAGTTGGGTCGTGGAGATAGTAAGTG CAATCT-3´
Ibuprofen aptamer sequence 2	5´- ATACCAGCTTATTCAATTGCGAACGACTTCATAAA ATGCTATAAGGTTGCCCTCTGTCAGATAGTAAGTGC AATCT-3´
Ibuprofen aptamer sequence 3	5'- ATACCAGCTTATTCAATTGGATCGGCGACGTGGG TGTCGTGATTCGGGGTGAGATAGTAAGTGCAATCT-3'
sequence for control (complementary to sequence 1)	5´- TATGGTCGAATAAGTTAATGTCATCACTCCCCAGG CAGCACCCCATCAACCCAGCACCTC TATCATTCACG TTAGA-3´

ii. Assay format 1 - Immobilisation on the streptavidin modified chip surface with 5´- end biotinylated modified aptamers

The first step in demonstrating an SPR-based assay format using biotinylated ibuprofen aptamers was to confirm their immobilisation to a streptavidin-coated SPR chip surface. After obtaining a stable baseline with a flow rate of 35 µl/min, each of the biotinylated aptamers and control were injected into one of the SA chip channels. Each injection was of 100 µl, made during 120 sec and each aptamer and control solution was at 1 µM. After all the injections and baseline stabilisation it was verified a change in the obtained RU. The ibuprofen 5´- end biotinylated aptamers and control were efficiently SA chip immobilised as seen by the obtained resonance units and sensorgrams, Table 5-2 and Figure 5-6.

Table 5-2 Obtained resonance units (RU) indicative of chip surface immobilisation of ibuprofen 5´- end biotinylated aptamers and control.

	Obtained RU
Ibuprofen 5´- end biotinylated aptamer sequence 1	1652.5
Ibuprofen 5´- end biotinylated aptamer sequence 2	1168.5
Ibuprofen 5´- end biotinylated aptamer sequence 3	1507.8
5´- end biotinylated sequence for control	1423.4

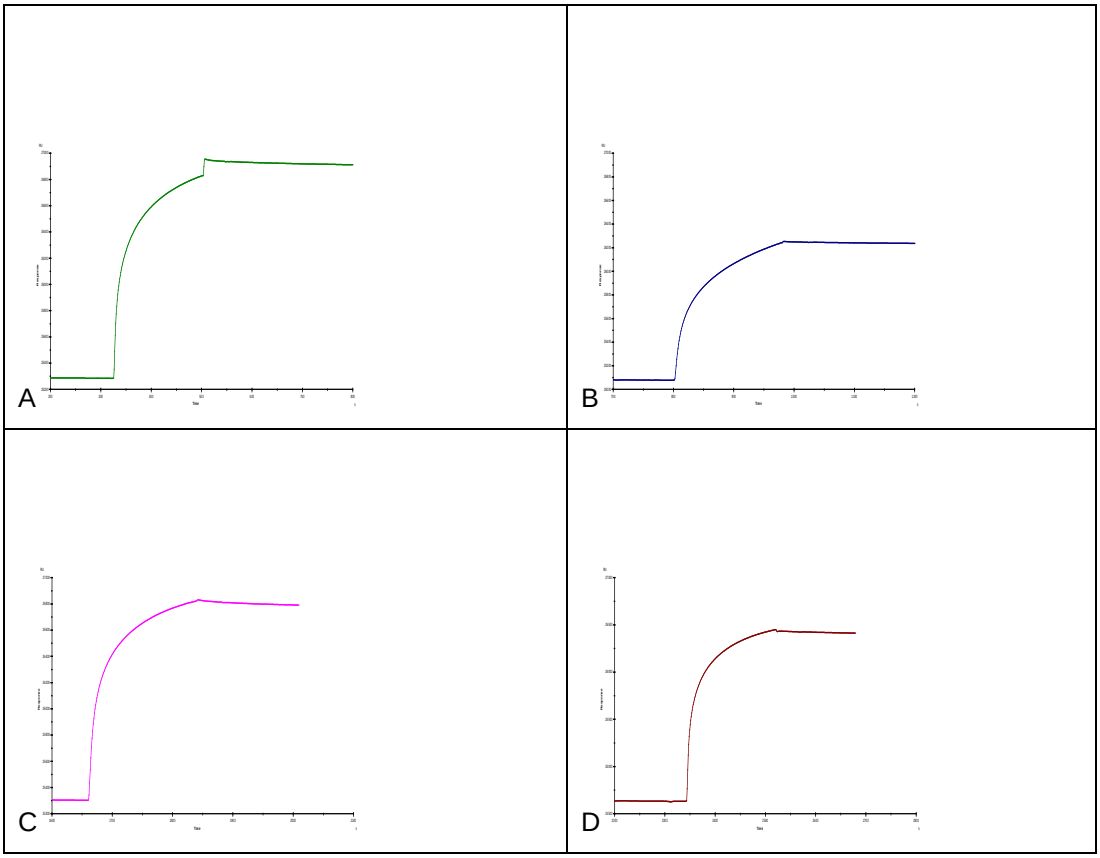


Figure 5-6 Sensorgrams (RU response vs. time) of SA-chip immobilisation of the ibuprofen biotinylated aptamers, A) sequence one, B) sequence two, C) sequence three and D) 5´-end biotinylated sequence for control.

It was obtained surface immobilisation of all the aptamers at a level that was suitable for subsequent study of ibuprofen free target binding.

iii. Assay format 1 - Binding from solution of ibuprofen to aptamers immobilised on SPR chips

Several concentrations of ibuprofen, (0.5, 1, 5, 10, and 20 µM), were injected sequentially and in parallel into each of the SA chip channels with immobilised biotinylated aptamers and the control sequence. The injections were 50 µl of ibuprofen solution with dissociation constant for 300 sec, at a flow rate of 35 µl/min. It was not verified in neither of the channels a change in RU after the injections and baseline stabilisation. The assay was repeated several times, and in all, no immobilisation signal was obtained.

It was necessary to take a different approach to that assay to study ibuprofen and aptamers binding affinities.

iv. Assay format 2 - Immobilisation on the streptavidin modified chip surface with biotinylated modified Ibuprofen

A new assay format was tested to study the interaction between ibuprofen and its aptamers. The ibuprofen was in house modified with the spacer maleimide-polyethylene glycol-biotin, (Peg_2-biotin) in order to bind to the SA chip. The control used was 5β-cholanic acid which was also in house modified with the same biotinylated spacer, (biotin-Peg_2-5β-cholanic acid).

After achieving a stable baseline with running buffer at 35 µl/ min, it was tested the surface immobilisation on a SA chip of biotinylated ibuprofen and control by injecting each to a SA chip channel at a concentration of 4.85 µM. Each injection was of 100 µl and made during 120 sec. One of the unmodified channels was used also as a control. After the injections and baseline stabilisation it was verified a change in the RU values in both of the modified channels. Biotin-Peg_2-ibuprofen and biotin-Peg_2-5β-cholanic acid were efficiently surface immobilised but with low RU values, this was expected because of their low molecular weight, Table 5-3.

Table 5-3 Obtained resonance units (RU) indicative of chip surface immobilisation of biotinylated ibuprofen and control.

	Obtained RU
biotin-Peg_2-ibuprofen	124.7
biotin-Peg_2-5β-cholanic acid	37.1

The efficient surface immobilisation allowed to proceed and test the addition of ibuprofen aptamers.

v. Assay format 2 - Binding of aptamers in solution to ibuprofen immobilised on SPR chips

The ibuprofen aptamers were injected into each channel at different concentrations (172, 17.2, and 1.72 µM). Each injection was sequential and made from the lowest concentration to the higher. The injections were made individually and passed by the three channels of interest. The conditions were 50 µl of aptamer solution with dissociation constant for 300 sec, at a flow rate of 35 µl/min. No specific binding was seen with any of the aptamers. In the case of aptamer one, unspecific signal was detected (~ 80 RU). When repeating the previous conditions in a new chip, the same unspecific signal was seen and no binding signal was detected. It was not found a reason for unspecific binding signal. Because it was not detected any interaction, it was taken a third approach, which considered the ibuprofen low molecular weight.

vi. Assay format 3 - Binding from solution of ibuprofen-protein conjugates to aptamers immobilised on SPR chips

It was considered the ibuprofen low molecular weight (206.28 g/mol) and decided to test the assay using biotin-PEG-ibuprofen bound to SA-HRP instead of free ibuprofen. This aimed to add molecular weight to ibuprofen and increase the RU response. The assay was similar with the biotinylated aptamers and control surface immobilised, but instead of adding ibuprofen, it was tested the protein conjugated ibuprofen in solution.

In the assay the running conditions and surface immobilisation occurred as explained previously. The biotinylated aptamers one and three, and the control bound to the SA chip as seen before with similar RU signals, and one channel was left unmodified. The biotinylated ibuprofen was mixed with a solution of SA-HRP and 100 µl of that solution was injected into the SA chip at a flow rate of 10 µl/min for 120 sec. The injections went thought the four SA chip channels in parallel and it was not obtained any change in the RU values once the baseline stabilised in three channels. Unspecific binding (~ 100 RU) was obtained in the unmodified channel. This occurred probably because there was an excess in solution of biotin-Peg_2-ibuprofen that was not bound to SA-HRP and bound to the SA surface.

b) Discussion

Two assay types were tested, one where the ibuprofen aptamers were surface immobilised and the other where ibuprofen was surface immobilised. In both cases the biotinylated species were efficiently surface immobilised as desired.

When ibuprofen was added as analyte in the first assay type, no binding signal was detected. It was considered that it could be that ibuprofen low molecular weight was preventing the detection. It was found in literature that SPR was not sensitive to small molecules and it was reported that molecules less than 400 g/mol were not detected, (Kim, Jung et al. 2007). In order to overcome this, the assay was tested with a protein modified version of ibuprofen. Again no binding signal was obtained. This might have been caused by conformation changes because both of the species were chemically modified; or the fact that the aptamer was attached to a surface might had interfered with the folding, or in the case the binding pocket was located near the chip surface steric hindrance might had happened.

At this stage it was decided to test the assay type two and add unmodified aptamer in solution to already surface immobilised ibuprofen. In this case the analyte molecular

weight was not a concern. Also the biotin modification made to ibuprofen was via its carboxylic group. This was considered to not interfere in the recognition because the ibuprofen aptamers were generated via FluMag-SELEX and ibuprofen was Dynabeads® immobilised via the same chemistry, (Kim, Jung et al. 2007). Once more no interaction was verified, leading again to the idea that the free aptamers conformation was affected somehow or that the binding area of the target was not exposed as necessary. To support this idea, that surface immobilisation seemed to have interfered with the aptamer-target complex; it was observed that the published affinity studies were all made in solution. Another reason might have been that the changes in the chip surface were so small that were not seen in the sensorgram, (Wang, Huang et al. 2011).

Summary and Conclusion

Interaction between ibuprofen and its aptamers were studied using SPR. Two types of a direct binding assay were tested. In the first the aptamers were SA chip immobilised and the ibuprofen was added in solution. Because no binding signal was seen it was decided to use a protein modified ibuprofen to increase the molecular weight. Again no interaction was detected. In order to overcome the problem the assay was transformed and the ibuprofen was surface immobilised and the aptamers added in solution. No binding signal was obtained besides the efficient surface immobilisation of the biotinylated molecule.

In response to the obtained results it was searched existing examples where aptamers and targets had been used in similar assay formats to the LMC and those were tested.

5.7.2. Further testing of aptamers in LMC relevant assays: aptamers against β-estradiol and Ochratoxin A

After testing ibuprofen aptamers under the LMC assay type and seeing no binding interaction between aptamer and target, it was pursued another aptamer examples against small molecules. In that case it was decided to test examples with published applications similar to what was required to the LMC assay. It was found two molecules with aptamer and surface immobilised assays. They were ochratoxin A (OTA) and β-estradiol. These were the only two examples found in literature, using low molecular weight molecules, with assays with the required surface immobilisation of one of the molecules. That showed that probably the implementation of an LMC type assay using aptamers against small molecules was more demanding than initially expected.

It was then tested the two examples under the LMC assay conditions.

β-estradiol aptamers tested into the LMC context

As seen before, it was found three published examples where β-estradiol aptamer was used in assays. In the first case the detection was made with an evanescent wave all-fiber biosensor, in the second by HPLC, and the third by an electrochemical biosensor. The first measured the bound fluorescent labelled aptamer to surface immobilised β-estradiol in a competitive assay, the second quantified the β-estradiol released after aptamer denaturation in direct binding, and the third the current changes also when direct binding occurred (Huy, Jin et al. 2011, Yildirim, Long et al. 2012, Kim, Jung et al. 2007).

a) Experimental and Results

i. Microplate assay to detect surface immobilised β-estradiol

In this work the detection was tested in a microplate assay, where β-estradiol was surface immobilised via conjugated BSA. The biotinylated aptamer was then added and expected to bind to the immobilised β-estradiol. The detection was made via the colorimetric HRP enzymatic product. Several concentrations of biotinylated aptamer and β-estradiol were tested, and also the time of interaction between them, Table 5-4.

Table 5-4 β-estradiol-BSA, and biotinylated aptamer tested concentrations and interactions time.

β-estradiol-BSA	1, 10, 50 (µg/ml)
Biotinylated aptamer 5´- GCTTCCAGCTTATTG AATTACACGCAGAGGGTAGCGGCTCTGCGCATTCAA TTGCTGCGCGCTGAAGCGCGGAAGC-3´ (Kim, Jung et al. 2007)	10, 20, 25, 50, 100, 150 (nM)
aptamer-target interaction time[5]	15, 30, 60 (min)

There was no evidence of binding in the tested assay conditions. The concentrations of aptamer and target, as well as the buffers and blocking solution used were similar to those published, (Yildirim, Long et al. 2012). No further tests were made because the starting point was to reproduce as close as possible the published conditions and there was no initial data for optimisation into an in house working assay.

[5] The time considered referred only to the incubation time between aptamer and target. The formed complex would be required to be stable during the full assay.

b) Discussion

The attempt to reproduce in a microplate assay the published work did not happen as expected. It was speculated that the addition of the enzymatic label interfered with the aptamer- β-estradiol complex stability. For this reason it was concluded that to run an LMC type assay both the aptamer and target required chemical modifications which did not appear to be compatible with the β-estradiol and its aptamer. For that reason another example of aptamer against small targets was pursued.

Ochratoxin A aptamers tested into the LMC context

OTA was a low molecular weight mycotoxin (403.8 g/mol) present in several foods and beverages such as cereal based products, dried fruits, coffee, cocoa products, liquorish, grape juice and fermented beverages, (el Khoury, Atoui 2010). OTA concentration present in those products was regulated so that the average consumer did not intake more than 120 ng/Kg per week, (European Union 2010).

Cruz-Aguado et al (2008) generated several aptamers able to bind OTA and used the OTA fluorescence to detect it in solution, (Cruz-Aguado, Penner 2008). Barthelmebs et al (2011) also generated aptamers against OTA, (Barthelmebs, Jonca et al. 2011). Several biosensors have been developed to quantify and to remove OTA from the contaminated products, (Barthelmebs, Hayat et al. 2011, Yang, Wang et al. 2011, Bonel, Vidal et al. 2011, Girolamo, Le et al. 2012, De Girolamo, McKeague et al. 2011). And a patented biosensor was also developed for commercial use, (Penner, Cruz-Aguado 2011).

a) Experimental and Results

i. Selection of specific aptamers to be used

In this work a group of published aptamers was tested. Those were biotinylated at their 5´-end and some were also tested biotinylated at the 3´-end instead, Table 5-5.

Table 5-5 OTA aptamers, from published work, tested biotinylated either at the 5´-end or at the 3´-end, and a random sequence used for control.

OTA aptamer H12 (Barthelmebs, Jonca et al. 2011) Two versions: Biotin modified at 5´- end and at 3´- end	5'- GGGAGGACGAAGCGGAACCGG GTGTGGGTGCCTTGATCCAGGGAGTCTCAGAAG ACACGCCCGACA-3'
OTA aptamer H8 (Barthelmebs, Jonca et al. 2011) Two versions: Biotin modified at 5´- end and at 3´- end	5'-GGGAGGACGAAGCGGAACTGG GTGTGGGGTGATCAAGGGAGTAGACTACAGAAG ACACGCCCGACA-3'
OTA aptamer H16 (Barthelmebs, Jonca et al. 2011) Two versions: Biotin modified at 5´- end and at 3´- end	5'-GGGAGGACGAAGCGGAACCGG GTGGGCGGGCTTGATCCAGGGAGTGGACAGAA GACACGCCCGACA-3'
OTA aptamer Sequence 1.12 (Cruz-Aguado, Penner 2008)	5'-Biotin-TGGTGGCTGTAGGTCAGCATCT GATCGGGTGTGGGTGGCGTAAAGGGA GCATCGGACAACG -3'
OTA aptamer Sequence 1.12.2 (Cruz-Aguado, Penner 2008)	5'-Biotin-GATCGGGTGTGGGTGGCGTAA AGGGAGCATCGGACA -3'
Random sequence for control[6]	5´- CACGCGAACACTATGTAACTATCTAG CGGAAACCAGTTTGATGCGATGGAAGAGCAGGG TAGAGG -3´

OTA binding assay was tested using several assay formats and surfaces. It was also tested different OTA modifications and ends of biotinylation in the aptamers.

ii. Microplate surface immobilised target (OTA-BSA) and soluble 5´- end biotinylated aptamers

The first assays were tested with H8, H12, and H16 OTA aptamers biotin modified at the 5´- end and OTA-BSA surface immobilised. The assay was tested under several conditions, such as OTA-BSA (maximum 50 µg/ml), biotinylated aptamer (maximum 1 µM), and SA-HRP at different concentrations (0.1; 0.5; 1 µg/ml). It was obtained binding signal but in all times, very high unspecific signal (not shown), therefore, in order to reduce the background, it was tested the presence of unspecific protein (1 mg/ml BSA), oligonucleotide (2 µM), or 0.01% Tween 20 in the SA-HRP buffer; several blocking agents (0.5% casein, 1 % BSA, 2 % milk powder, 0.5 % Hammerstein casein, either in PBS or Binding buffer 1); and different incubation times (maximum 1 hour) and temperatures (4 °C , RT and 37 °C). The unspecific signal was still high and the assay binding signal not very reproducible. It was tested the effect of 0.01% tween 20 in the Binding buffer 1 during target-aptamer interaction. Again no major improvements were seen so it was decided to keep using tween 20.

[6] The random sequence was generated with f (x) = rand () and keeping the percentage of each base present on sequence H12.

iii. Microplate surface immobilised target (OTA-BSA) and soluble 3´- end biotinylated aptamers

It was decided to use the same assay format but with biotinylated aptamers at the 3´-end. This was because after re-analysis of the published work from Barthelmebs et al (2011), (Barthelmebs, Hayat et al. 2011), it was not clear at which end the aptamers have been modified so it was tested again the same aptamers but biotinylated at the 3´- end. At this stage it was tested aptamer temperature pre-treatment (90 °C of 1 min and ice for 10 min, as done in SPR ibuprofen work, (Polonschii, David et al. 2010). No major changes were seen in the assay behaviour, Figure 5-7.

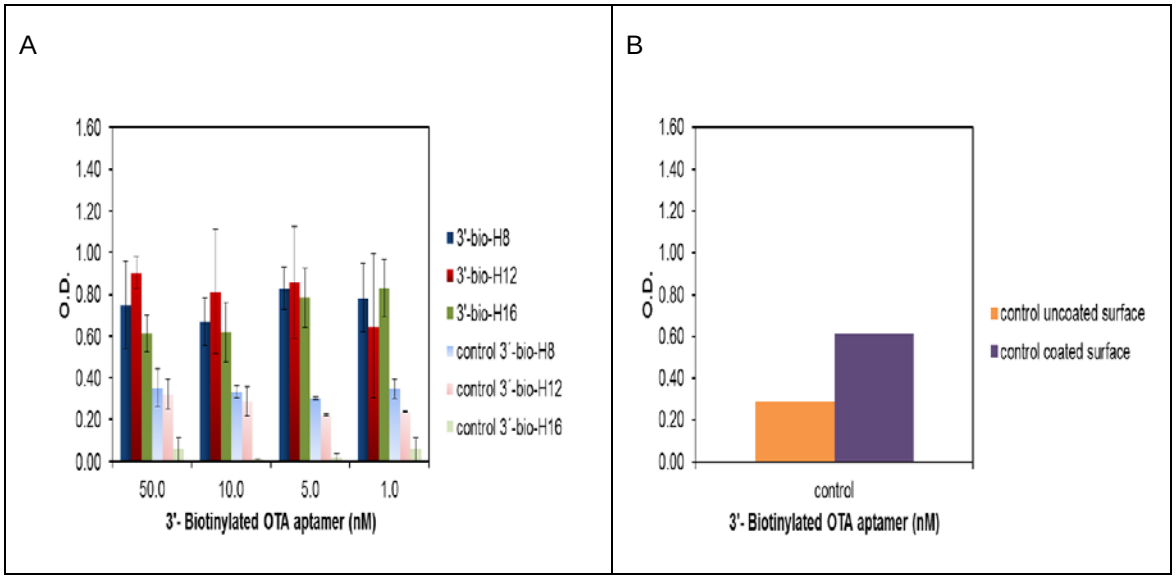


Figure 5-7 Assay with immobilised OTA-BSA (50 µg/ml) and three different 3´end-biotinylated aptamers at different concentrations.

The results obtained showed once more binding and the background was slightly lower, but once it was attempted to reproduce the results the background values increased once more. The assay was not consistent and the background signal was not reduced. Because of that the assay was tested under the opposite conditions, with immobilised aptamer and HRP modified OTA.

iv. Microplate surface immobilised aptamer (biotinylated aptamer) and soluble target (OTA-HRP)

The assay was tested with 5´-end and 3´-end biotinylated aptamers, surface immobilised and OTA-HRP directly bound, Table 5-5. Although this assay format was referred in literature as the one that gave better results it was not possible to reproduce this work. Because it was not seen binding signal; it was tested SA Dynabeads® as

solid surface, to see if those could be an alternative surface for the assay and to understand if the interaction in solution would give positive results.

v. SA Dynabeads® immobilised aptamer (3´-end biotinylated aptamer) and soluble target (OTA)

Using the SA Dynabeads® as surface, the 3´-end biotinylated aptamers were immobilised and OTA in solution was added. Because OTA was fluorescent, after washing the excess of OTA the Dynabeads® were heated to promote aptamer denaturation. The released OTA was collected, detected, and the quantity extrapolated via a calibration curve (not shown). The obtained difference between the quantity of OTA present in the last wash-liquid and the one obtained after aptamer denaturation was less than half unit, which was considered insufficient to prove binding and not unspecific interaction. Based on the obtained results it was decided to take a novel approach to test the aptamers interaction with OTA.

vi. Soluble target (OTA-BSA) and soluble aptamer (3´-end biotinylated aptamer)

The 3´-end biotinylated aptamers were mixed in solution with OTA-BSA at different concentrations, and the formed complex was surface immobilised via BSA adsorption into a microplate. The signal was obtained after oxidation of bound SA-HRP to the biotinylated aptamer.

From all the OTA aptamers, the aptamer that showed better results was the H12 biotinylated at the 3´-end. It was then used to perform the assay with OTA-BSA both in solution. It was possible to see that the lowest concentrations of OTA-BSA gave better results than the higher ones. This was perhaps higher concentrations did not allow better interaction between aptamer and target and the lowest did. That phenomenon occurred in both of the two assays performed (A and B). The controls for detection of unspecific binding indicated that there was binding interaction between the aptamer and OTA (A´and B´). In the first assay it was seen a higher unspecific signal from the biotinylated aptamer itself, which did not occur on the second assay replicate. In both cases the unspecific signal was lower than the binding signal, Figure 5-8.

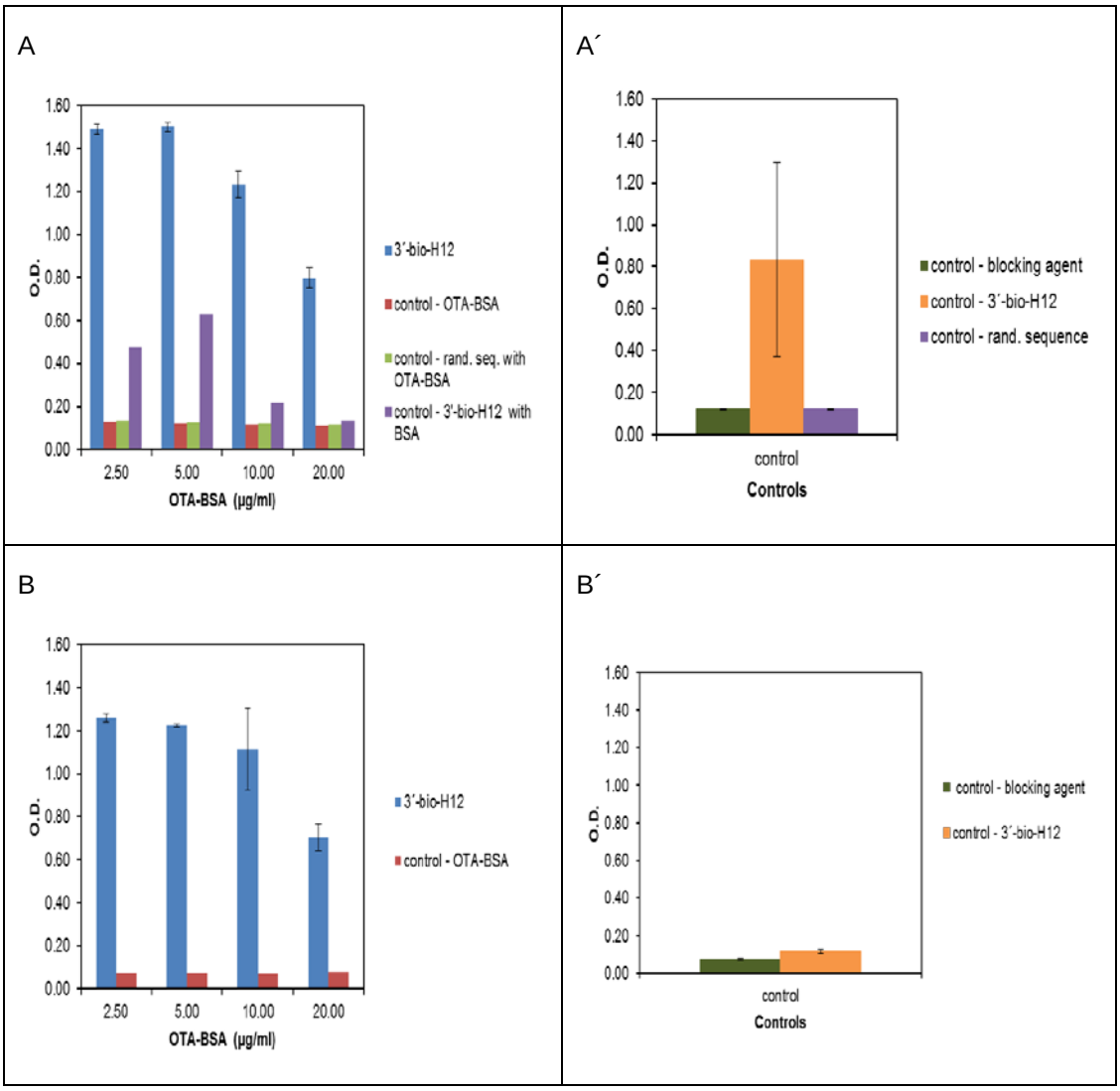


Figure 5-8 Assays (A and B) of OTA-BSA at different concentrations and 3´-bio-H12 aptamer both in solution, that after complex formation were surface adsorbed. A´and B´presented the assay controls for unspecific signal.

Afterwards it was attempted to obtain a binding curve with different concentrations of OTA-BSA, starting at 2.50 µg/ml, because it was the OTA-BSA concentration that gave the highest signal. The assay format was kept but the obtained signal was low and it was not possible to mimic the previous results. There was evidence of binding proportionality according to OTA-BSA concentration, but always very low binding signal. Higher concentrations of OTA-BSA were tested but no improvements were seen, Figure 5-9.

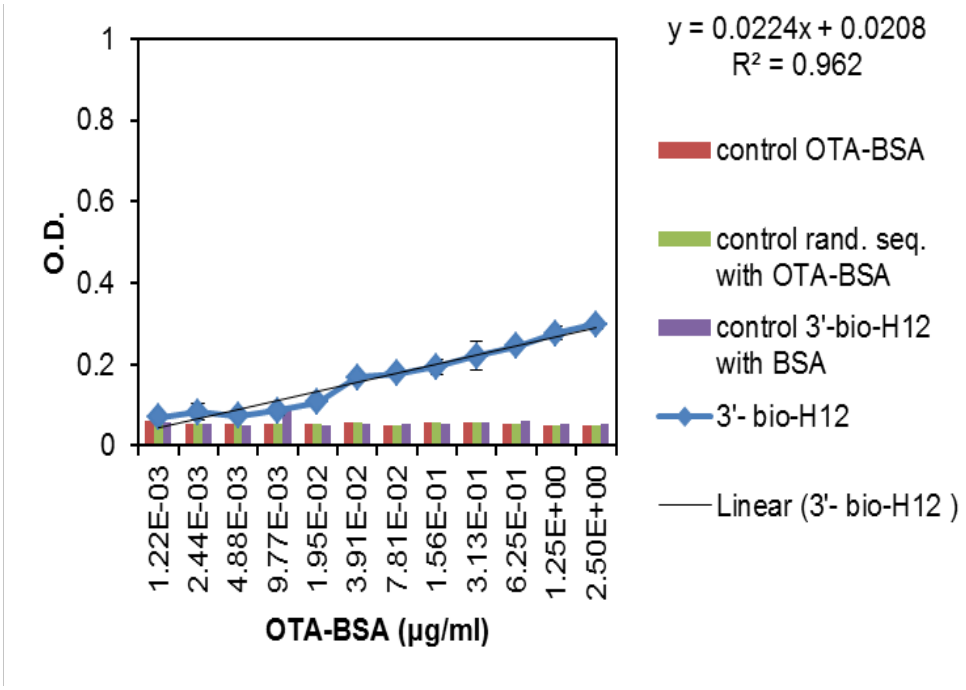


Figure 5-9 Direct binding curve of OTA-BSA and 3´- biotinylated H12 aptamer, obtained after complex formation in solution and surface adsorption.

b) Discussion

Several published OTA aptamers were tested under three assay types. The objective was to obtain direct binding between the aptamer and OTA. In the first assay type, the OTA-BSA was surface adsorbed and a biotinylated aptamer was added in solution. To the biotinylated aptamer it was added SA-HRP to give origin to an optical detected signal that would indicate complex formation. There was evidence of binding but a high background was always present and it was not possible to obtain a calibration curve. Also Bonel et al (2011) reported that their assay when surface immobilising OTA-BSA was not robust and presented low reproducibility, (Bonel, Vidal et al. 2011). Another interesting point was the fact that immobilised aptamers via their 5´-end performed worse than when biotinylated by their 3´-end. This showed that biotinylation had an impact either on the aptamer folding or in the binding site.

The second assay format required biotinylated aptamer surface immobilisation. It was tested microplates with OTA-HRP in solution and SA Dynabeads® with OTA in solution. No signal was obtained with OTA-HRP and the fluorescent signal from OTA obtained after aptamer denaturation was not sufficient to confirm binding. This was not expected but no reason was found for these results. It was argued that the OTA

modification might have not been efficient but no detailed investigation was made under this subject.

A third assay format was tested; this was a different approach where OTA-BSA was incubated in solution with the 3´-bio-H12 aptamer and then the complex was surface adsorbed. 3´-bio-H12 was the aptamer that showed best results under this approach. Initially the lowest concentrations of OTA-BSA gave the best binding signal, but after running a binding curve it was not possible to obtain the same value range and lower binding signal was seen consistently. Because of this it was concluded that the assay was working but it was not very robust.

Summary and Conclusion

The β-estradiol assay did not present any binding signal. It was concluded that probably the addition of the label interfered with the complex. Because of this it was seen as an unsuitable assay for further studies. The second example found in literature where similar assays to LMC had been developed, was the OTA aptamers. The assay presented very high unspecific signal when OTA-BSA was surface immobilised, so it was tested surface immobilisation of the aptamers and direct binding to OTA-HRP. In that case no binding signal was obtained. It was also tested the direct detection of OTA after interaction with the immobilised biotinylated aptamer onto the surface of Dynabeads®. The obtained OTA was not conclusive if it was caused by unspecific or specific binding.

In order to confirm the initial interaction between the aptamer and the OTA-BSA, a novel approach was tested. The aptamer and OTA-BSA were incubated in solution and then surface adsorbed. In that case it was possible to run several assays, including a direct binding curve. It was seen a decrease in signal that was not possible to recover. This might have been caused by aptamer or OTA-BSA degradation.

Because the obtained data was not robust as required, it was decided to test an aptamer against a protein and learn more about aptamer interaction with their targets and the impact of surface immobilisation in their interaction.

5.7.3. Additional testing of aptamers in LMC relevant assays: aptamers against a protein target - thrombin

The thrombin aptamer was used because it was a well-known aptamer and it was applied in several assays formats and biosensors. The test of the thrombin aptamer in this work had the aim to obtain a more robust working assay than the tested with small molecules. In special because it was seen that aptamers against small molecules were used in solution and that in the few published examples where aptamers or targets were surface immobilised, their reproducibility was not as good as expected. Also the test of aptamers against small molecules and against a protein allowed a better understanding on how aptamers could interact with their targets and the impact that surface immobilisation could cause.

Thrombin Aptamers tested into the LMC context as alternative to small molecules

Human thrombin had two published aptamers, one 15 oligomers long and another 29. The 15 oligomers long was modified with a poly T tail 20 oligomers long. This was used because it was previously published with the modification, and it allowed the understanding of the impact of having a poly tail in aptamers interaction with their target. The two aptamers were known to bind two different epitopes in thrombin, (Centi, Messina et al. 2008).

a) Experimental and Results

i. Thrombin aptamers to be used

In the assays it was used negative controls, so it was randomly generated two sequences. This was done similarly to OTA random sequences in the previous point, Table 5-6.

Table 5-6 Thrombin aptamers and random sequences used in the assays.

Thrombin Bio-Aptamer poly A 15-oligomer 5'-Biotin-TTTTTTTTTTTTTTTT TTTGGTTGGTGTGGTTGG -3'	Random sequence poly A 15-oligomer 5'-Biotin-TTTTTTTTTTTTTTTTTTT ATGCGGGGCGGAGTT-3'
Thrombin Bio-Aptamer 29-oligomer 5'-Biotin-AGTCCGTGGTAGGGCAG GTTGGGGTGACT -3'	Random sequence 29-oligomer 5'-Biotin-ATGCATTGTCGTACGACG CTCGACCAGGA -3'

Thrombin was biotinylated to allow streptavidin surface immobilisation or to bind to HRP also modified with streptavidin. The assay principle was the same as in the previous points, either the aptamer was surface immobilised or the target, and the biding was direct.

In the assay optimisation, the aptamers were tested at several concentrations, and the biotinylated thrombin was diluted several factors using the stock solution as initial parameter (50 U per µl). It was tested two surfaces, SA Dynabeads® and SA microplate, and two different methods.

ii. SA Dynabeads® versus microplate for immobilisation surface

As published by Centi et al (2008), (Centi, Messina et al. 2008) the 15 oligomer biotinylated aptamer was SA Dynabeads® immobilised but in this case the detection was made using the enzymatic oxidation of TMB. The colorimetric product was transferred from the solution with Dynabeads® to a clean microplate for optical reading. It was obtained high unspecific background. When tested the same conditions in a SA microplate, the background was lower and the assay clearer. Because of this the assay was moved to a microplate, Figure 5-10.

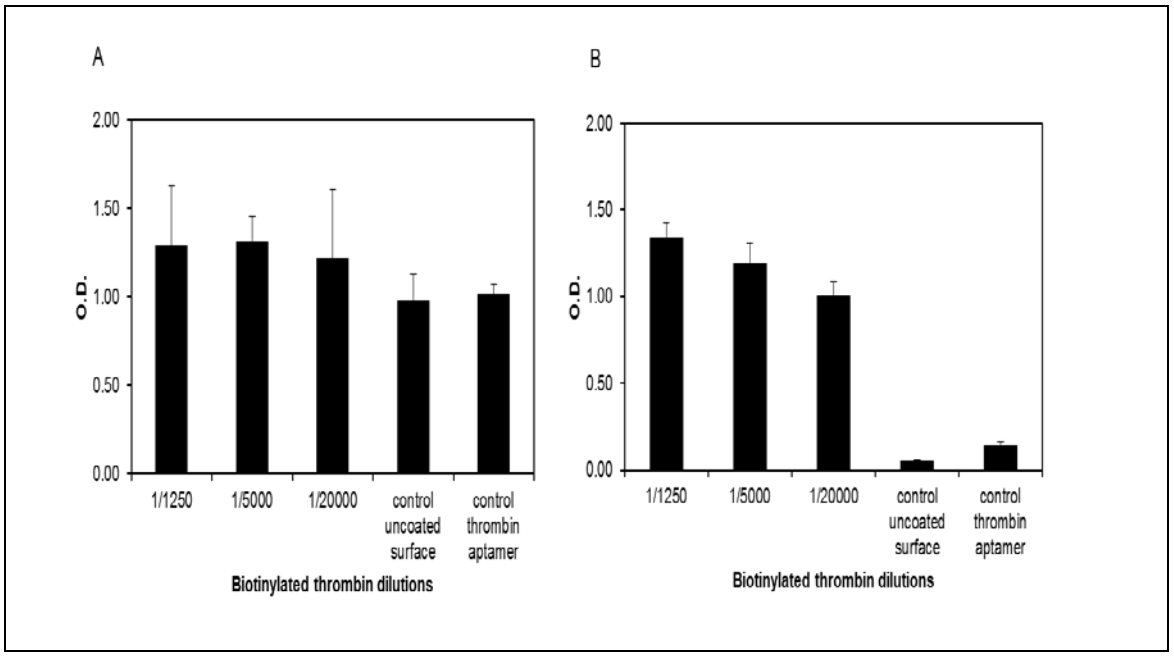


Figure 5-10 Comparison assay between A) Dynabeads® and B) SA microplate for immobilisation surface.

When repeating the assay in the microplates, the binding signal decreased and the assay did not show stable binding signal among the different thrombin dilutions. In order to avoid this, this protocol was compared to a new one.

iii. Comparison between two different protocols for the biotinylated thrombin microplate assay

The two protocols tested were based on published work, (Baldrich, Acero et al. 2005, Centi, Messina et al. 2008). The protocols were different, as the concentration of biotinylated aptamer changed from 1 μM to 50 nM, different buffers (see previous point), and different incubation time and temperatures. The biotinylated thrombin dilutions were the same as the detection, Figure 5-11.

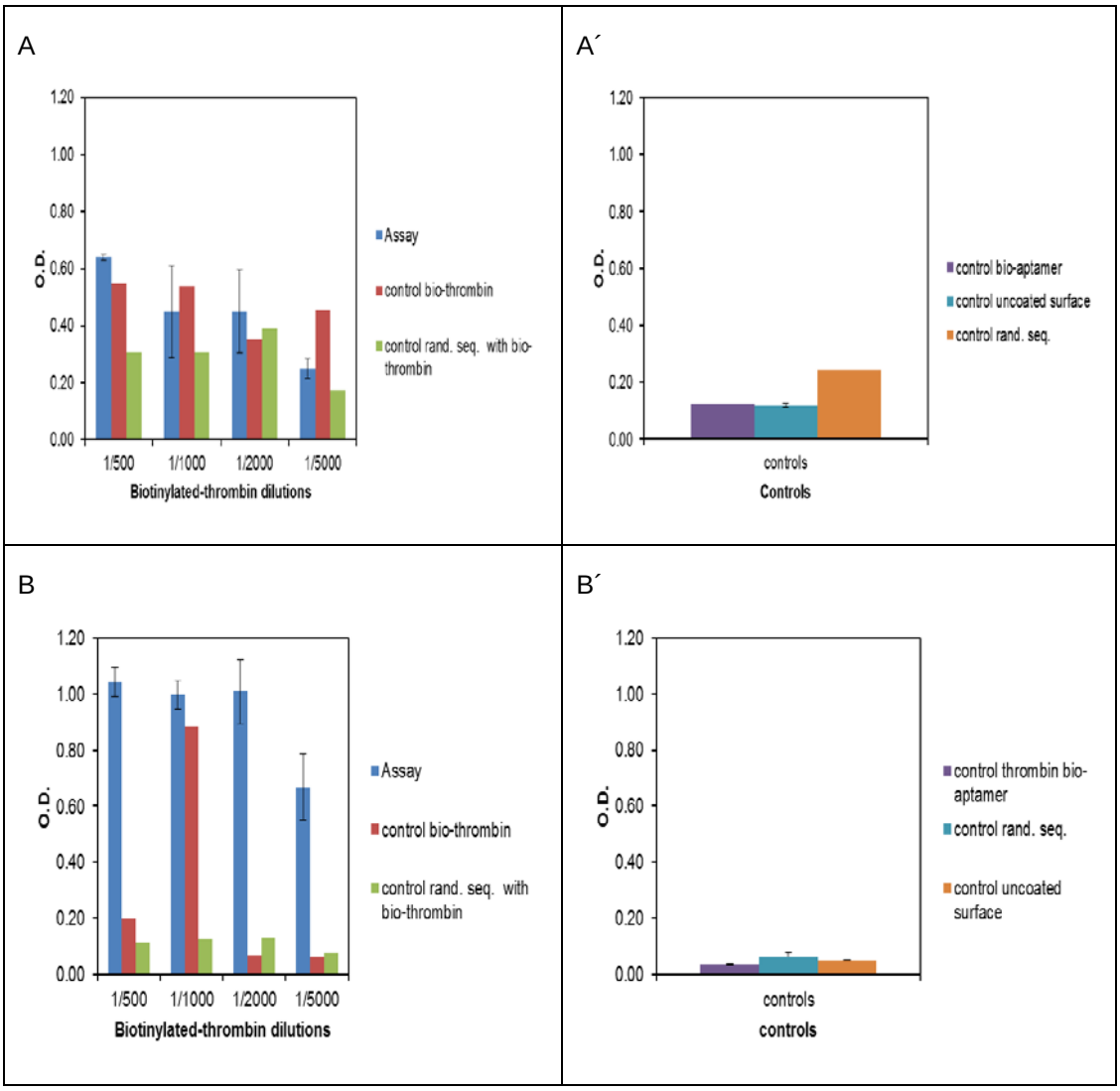


Figure 5-11 Comparison between two assay protocols, A and A´) based on Centi et al (2008), and B and B´) based on Baldrich et al (2005). A´and B´represented the assay unspecific binding, (Baldrich, Acero et al. 2005, Centi, Messina et al. 2008).

The results obtained with the protocol based on Baldrich et al (2005) presented lower unspecific signal and a higher binding signal. For that reason was immediately adopted. It was then tested the performance of the two thrombin aptamers.

iv. 15 oligomer versus 29 oligomer aptamer to bind biotinylated thrombin

All the previous assays were performed using the poly T 15 oligomer aptamer against thrombin. It was decided to test also the 29 oligomer with the corresponded controls. When running an assay with both the aptamers, no binding signal was obtained for the 29 oligomer. This was repeated and again no binding signal was found. Because of this the 29 oligomer was not used in the following thrombin assays.

v. Impact of pH and working buffers in the assay performance

It was decided to test the impact of the working buffers on the assays. In order to do so two different tests were made. In the first the biotinylated aptamer was in different buffers and the biotinylated thrombin in HEPES as before, in the second test the biotinylated thrombin was in different buffers and the biotinylated aptamer in PBS as before. The buffers were HEPES, HEPES with KCl and an ion rich buffer, Table 5-7.

Table 5-7 Buffers tested in the assay to study the impact on aptamer and target binding.

Poly T 15 oligomer thrombin aptamer	PBS pH 7.4 or 10 mM HEPES with 50 mM KCl, pH 8.0, adapted from Baldrich et al (2005), (Baldrich, Acero et al. 2005) Buffer C (5 mM Tris-HCl, 0.5 mM EDTA, 1 M NaCl) pH 7.5, adapted from Centi et al (2008), (Centi, Messina et al. 2008, Baldrich, Acero et al. 2005)
Biotinylated thrombin	10 mM HEPES pH 8.0 or 10 mM HEPES with 50 mM KCl, pH 8.0, adapted from Baldrich et al (2005), (Baldrich, Acero et al. 2005) Buffer D (50 mM Tris-HCl, 140 mM NaCl, 1 mM $MgCl_2$, 0.1% BSA) pH 7.4, adapted from Centi et al (2008),(Centi, Messina et al. 2008)

In the first example, where the only change was the buffer in which the aptamer was in, it was seen similar binding results between the different buffers, but the background was lower with the PBS buffer. In the second test, it was seen a slightly lower binding signal with buffer D, a higher background with HEPES and an improved signal with HEPES with 50 mM KCl. Despite the better binding signal, the background was also high, similar to the HEPES. Because it was not possible to develop these tests to a full understanding of the impact of KCl ions in the aptamer conformation and because the buffer D was rich in ions and did not show such binding improvement, it was decided to maintain the original buffers, the PBS for the biotinylated aptamer and HEPES for the biotinylated thrombin.

vi. Aptamer pre-heating and its influence in binding ability

Once more it was tested if the temperature influenced the aptamer performance or not. The aptamer was pre-heated as before; 94 °C for 10 min and then put on ice before being added to the assay. The results obtained did not show any major differences from the assays obtained with a lower binding signal. There were sometimes assays with lower binding signals than others. This might have been due to aptamer poor conformation or any internal change, room temperature or enzymatic degradation, which interfered with the aptamer correct folding. Or even the distribution of analyte was not presenting the binding site as well as in other assays. If any change was seen due to temperature pre-treatment, it was the obtained higher standard deviation than in the previous assays. As seen previously the pre-heating treatment did not increased binding signal or decreased background, for this reason it was not considered to be used as a routine step for this assay, Figure 5-12.

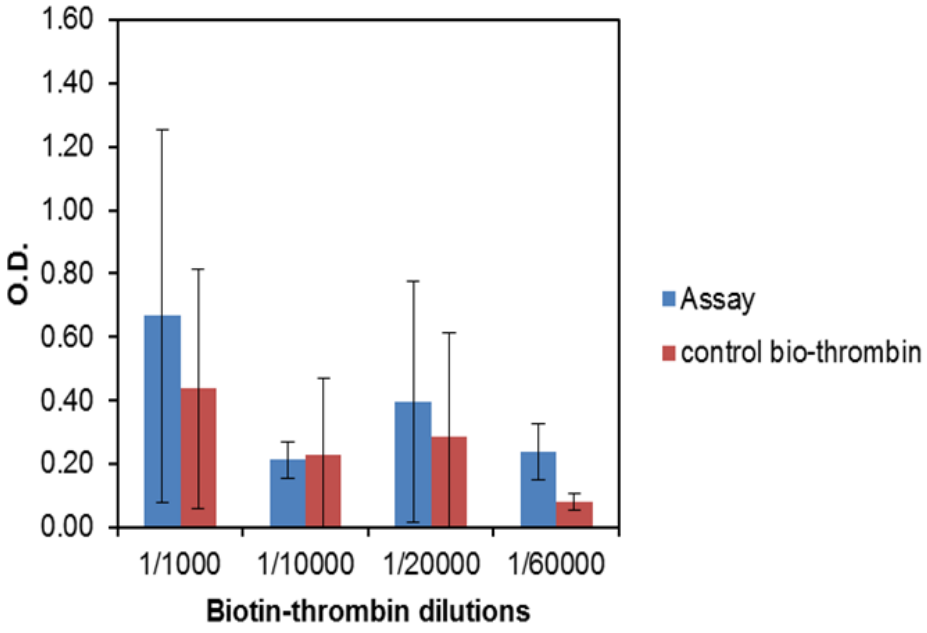


Figure 5-12 Assay with pre-heated aptamer at 94 °C for 10 min and then ice cooled.

vii. Direct binding assay and competitive binding assay

It was decided to perform a direct binding assay under the same conditions published by Baldrich et al (2005), (Baldrich, Acero et al. 2005). A direct binding curve was obtained with biotinylated thrombin dilutions from 1:1000 to 1:20000 and the biotinylated poly T 15 oligomer aptamer at 50 nM. The assay controls showed

unspecific binding of bio-thrombin at the lower dilutions, in special at 1:2000. This unspecific signal might be due to the binding of bio-thrombin to SA-HRP. This could have happened because of inefficient washing steps or poor blocking. When analysing the signal obtained in the controls for the biotinylated aptamer, the random sequence and the uncoated surface, the values were similar to what was seen in Figure 5-11, (data not shown). The control, to see unspecific aptamer binding, showed again higher values in the lowest dilution of biotinylated-thrombin. Despite the unspecific signal at the lowest dilutions of analyte it was obtained a binding curve with an r^2 of 0.94, Figure 5-13. It was also seen that lower dilutions of biotinylated thrombin gave lower binding signals; this probably indicated a saturation of analyte that prevented aptamer interaction.

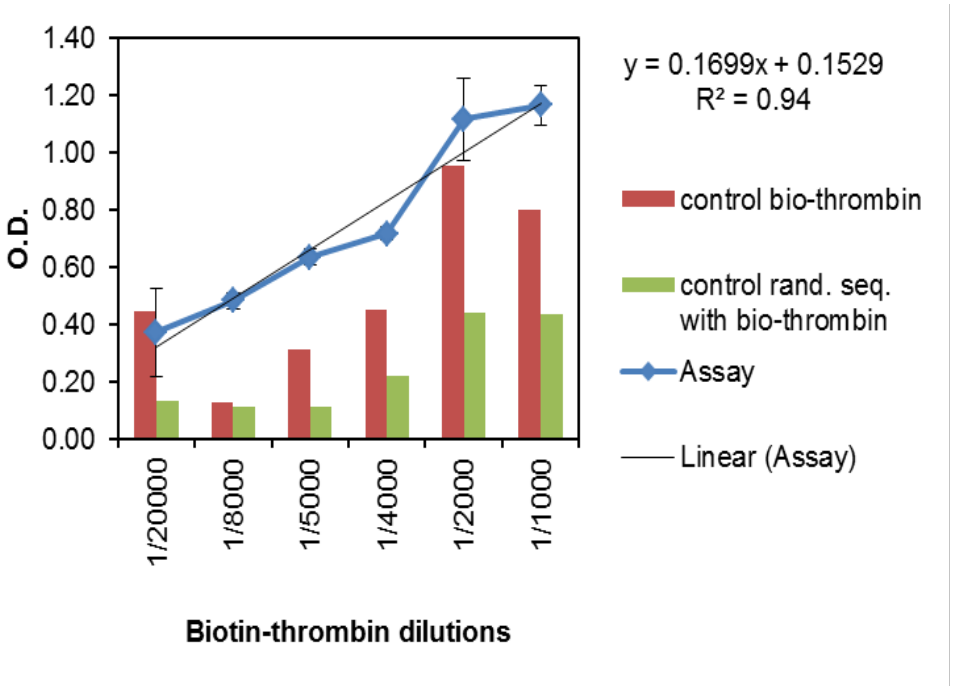


Figure 5-13 Direct binding curve with controls of biotinylated thrombin and biotinylated poly T 15 oligomer aptamer.

Afterwards a binding competitive assay was attempted with different dilutions of thrombin competing with biotinylated thrombin at 1: 1000. This value was chosen because it gave higher binding signal in the direct binding curve.

The obtained competitive binding assay signal was very low showing the preference to bind free thrombin instead of its biotinylated version. In the highest dilutions of free thrombin the highest competition signal obtained was of 0.22. This was so low that the competition assay was considered very difficult to obtain under this detection method.

a) Discussion

As a well-known assay the thrombin assay showed how sensitive aptamers were to assay working conditions. It was expected to be a straightforward and robust assay, simple to perform. The assay fundamentally worked since the first run, but it was difficult to reproduce the assay consistently. It was reported by Baldrich et al (2005) that thrombin was very unstable in solution; this could also have been one of the reasons for the signal variability, (Baldrich, Acero et al. 2005).

Two biotinylated aptamers were tested against biotin modified thrombin. The 29 oligomer aptamer did not show any binding signal. It was assumed that the thrombin binding site was used for biotin modification. It was considered that binding of the 15 oligomer aptamer was result of using a poly T tail to add length between the immobilising surface and the binding place. It was expected that the aptamer folded more freely, without any steric hindrance, and in that way bound the target efficiently. Musumeci et al (2012), described the three binding sites of thrombin and referred the interactions of the two aptamers with the target. The 29 oligomer interacted with thrombin hydrophobically to its heparin region while the 15 oligomer bound, via the G-quadruplex structure and electrostatic interactions, to the fibrinogen region. The same study mentioned that thrombin aptamers were very sensitive to enzymatic degradation and that should be chemically modified to prevent their denaturation, (Musumeci, Montesarchio 2012). It was attempted to understand if the biotinylation affected the heparin binding site but no detailed covalent chemistry information was found about the subject.

It was found in literature that K^+ ions gave higher stability to quadruplexes structures than Na^+ ions. Perhaps this was the reason why it was only seen an improvement in the binding signal in the buffer with K^+ in comparison with the other buffers,(Sigel, Sigel et al. 2012). It was also found in literature the interaction between the thrombin and the 15 oligomer aptamer was helped with K^+ and Na^+ ions, (PDB 2005, Russo Krauss, Merlino et al. 2012). Despite this it was reported that the aptamer in an assay performed at 4 °C (it was the adopted situation), was not depended on K^+ ions to achieve a G-quadruplex conformation, (Baldrich, Restrepo et al. 2004). Nevertheless more investigation would be necessary to understand why the presence of divalent ions did not show any major improvements in the binding interaction while K^+ ions did and if that effect was real or a false positive result.

Centi et al (2008) reported no binding in an assay with thrombin surface immobilised and the addition of aptamer. This was not tested here because of those reported results, but it was interesting to verify that the interaction of a protein as thrombin suffered when surface immobilised, and that the obtained results in this case showed binding only with the aptamer that presented a poly A tail that added length from the surface.

Summary and Conclusion

The thrombin assay was tested in order to use a well-known example to study aptamers in the LMC assay type. It was found in literature that the thrombin aptamer did not bind to surface immobilised thrombin and the assay was developed with immobilised aptamer and addition of biotinylated thrombin. The obtained biding signal occurred with the aptamer modified with a poly T tail. It was possible to run a direct binding curve but when testing a inhibition ELISA the highest signal obtained was very low, showing that the affinity to the free thrombin was higher and it was necessary to obtain a more robust direct binding assay. This was not possible to pursue within the allocated time frame.

5.8. Discussion to identify the aptamer (s) that was adequate to run LMC microarray plate assays

Finding examples of aptamers against small molecules was challenging because the majority of the targets were not as simple as the LMC targets and it was chosen to work with DNA, and some of the targets of interest had RNA aptamers. When searching for examples within the DNA aptamers against small molecules, where the aptamers had been used in detection assays it was found only two examples with surface immobilisation. It was found that kinetics studies and biosensors with aptamers against small molecules were made, in their majority, in solution.

The LMC assay was a competitive assay with immobilised target derivatives and labelled aptamers. This meant that an aptamer would have to bind to a target in solution as well as to its immobilised derivative. It was important to considerer that the LMC targets had low molecular weight and for that reason their chemical modification could interfere in their binding sites availability. As seen before, aptamers bound preferentially to regions rich in ions or complex structures as aromatic rings. The requirement of the LMC assay to surface immobilisation of target derivatives, in the

majority of times was made via the target “richer interaction” site. This was expected to have a negative impact on the aptamer-target interaction. Other point of change was that the aptamer was labelled or modified somehow at one of its extremities. Those modifications were proteins which introduced new chemical interactions within the aptamer and that might have interfered with the aptamer initial folding structure.

Also when adding the SA-HRP to the biotinylated analyte that formed the aptamer-target complex, the complex could be destroyed due to the new covalently attached label. Although Batchelor-McAuley et al (2009), reviewed the applications of DNA into electrochemistry detection methods, and mentioned that HRP and biotin were the most common labels used for detection, (Batchelor-McAuley, Wildgoose et al. 2009). The ideal would have been to use directly modified analyte with HRP. This was tested with OTA without positive results. It would be interesting to understand the impact of this modification and binding interaction of the enzymatic label in the formed complex. The published example where it was run a inhibition ELISA with β-estradiol aptamer, the aptamer was directly labelled. Maybe the post-complex labelling in this case interfered with the target-aptamer interaction. In the case of the ibuprofen aptamer, tested in the label-free detection system, SPR, the detection system was not sensitive enough for such low molecular weight target and the modification of it to increase mass and subsequently the index of refraction might have modified too much the target and prevented binding.

Mitchell et al (2005) suggested the use of a water soluble linker to surface immobilise progesterone into a dextran CM5 chip, (Mitchell, Wu et al. 2005). The same authors also suggested the use of gold nanoparticles to enhance the binding signal after complex formation. This was done in an immune assay so no reference to aptamers modification was made. A similar method was also used by Wang et al (2008), but in that case using aptamers, (Wang, Zhou 2008). Gopinath (2010) reviewed the SPR applications with aptamer and small molecules. The author reported only two examples between low molecular molecules and DNA aptamers without any modification to enhance the binding signal in SPR (Gopinath 2010).

When observing the results obtained and comparing it with the published work, it was clear the challenge in obtaining a LMC type working assay. In the examples tested the target surface immobilisation and addition of aptamer in solution only occurred for OTA and presented high unspecific signal maybe due to incompatibility with the chosen detection system. It would be interesting to test a different label system and verify if the

unspecific signal would decrease. The obtained data was similar to what was reported in literature. Bonel et al (2010) mentioned that the assay type with surface immobilised OTA was unstable and difficult to reproduce, (Bonel, Vidal et al. 2011). This was not expected because the OTA aptamers were generated using OTA surface immobilised, and for that reason it was initially assumed that if the surface immobilisation was made at the same position there would be aptamer interaction.

It was also found that the thrombin surface immobilised did not bind to the aptamer in solution, (Centi, Messina et al. 2008), because of this it was not even tested in this work the thrombin surface immobilisation.

When surface immobilising the biotinylated aptamer and adding in solution the target, in the tested examples it was obtained results for the thrombin aptamer. It was not possible to obtain a binding signal with OTA, maybe because of a poor chemical modification with the enzyme HRP. This was not expected as examples were found in literature where OTA-HRP or OTA bound to immobilised aptamer, (Wu, Hu et al. 2011, Prieto-Simon, Campas et al. 2008, Barthelmebs, Jonca et al. 2011), the modification made was confirmed to be in the same position as the one published, (Worsaae 1978). It was found several examples were OTA bound to aptamer immobilised into gold nanoparticles or magnetic particles, (Yang, Wang et al. 2011, Ma, Yin et al. 2013). It was expected also to obtain a clearer difference between the wash-liquid and the eluted OTA solution when the assay was tested in SA Dynabeads®. That did not happen perhaps because the aptamer denaturation was not very efficient.

The immobilisation of the aptamer-OTA-BSA after interaction in solution was a novel approach and it was possible to run a binding curve. Despite this the obtained binding signal was low and it was not run a competitive assay.

It was relevant to observe that the initial aptamer kinetics studies were made in solution for the tested aptamers against small molecules and that it may show that the aptamer and target expected interaction could be affected by any kind of surface immobilisation or chemical modification, (Cruz-Aguado, Penner 2008, Kim, Hyun et al. 2010, Barthelmebs, Hayat et al. 2011, Kim, Jung et al. 2007).

The presence of ions was known to be important for aptamer conformation and stabilisation. This was referred by Cruz-Aguado (2008) for the OTA aptamer and the used buffers were rich in divalent ions as Mg^{2+} or Ca^{2+}, (Cruz-Aguado, Penner 2008). The thrombin aptamer as a well-known G-quadruplex structure showed increased

binding response when in presence of KCl but no further investigation was made and the HEPES buffer was used without any ionic additives. This was chosen because it was obtained better results with this buffer than with an ions-rich buffer and it was not understood why it happen. Because of that it was kept the initial working buffer conditions, this decision was supported by Baldrich (2011), who reviewed that in the majority of times the assay buffer was similar to the selection one, (Baldrich 2011).

5.9. Summary and Conclusions

It was found in literature ten examples of DNA aptamers against small molecules that presented similar physiochemical characteristics with the LMC targets. Within those examples it was only found two of them where the aptamers had been integrated into LMC assay or similar formats. It was considered that if an inhibition LMC assay would be developed with one of the published aptamers against its small target, it could be assumed that aptamers generated against LMC targets would behave similarly. It was also, based on the few examples found in literature, considered that it would be probably difficult to develop a LMC working assay.

In addition to the two examples found in literature, it was add an extra small molecule had been used as positive control in the previous chapter and had published aptamers against. In summary, three examples of aptamers against small targets were tested and two of them had published work where the aptamers had been used in assays with surface immobilisation. Because in this work it was not achieved a developed assay where it was possible to run an inhibition assay with the aptamers against small molecules, it was also studied the thrombin aptamer. The Figure 5-14 summarises the assays tested with the four targets and their aptamers.

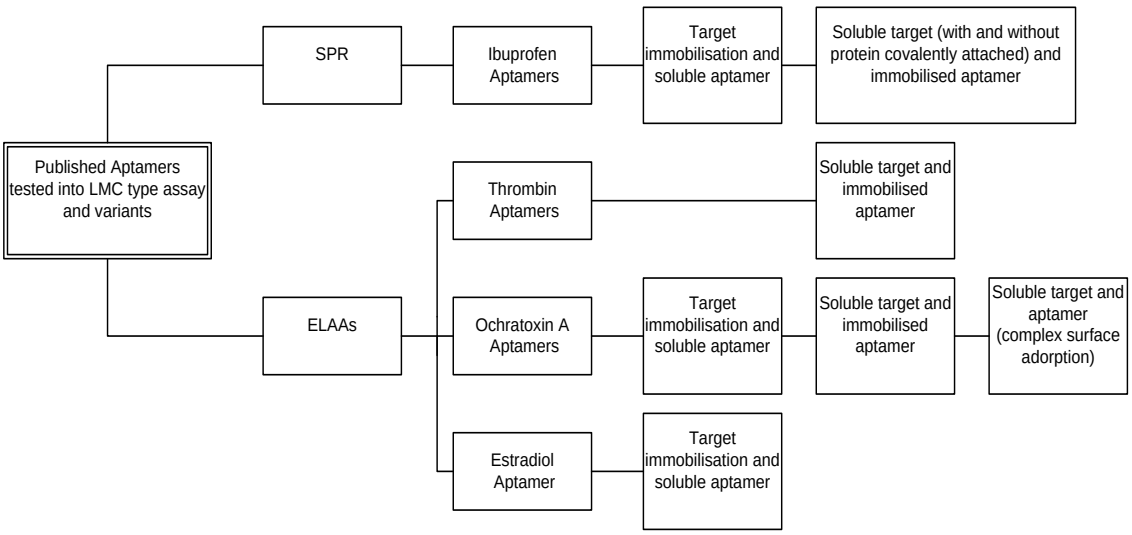


Figure 5-14 Diagram with the aptamers and assay formats tested in this chapter.

It was discovered on aptamer work against small molecules several points that required further work to fully understand their impact on the aptamers integration onto the LMC assay. The first point was about the aptamers against small molecules sensitivity to targets surface immobilisation showing unstable binding results. It was also referenced in literature that the thrombin aptamer did not bind to surface immobilised thrombin. Another concern was regarding the addition of the enzymatic label, which could interfere in the complex interaction. The low interaction could probably be overcome using a different detection method (test the released ions instead of the enzymatic HRP colorimetric product, for example), or label directly the aptamers not introducing an extra structure to the fragile aptamer-target complex. The final consideration was regarding the addition of a spacer between the surface and the target that could expose better the target into solution, reducing possible surface effects of steric hindrance.

Based on the previous points, it was concluded that it was very difficult to integrate aptamers against small molecules in the LMC assay format within the LMC allocated resources and timeframe.

Chapter 6. Initial considerations of Planetary Protection and Contamination Control for the LMC

6.1. Chapter Introduction

The LMC instrument as part of a payload within a planetary exploration mission to Mars had to obey to a certain number of requirements and parameters. Two of those requirements were the prevention of Mars contamination with Earth´s microbial or organic contaminants, and the prevention of detecting contaminants on the Martian sample, (false positive results). In order to prevent both of the situations; it was developed the Planetary Protection and Contamination Control (PP&CC) regulations.

In this chapter it was described an opportunity that arose to demonstrate instrument designs and handling protocols associated with PP&CC via a stratospheric balloon flight to search for life in the extreme environment of the Earth's Stratosphere (the CASS•E experiment) and from which design and handling protocol lessons relevant to the LMC were drawn.

Also in the context of aptamers as alternative bioreceptors for the LMC, it was considered in this chapter the compatibility of aptamers with sterilisation techniques that were required if the LMC instrument wanted to meet the PP&CC requirements.

6.2. Literature Review on Planetary Protection and Contamination Control

6.2.1. Importance of microbial control in space missions

PP&CC concept was developed with the objectives of control the presence of biological burden (bioburden) transported to other planets in space missions, and by doing so, protect the explored planets from Earth-life-like contamination. Also to protect our planet, in the case of return missions, from extra-terrestrial matter, and to prevent detection of false positives. All of this was made by controlling and reducing the microbial presence on the mission spacecraft and in special on the hardware that collected and analysed the Martian samples.

In order to regulate the space missions and to generalise good practices among the several global space agencies, the Committee of Space Research, (COSPAR) was

created. This committee has the responsibility to regulate and oversee the PP&CC implementation on each mission.

COSPAR and PP&CC regulations- Principles of PP&CC

The implementation of PP&CC was standardised by the European Cooperation for Space Standardization (ECSS) and NASA´s office of Planetary Protection. The Committee of Space Research (COSPAR) regulated the Space research, policies and the practices implementation. The PP&CC objectives were to prevent extra-planetary contamination with Earth´s microorganisms or organic molecules by controlling the presence of bioburden on the spacecraft hardware. The major ways of meeting PP&CC requirements were to reduce the levels of viable microorganism contaminating the spacecraft or specific instrument, and to control and prevent microorganisms' re-contamination. The major concern was the microorganisms, (such as extremophiles or encapsulated forms of bacteria or fungus) that could survive the implementation of sterilisation processes associated with meeting PP&CC regulations. Those resistant microorganisms that maintained their viability, could recover from a dormant state once the environmental conditions became favourable. The PP&CC was applied with the objective to decrease the microbial presence, at least, until the maximum value accepted by the COSPAR regulations. This was considered as the sterility assurance level (SAL), which was the estimated probable value of microorganisms present after PP&CC implementation, (Barengoltz 2010). The bioburden could be found on the aircraft surface, mated or encapsulated. The location could influence the effectiveness of reduction but also the release into a new environment. The bioburden reduction was achieved by following standard processes that could either decreased the presence of microorganisms or prevent re-contamination. Those were dependent on the microorganisms' location, the efficiency of the process and the initial biodiversity present. As seen in section 2.6 (Chapter 2), the space missions were classified into five categories and sub-categories. The mission was categorised according to its aim and destination. The LMC instrument being part of the ExoMars mission which had a category of IV-b (as seen in section 2.6) required a limit of 2 $x10^5$ bacteria spores / m^2 on the spacecraft exterior and less than 300 bacteria spores / m^2 on the LMC interior, (Pillinger 2009).

6.2.2. PP&CC implementation

PP&CC implementation required a group of techniques that reduced the bioburden and controlled the biological recontamination. That required the assembling into specific controlled environments (cleanrooms), team work (constant control of personnel contamination), microorganism's physical removal and monitoring, sterilisation procedures, the use of materials compatible with the previous procedures, and spacecraft or instrument design that allowed PP&CC implementation and helped preventing hardware re-contamination after cleaning and sterilisation procedures had been implemented. The PP&CC implementation also required the validation procedures to access cleanliness and sterility levels.

Cleanrooms classes and personnel working preparation

Cleanrooms were air cleanliness controlled areas used for assembling spacecraft and were classified according to the number of particles present per cubic meter. The PP&CC implementation required at least an ISO 8 cleanroom category (ISO 14644-1:1999), which had a maximum of 100^3 particles per cubic meter of air. Cleanrooms bioburden was controlled applying the standard ECSS-Q-ST-70-58C. That standard regulated the cleanroom design, controls of cleanliness, how to operate inside a cleanroom, the garments requirements and the necessary training to operate inside a cleanroom. The cleanliness and contamination control were regulated following the standard ECSS-Q-ST-70-01C, (ECSS 2012, Debus 2006).

The space organization of the cleanroom should be in such a way that the personnel movement was made from the last clean area to the cleanest. This could include different areas within the cleanroom with different levels of cleanliness. The cleanroom air should be filtered with a high efficiency particulate air filter (HEPA) and the floor and walls mopped and wiped before use. The HEPA filters also had different levels of filtration and were classified (EN1822:2009) according to their efficiency and particle size at which the filtration was lower. The personnel had to wear special garments that minimized their contamination of the surrounding area. Personnel were the major source of contamination inside a well prepared cleanroom. Considering this, the use of garments, gloves, masks and shoes-covers minimised as possible the shed of skin cells, hair and microbial or particle transport. Personnel had also to receive appropriated training about how to move and work inside the cleanroom in order to minimize the particle shed, had to be submitted to health checks and had to have

hygiene habits (e.g. minimum facial hair, no cosmetics of any source) that assured the minimum contamination possible. Moreover microbial control had to be performed regularly and if above the desired levels action for its reduction should be taken, (Boni, Clark 2008, Cheng, Liu et al. 1998, Braun 1998).

Microbial control and reduction techniques

The microbial examination taken inside a cleanroom was regulated in the ECSS-Q-ST-70-55C. That included airborne, surface and personnel microbial control. That control was made using microbiological techniques to collect samples. That was obtained by active air filtration, surface swabbing or wiping, and contact plates. The aim of collecting the samples was to identify and control the presence of microorganisms. The sample collection should have been taken always from the same place for microbial tracking and comparison. The filter or swab material could be analysed directly by its DNA content or prior to it by cell culturing. Only a very low percentage of existing microorganism in nature were cultivable (less than 1%) and because there was no initial information about the collected microorganisms, several cultivating conditions had to be tested, which made it a very long and complex task. There were two approaches taken to identify the microorganisms, either by initially culturing them, or by direct detection of genomic DNA content of the bacterial 16S rRNA gene, in both cases they were classified taxonomically after DNA analysis, (Vaishampayan, Osman et al. 2010). The schematic present in Figure 6-1 presented the standard procedure taken to collect and cultivate the obtained swabbed material,

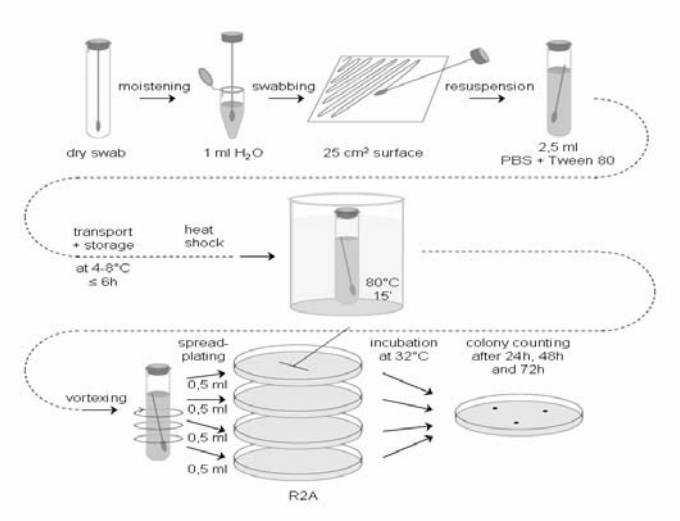


Figure 6-1 Recommended standard swab assay procedure for microbiological sample collection by swabbing a specific area and transferring the collected material into culture media for microorganism analysis, adapted from page 22, ECSS-Q-ST-70-55C, (ECSS 2012).

The decontamination methods were essentially cleaning methods. Those intended to remove physically and to destroy most of the present microorganisms. There were several options but ideally the chosen cleaning liquids would be efficient either in bacteria and in fungi, do not be inflammable, toxic, corrosive or expensive. The usual cleaning liquid chosen was sterile isopropanol alcohol (IPA) in a 70 % aqueous solution, this concentration was the most effective to destroy microorganisms, by being a disinfectant and interfering with cells protein structures present in the cellular walls and enzymatic activity. IPA was a cheap consumable easy to use and have access to, with a low level of toxicity when compared with other chemicals. IPA was used in solution applied directly onto surfaces, used to immerse components or to impregnate cleaning wipes, (Whyte 2001).

Besides destroying and physically removing the maximum possible content of microorganisms, PP&CC required the implementation of sterilisation.

Sterilisation - Dry Heat microbial reduction (DHMR)

After implementing the cleaning procedures, and in order to eliminate the viable microorganisms and achieve the required COSPAR acceptable level of bioburden, sterilisation was necessary. The implementation of PP&CC in space missions had been developed along the years which gave the possibility to learn by heritage and to understand which conditions were better to reduce the bioburden, the next table

explained the types of sterilisation methods and in which missions were applied, Table 6-1.

Table 6-1 Sterilisation types used in past space missions, adapted from page 19 ECSS-Q-ST-70-53C, (ECSS 2012).

Type	Methods	Sterilisation type		Heritage	
		Surface	Bulk	Studied	Studied and Used
Chemical	Formaldehyde gas	x	-	Space components	-
	Ethylen oxide	x	-	-	Ranger 1961/62
	Sporicide solution (TBD[7])	x	-	Mars 96	Mariner Mars 1971
	Hydrogen peroxide	x	-	-	Mars 96, Beagle 2, DS 2
Thermal	Dry heat	x	x	-	Viking, Mars 96, Pathfinder, Beagle 2, MER, Phoenix, MSL
Steam	Steam (space hardware excluded)	x	-	-	Only compatible components, garments
Radiative	Gama / Beta radiations	x	x	-	Mars 96, Beagle 2

The method of sterilisation by DHMR allowed the reduction in the level of viable microorganisms both in surface and bulked. DHMR was not always compatible with certain hardware components as heat sensitive electronics or with biological components as bioassay reagents.

DHMR used in space missions was regulated in the document ECSS-Q-ST-70-53C, (ECSS 2012). DHMR consisted in heating, under controlled humidity (lower than 1.2 g/m^3) and pressure, for a constant temperature and a certain period of time the desired piece of material, Table 6-2.

Table 6-2 Time and temperature required to reduce the presence of microorganism encapsulated or in free surfaces, adapted from ECSS-Q-ST-70-53C, (ECSS 2012).

	Temperature (°C)			
	110	115	120	125
Free and mated surfaces	32 hr	18 hr	11 hr	6 hr
Encapsulated surface	156 hr	90 hr	52 hr	30 hr

As explained in Table 6-2, according to the applied time and temperature the microorganisms were reduced. The encapsulated ones required more time or higher temperatures to achieve the same levels of reduction than the free ones. The

[7] Usually those solutions had a mixture of hydrogen peroxide and acetic acid, or sodium hypochlorite, (ECSS 2012).

described table was a summary of a logarithmic proportion between the time of implementation, initial number of microbial population and the microbial reduction obtained, using as standard the spore of *Bacillus atrophaeus* (Figure A-22, Appendix A), (ECSS 2012).

In the LMC, the chosen bioreceptors (antibodies) were not compatible with DHMR implementation, and for that reason the assay assembling would be made aseptically, after instrument cleaning and sterilisation and antibodies sterile filtration (Cullen, Mark 2007, Sims, Cullen et al. 2012). If the LMC bioreceptors were compatible with DHMR it would be possible to assemble the assay into the instrument and then implement sterilisation, reducing costs and time. Therefore it would be relevant to known if aptamers, as alternative candidates to LMC bioreceptors, would survive DHMR implementation.

Design impact in PP&CC implementation

The implementation of PP&CC required the compatibility of the used materials with the cleaning process (smooth surfaces and resistant to used cleaning chemicals), with microbial sample collection (to validate cleanliness, sterilisation procedures, and final assay / collection), and with sterilisation (e.g. DHMR). The compatibility of materials with DHMR had been documented (ECSS-Q-ST-70-53C), and listed which materials were compatible and which were not. Besides choosing PP&CC compatible materials it was also necessary to develop a design for the spacecraft that was compatible with PP&CC implementation. The aircraft design had to contemplate the need to have easy access to the experiment to implement the cleaning and validation techniques and to be compatible with DHMR, (e.g. changes in pressure and temperature), (ECSS 2012).

An answer to the problems of having different hardware tolerance, allowing easy access to PP&CC implementation, and reducing the difficulty of meeting PP&CC bioburden levels requirements, was the experiment division in specific areas. The hardware that would be in contact with biological sensitive areas in the extraterrestrial planet was located in an ultra-clean-zone (UCZ). The UCZ was a sealed / separated area from the rest of the aircraft. In that case only the UCZ suffered the most rigorous implementation of PP&CC, making the implementation less expensive and faster in comparison with meeting the same requirements in the full instrument, (Sims, Cullen et al. 2012).

The design of the instrument also contemplated the re-contamination of the experiment after implementation of PP&CC. In order to prevent re-contamination until before flight, the experiment was sealed after assembling in the cleanroom, was bagged every time and stored appropriately, and had a remove-before-flight cover. Another specific design implementation to prevent re-contamination after launch and until sample collection was the biobarriers. Those were implemented on the experiment and prevented the passage of microorganisms from the surrounding environment to the experiment, and could only be breached when appropriated, (Salinas, Zimmerman et al. 2007).

Validation of cleanliness and sterility

Knowing the level of microbial presence was critical to implement adequately the PP&CC protocols. Validation included the detection and quantification of microorganisms present on the hardware surface and based on that it was possible to predict the required level of PP&CC implementation for an efficient bioburden reduction. Validation allowed understanding if the implemented cleaning procedures were working, and also to predict the necessary level of sterilisation implementation. For example when implementing DHMR in standard space missions, it was necessary to estimate the amount of bioburden present to implement the required time and temperature to achieve the highest reduction possible (Figure A-22). Validation was also extremely important to give confidence on the data obtained from the experiment after sample collection and analysis. Validation methods were described on ECSS standards as microbial growth (as explained in Figure 6-1), and DNA analysis. Other techniques that could give an indication of bioburden presence could also be used, as for example the ATP detection, (Davidson, Griffith et al. 1999, Shubert, Kazarians et al. 2003).

6.2.3. PP&CC case study examples

The implementation of PP&CC requirements is an on-going process and past space missions gave input and allowed improvements to present and future missions. Examples of relevant lander missions to PP&CC implementation were the Mars 3, the Viking and the Phoenix because added or improved its design in order to better implement PP&CC requirements. In the ExoMars mission was planned the implementation of a novelty in design to aid in the PP&CC protocols execution.

USSR Mars 3 (1971) lander was the first successful lander to reach Mars surface and it was referred the implementation of sterilisation with methyl bromide and the validation made by microbial viability assays for bioburden control (DeVincenzi, Race et al. 1998).

After Mars 3, the NASA Mars Viking landers 1 and 2, (1975), were the following mission with successful landing on Mars. The Viking was classified as category IV-a, with 5 x 10^5 as maximum value acceptable for the presence of total bacterial spores. One of Viking aims was to detect evidence of life on Mars. The implementation of PP&CC on the Viking mission established standard cleaning and assessing cleanliness procedures and implementation of sterilisation, (Puleo, Fields et al. 1977, Daspit, Stern et al. 1975).

Another important example developed to improve the effectiveness of PP&CC was in the NASA Phoenix lander (2008, category IV-c) where it was implemented a biobarrier around the robotic arm that drilled and collected Martian soil samples. The biobarrier protected the arm avoiding microbial contamination during the launch and until it collected the samples, (NASA 2012).

The ESA ExoMars rover (under development) was designed with a specific compartment, named UCZ, which was a comprising surfaces zone which would be in contact with collected samples and into which ultra-high sensitivity instruments (organic biomarker detectors) for collection and analysis would be placed or interfaced and for which there was a stringent requirement on cleanliness. Therefore the resulted design of an UCZ allowed a more efficient implementation of PP&CC because it was a separate sub-system that could be cleaned to a higher level than the other rover sub-systems that did not contact the collected sample; and also at the same time was isolated to prevent re-contamination. Having a UCZ removed the problem of having to implement the whole rover to the same level of cleaning and protection from recontamination and therefore reduced the overall cost and complexity of the mission, (Sims, Cullen et al. 2012).

6.2.4. PP&CC in the context of the LMC

The LMC as an instrument initially designed to be part of the ExoMars mission, had to be compatible with the PP&CC requirements of a category IV-b mission, (Pillinger 2009).

Status of PP&CC within the context of the LMC

Initial PP&CC plans were developed for the LMC. It included a plan for contamination control, for instrument bioburden control and reduction, prevention of recontamination and procedures to take during the mission different stages (assembling, integration, verification and launch). It was described the cleanrooms procedures and personnel requirements, how to obtain microbial samples from specific areas of the LMC, the intended statistical treatment of obtained data and the consequent estimation level of bioburden. It was planned to start PP&CC implementation after the critical design review (CDR) but currently the LMC was removed from the ExoMars, so PP&CC implementation will be made for future missions.

PP&CC considerations for molecular receptor assays

The LMC PP&CC plan described the sterilisation of LMC subsystems by the most appropriate approach (e.g. sterile filtration), and then aseptically assembly the sterilised subsystems into the final LMC instrument. This was driven by the fact that the LMC bioreceptors (antibodies) would not survive DHMR. The LMC bioreceptors would suffer sterile filtration prior to aseptic assembly. Nevertheless it would be desirable to be able to clean the LMC subsystem, then assemble it and sterilise the final instrument by DHMR, (Cullen, Mark 2007). If aptamers could survive DHMR, this would allow the LMC (if implementing aptamers) to be sterilised post assembly by DHMR thereby reducing the complexity and costs of building the LMC

6.2.5. CASS•E - a stratospheric life detection experiment as a proxy for implementing PP&CC approaches relevant to the LMC

The opportunity of implementing the principles of PP&CC appeared with the chance to develop a small scale experiment with the aim of collecting stratospheric microorganisms and particles.

Cranfield Astrobiological Stratospheric Sampling Experiment - CASS•E

The ESA, the German Aerospace Center (DLR), the Swedish National Space Board (SNSB) and the Swedish Space Corporation (SSC), developed and promoted educational programmes among the new generations. One of those programs was the rocket and ballon experiments for university students (REXUS/BEXUS), where rockets and stratospheric balloons were launch giving opportunity to students to develop

experiments to fly in them. The program involved, during one full year, a sequence similar to the one found in the space industry where the aircrafts needed to go through several established evaluation periods before they actually flight, (REXUS/BEXUS 2012).

The aims of CASS•E were to attempt collection of microorganism and particles present in the Stratosphere and to demonstrate the implementation of PP&CC procedures during the experiment development. The implementation of PP&CC procedures was done to prevent false positive results and to demonstrate the implementation of the protocols under a small experiment that could be scaled up for missions like the LMC and to take conclusions that could be applied in the future. CASS•E design included two important features, an UCZ in which the samples were collected and biobarriers that were only open before sample collection. Besides the novelty of using an UCZ for the sample collection area, it was also tested the use of fluorescent beads to track microbial contamination pathways in order to validate the collected samples. CASS•E travelled to the stratosphere twice in a stratospheric balloon gondola in the BEXUS flight 10 first and then on BEXUS flight 11. That occurred because in the first flight the opening of one of the biobarriers did not happen and the other was destroyed during landing. During the first flight it was not possible to verify air flow through the pumps. Flying on BEXUS 11 was a special second opportunity given because it was impossible to conclude about the PP&CC implementation on the first flight. On the second flight it was possible to verify air flow through the pumps and CASS•E was recovered in good conditions with both of the biobarriers breached and the UCZ sealed. It was possible to open inside a cleanroom the UCZ and remove the sample collection filters and analyse them, (Juanes-Vallejo, Grama et al. 2011).

6.3. Chapter Aims and Objectives

In this chapter it was used CASS•E as an opportunity to demonstrate PP&CC methodologies, and aptamers to study their compatibility to PP&CC sterilisation.

Specifically the aims of this work were:

- To consider design characteristics for PP&CC implementation.
- To implement an new method to track microbial contamination.
- To extrapolate, if possible, the learned lessons into the LMC.

- To study preliminary the aptamers resilience to DHMR conditions and discuss the implementation of DHMR if aptamers were chosen bioreceptors within the LMC.

These aims resulted in the following objectives:

- To implement UCZ and biobarriers as design modifications for PP&CC requirements.
- To design and implement an AIV plan to decide on how to build CASS•E enabling the PP&CC requirements.
- To implement standard cleaning PP&CC protocols for microbial reduction.
- To assess cleanliness using an emerging procedure (ATP bioluminescence assay).
- To implement sterilisation with DHMR.
- To use fluorescent beads to track microbial contamination pathways.
- To demonstrate resultant experiment in flight conditions.
- To test aptamers resilience to DHMR at different conditions.
- To conclude about the implementation of PP&CC, the design changes, the use of fluorescent beads, and extrapolate lessons learned into the LMC along with the considerations made about the aptamers resilience to DHMR.

6.4. Chapter experimental rationale

In space life search missions, it is necessary to implement several procedures to reduce the biological and organic content transported to other planets. Those procedures while reducing the presence of microorganisms and organic content can also damage the bioreceptors used for targets detection. In the work described in this chapter it was possible to use a small scale experiment to demonstrate the implementation of standard planetary protection procedures, as cleaning and sterilising, and at the same time demonstrate the novel design implementation of a UCZ, and also a new use of fluorescent beads as a validation technique to track contamination. Those changes reduced the mission costs by improving the implementation of the planetary protection techniques because they were confined to one specific area, and the confidence in the obtained data. In the same way as it was implemented a sterilisation procedure in the small scale experiment, aptamers were also preliminary tested if were compatible with the sterilisation procedure and in that way reduce the costs to space missions. Usually bioreceptors are treated differently

than the instrument and only assembled at a later stage. If aptamers could survive sterilisation procedures could be implemented earlier and reduce time and costs. The aptamers were freeze-dried and kept at 110°C for 32hr. A second test, in harder conditions, was also made with aptamers at 120°C for 52hr. After suffering the sterilisation procedure the aptamers integrity was accessed by UV and agarose gels. It was seen that aptamers could be easily used as bioreceptors in space missions because could be integrated in the instrument prior to sterilisation and that the implementation of a UCZ in the space instrument design as well as the use of validation methods improve the confidence in obtained data and decreased the overall mission costs.

6.5. Use of PP&CC approaches within CASS•E as a case study for the LMC

CASS•E was a small scale space project, that had to obey to procedures and requirements of a standard space mission. By having the aim of collecting stratospheric microorganisms or particles, it was necessary to implement the same principles of a life-search space mission. This was because it was important to confirm that the collected samples were not contamination. PP&CC protocols were implemented as well as design specificities such as an UCZ and biobarriers, and on top of that a novel validation technique to verify microbial contamination pathways.

Author's role within the CASS•E project

CASS•E was a student-led team project within the ESA BEXUS programme. Each team member was allocated a number of specific roles. The roles of the author was to implement standard PP&CC cleaning protocols, assess cleanliness levels including CASS•E UCZ and components, and be responsible for the overall PP&CC implementation and compliance within the CASS•E programme. This role included contribution to the design of UCZ and biobarriers and the design and implementation of the fluorescent beads protocol to track microbial contamination. It was noted that the latter tasks were led by other team members. The author's contribution to the latter two activities is estimated to be 25% in each case.

6.5.1. Demonstration of an Ultra Clean Zone concept within trace-life detection / collection instruments

The implementation of a UCZ solved the problem of incompatibility of certain hardware to the implementation of PP&CC, and allowed the separation of sample collection into a specific area that could be rigorously clean and sterilised. Also implementing rigorous PP&CC in only an area on the spacecraft reduced time and cost. It was implemented on CASS•E an UCZ with the aim of demonstrating the concept. The UCZ was an isolated sealed rectangular area in which was located the sample collection filters. CASS•E´s UCZ was made of aluminium alloy (compatible with cleaning, sterilisation and validation methods) and was sealed on its top face with Tyvek and a transparent polypropylene sheet (Tyvek was porous and allowed UCZ internal pressure equalisation during sterilisation and flight). UCZ was an independent piece that was mounted on the experiment main support frame before flight. The UCZ was connected to pumps on one side and inlet tubes for sample collection on the opposite side. Inside the UCZ was located, as explained, the sample collection filters, but also barrier filters, valves, temperature sensor, foil heaters and an electric panel connector, Figure 6-2.

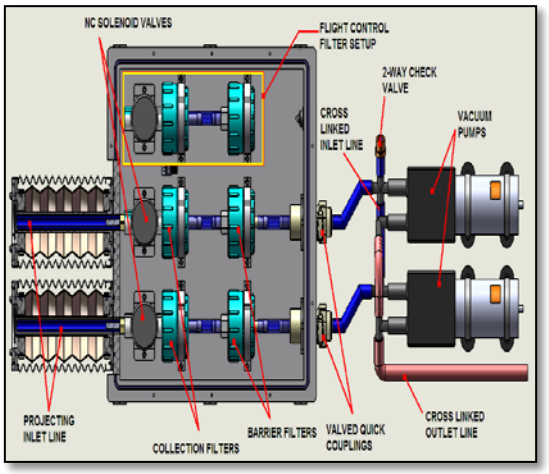


Figure 6-2 CASS•E sample collection schematics showing biobarriers, UCZ and pumps, (Juanes-Vallejo, Grama et al. 2011).

As seen in the figure, inside the UCZ there were three filters sets, two for collection (for redundancy) and one for control. Each set had a collection filter and a barrier filter. In front of each collection filter it was located a valve, the valves and the barriers filters protected the collection filters from contamination after the biobarriers opening and

from the pumps. The UCZ was connected with two projecting inlet tubes each covered with a bio-barrier, Figure 6-3.

Figure 6-3 UCZ after being assembled inside a cleanroom and before being sealed with a top Tyvek and polypropylene sheet.

The UCZ materials and design emerged from the requirements defined for an experiment with the aim to collect stratospheric microorganisms that mimicked a life-search mission as much as possible regarding PP&CC implementation. The UCZ had to be compatible with the cleaning, sterilisation and validation methods. In Table 6-3 it was discriminated the requirements that lead to the UCZ design.

Table 6-3 Design requirements for UCZ and components, adapted from Juanes-Vallejo et al 2011 (Juanes-Vallejo, Grama et al. 2011).

DR16	The filter shall withstand the vibrations created by the pumps.
DR17	The UCZ *should* meet the requirements of a COSPAR category IVa mission *i.e.* bio-burden at launch should be no greater than 300 spores per m^2 pre-sterilisation (this can be assessed by swabbing and culturing)
DR18	All components inside the UCZ shall be compatible with 70% isopropyl-alcohol (IPA) for immersion and/or wiping for cleaning.
DR19	All components outside the UCZ should be compatible with IPA wiping for cleaning
DR20	All components contained within the UCZ shall be compatible with Dry Heat Microbial Reduction (DHMR), *i.e.,* 110°C for a minimum of 32 hours -as per the requirements for a 10^4 reduction in bio-burden by DHMR on free and mated surfaces

a) Discussion

The design requirements for the UCZ gave origin to a rectangular shape confined area, made of aluminium alloy which was compatible with cleaning, sterilisation and the validation implemented methods. The smooth surface, the simple structure and easy access to the UCZ interior before top sealing made UCZ a practical and functional area for PP&CC implementation.

Summary and Conclusion

It was demonstrated with CASS•E a new design concept that reduced overall cleaning sterilisation and validation time and costs. The UCZ improved the PP&CC procedures implementation and at the same time reduced the possibility of re-contamination after assembling because it confined the sample collection area to a specific zone.

6.5.2. Demonstration of Bio-barriers to prevent experiment re-contamination within trace-life detection / collection instruments

As explained before it was implemented on CASS•E design a UCZ that was protected after cleaning from re-contamination with biobarriers. The biobarriers were divided in two major parts, the one that covered the top of the UCZ and the one that protected the collection tubes until sample collection. The biobarriers developed were made in order to respond to design requirements such as the prevention of UCZ re-contamination with particles greater than 0.3 microns after implementation of PP&CC cleaning and sterilisation methods. The requirements were explained in detail in Table 6-4, and included besides the prevention of re-contamination, the resistance to water and impact landing.

Table 6-4 Design requirements for biobarriers, adapted from Juanes-Vallejo et al 2011, (Juanes-Vallejo, Grama et al. 2011).

DR21	The UCZ shall be protected using a bio-barrier to ensure it remains clean after sterilisation and during assembly, testing and integration.
DR22	The re-sealed bio-barrier shall retain 99.7 % of all particles or organisms greater than 0.3 µm in size - NASA planetary protection standards for sealing (this can be assessed by spraying the sealed bio-barrier with fluorescent beads).
DR23	The experiment (specifically the re-sealed bio-barrier) shall withstand landing shocks of up to 35 g.
DR24	The experiment (specifically the re-sealed bio-barrier) shall withstand landing in water.
DR25	The experiment (specifically the re-sealed bio-barrier) shall survive temperatures of -15 °C for up to 48 hours (conditions of gondola whilst awaiting recovery)

The biobarriers had to cover the inlet collection tubes and were made of Tyvek because it allowed gaseous exchange, which was essential for the atmospheric pressure changes during the flight and during the sterilisation procedure. The biobarriers located around the inlet collection tubes had to have an opening mechanism, which could only open in the stratosphere. That was basically a burning wire that was in contact with the Tyvek and that once heated burned the Tyvek, Figure 6-4. The biobarriers design was changed between flight 10 and 11 in order to improve the burning wire contact to Tyvek, Figure 6-5. In the improved design the burn wire was located closely to Tyvek, Figure 6-6.

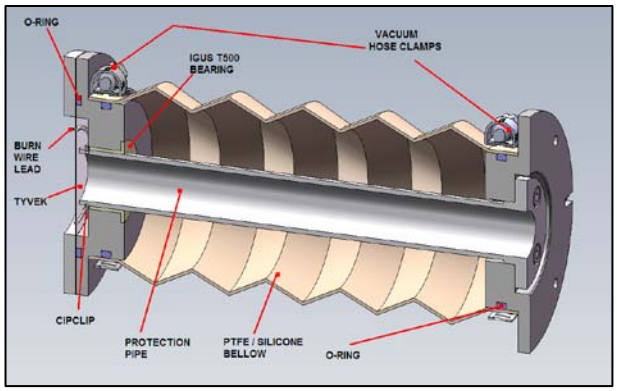


Figure 6-4 Initial biobarrier model designed for CASS•E.

As mentioned before CASS•E had two balloon flights because in the first one it was not possible to confirm air flow for sample collection during flight and one of the biobarriers

did not open and the other was destroyed during landing not allowing to understand if it had opened or not.

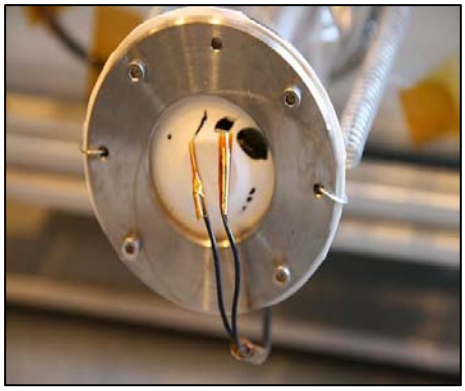


Figure 6-5 Unopened biobarrier on BEXUS flight 10 because of poor contact between Tyvek and the burn wire.

For the second flight the biobarriers opening system was improved and opened during flight. That was confirmed by the increase in registered air flow during flight. The pumps collected stratospheric air for 2 hours before the UCZ was sealed by the valves (data not shown).

Figure 6-6 Re-designed biobarrier prototype breached by burn wire

a) Discussion

The choice of Tyvek as material for biobarriers showed to be suitable to the design requirements regarding PP&CC methods compatibility. The implementation of biobarries had the disadvantage of requiring an opening mechanism that would need to open only at the sample collection phase. In the described case the mechanism failed to open in a first flight but after design improvements worked as expected.

Summary and Conclusion

Biobarriers were implemented into the design to prevent re-contamination of the UCZ after cleaning and sterilisation implementation. Biobarriers had also the aim to open only at the collection site exposing the sample collection tubes, and to resist hard landing conditions. The biobarriers were divided in two major areas, one covering the top side of the UCZ and the other the inlet tubes. In the first flight (BEXUS 10), the opening of the biobarriers failed and it was not possible to validate the experiment. It was given a second opportunity and the biobarriers design was improved in order to prevent the same failure. On the second flight (BEXUS 11) the biobarriers opened and allowed sample collection as desired.

6.5.3. Assembling, Integration and verification plan (AIV) and its implementation on CASS•E

An assembling, integration and verification (AIV) plan was made in order to implement PP&CC during all the phases of CASS•E development. The components were divided in compatible and non-compatible with PP&CC, and were treated accordingly. The PP&CC compatible components suffered cycles of cleaning and cleanliness assessment during assembling, were sealed and sent to sterilisation by DHMR procedure, Figure 6-7.

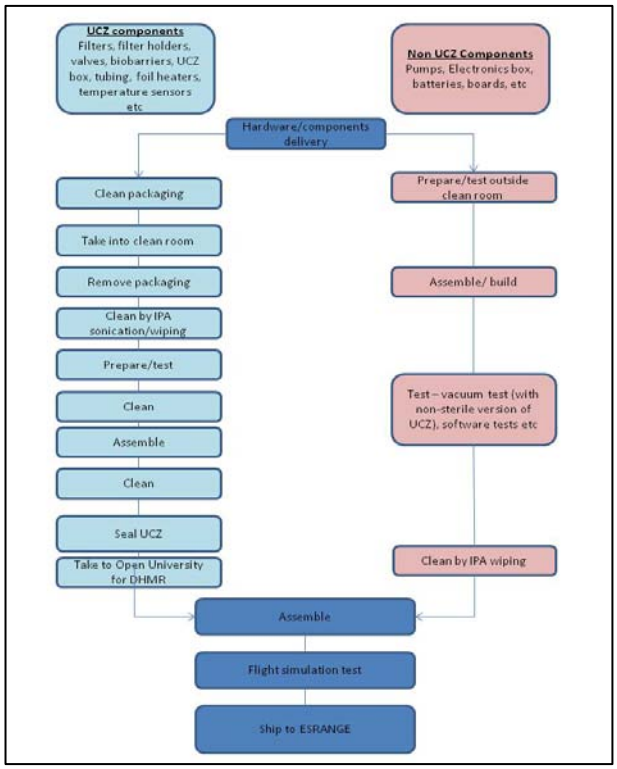


Figure 6-7 AIV plan for PP& CC implementation on CASS•E, adapted from Juanes-Vallejo et al 2011, (Juanes-Vallejo, Grama et al. 2011).

The recognised PP&CC method to validate cleanliness procedures was to swab or wipe the area of interest and then cultivate the obtained microorganisms. This process was slow because organisms needed a minimum of 24 to 48 hours to grow and several testing growth conditions. In this case, because of time, budget and personnel constrains, it was used another way of verifying the presence of microorganisms by detecting the presence of adenosine triphosphate (ATP). ATP was a molecule only synthesised by viable microorganisms and used as an indicator of their presence in food industry. ATP was determined by measuring the bioluminescence obtained from a biochemical reaction catalysed by the enzyme firefly luciferase[8]. The lower the value of ATP detected the better, as it represented the lowest presence of microorganisms on the material surface. However this method had limitations, as many small components were difficult to swab and ATP detection was not ideal for the detection of spores, since spores were dormant and therefore may not had detectable levels of ATP present. Based on the fact that it gave faster results, was cheap and easy to use, it was chosen

[8] Luciferase catalysed the reaction between ATP and luciferin and was expressed by $D-luciferin + O2 + ATP \rightarrow oxyluciferin + CO2 + \boldsymbol{AMP} + diphosphate + light$ adapted from (Biocontrol 2005).

to assess cleanliness during CASS•E´s AIV. There were a number of commercially kits available for ATP detection, which was commonly used in food and healthcare industries as a measure of cleanliness, (Fulford, Walker et al. 2004, Hunter, Lim 2010). Part of the AIV plan included operations procedures to follow before and after launch, (Table A-3, Appendix A), with the aim to track and prevent contamination.

a) Materials and Methods

i. Materials and Chemicals

Liquid sterile 70% IPA, 5 L (CG100-5S), sterile 70% IPA impregnated wipes (71108), sterile 18 MΩ.cm water, 5 L (71919S) from Guardline, ATP luminometer lightening MVP (75005) and ATP swabs (64003-100), from Biocontrol, ultrasonic bath, cleanroom garments, sterile gloves, face-masks, disposable bouffant hat, cleanroom overshoes, cleanroom mat, nitrile gloves, glass beakers, aluminium foil, mops, mopping buckets, vacuum cleaner with HEPA filter, laminar flow cabinet

ii. Method for assembling in an ISO8 cleanroom

The cleanroom had to be prepared by wiping the walls and vacuuming and mopping the floor. Those were made following specific criteria described by Whyte, W. (2001). The same author described the procedures to take to enter and to exit the cleanroom, (Whyte 2001). The cleanroom was organised into areas according to its cleanliness. It was introduced in the cleanest area (far from the entrance) a laminar flow cabinet to be the assembling area. All the movements inside the cleanroom were made at a slow pace to reduce the particles spread.

iii. PP&CC cleaning methods of UCZ, tools, components and cleanroom

The cleaning procedure was made before entering the cleanroom, and again inside. The tools that could be immersed in solutions were treated differently than the ones that could not, as for example the UCZ, due to its size, it was only wiped. The tools/components were cleaned outside the cleanroom by being immersed for 5 min in sterile 70 % IPA, and then rinsed with sterile 18 MΩ.cm water. Allowed to dry and assess cleanliness. If the levels of cleanliness were the desired, the tools were wrapped into aluminium foil or bagged. If the tools size allowed during the liquid immersions, the tools suffered an ultrasonic bath. The process was repeated until the levels of cleanliness were within the desired level. In all the steps it was avoided to touch the tools/components. Before entering the cleanroom all the bags with tools were wiped with 70 % IPA impregnated wipes, and once inside the cleanrooms, the

tools/components were cleaned again in the same way as before and re-bagged until use.

iv. Method for ATP detection to measure cleanliness

ATP detection was made before and after the cleaning, to determine cleaning effectiveness. The ATP swab was taken according to manufacture recommendations, (Biocontrol 2005), and the cleanliness level assessed according to the obtained relative fluorescence units (RFU). Below 100 RFU (flight BEXUS 10) and below 400 RFU (flight BEXUS 11), it was accepted as clean, if not a new cleaning cycle was required.

b) Experimental and Results

i. Assembling in an ISO8 cleanroom

The cleanroom was clean and assembled and divided into areas of cleanliness. The personnel had to move in one direction and prevent from returning to a “clean” area after being in a “dirty” area, Figure 6-8. The cleanest areas were more often monitored and cleaned.

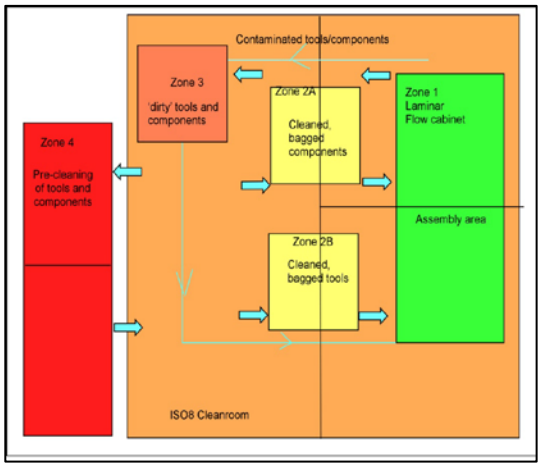


Figure 6-8 Cleanroom organisation diagram of working areas and their degree of cleanliness, from red (less clean) to green (cleaner).

ii. PP&CC cleaning method of UCZ, tools, components and cleanroom

The cleaning procedures implementation was assessed by ATP detection. The implementation occurred as expected. It was necessary to implement the procedures for each flight.

iii. Confirmation of cleanliness by ATP detection

The ATP measurements were done to every tool, area or surface that needed to be monitored and the results were displayed in RFU. In that case the obtained data was interpreted as a qualitative indicator of cleanliness. It was initially assumed as acceptable RFU values above 100, but during the preparation of the second flight it was accepted values above 400. This was because of the available period of time. It would have been possible to use ATP as a quantitative detection method if it had been defined the existence of standards as “clean”, or with known cleanliness values. This was not made because it was not found such standard with known amount of contamination that could be used and denominated as clean. Instead the aim was to decrease the obtained ATP values down to values near the instrument background (around 100 RFU). This consisted in reading luminescence of an unused (clean) swap and the obtained value used as baseline for that day reading batch, to which every measurement was normalised accordingly.

- Use of ATP to access materials and tools cleanliness during AIV

ATP showed the cleaning efficiency presenting lower reading values after each cleaning cycle. It was noticed that in the implementation of wiping, sometimes it occurred an increase of contamination. This was caused by incorrect wipe folding or wiping technique implementation, leading to spread of microorganisms instead of physical eradication.

It was import to confirm that the implementation of cleaning procedures was reducing the microorganism and consequently the ATP content decreased. That was verified in three examples of required cleaning materials. The implementation of cleaning procedures on two valves and one fix flange showed a decrease of ATP values after each cleaning cycle. It was also seen that in three or four steps it was achieved the required level of cleanliness (below 100 RFU), Figure 6-9.

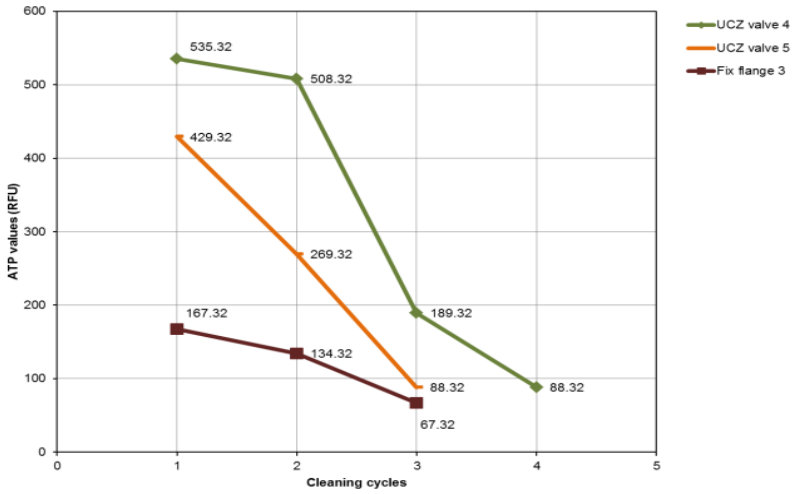


Figure 6-9 Example of ATP decreasing values after implementation of a new cleaning cycle in two valves and one fix flange.

Similar action was taken to the UCZ. Several points in the UCZ were tested and used for cleanliness comparison. In some exposed places it was necessary to clean up to eight times to confirm ATP values below, or just above 100 RFU. The following data referred to the UCZ 1 prepared for the BEXUS flight 10, Figure 6-10.

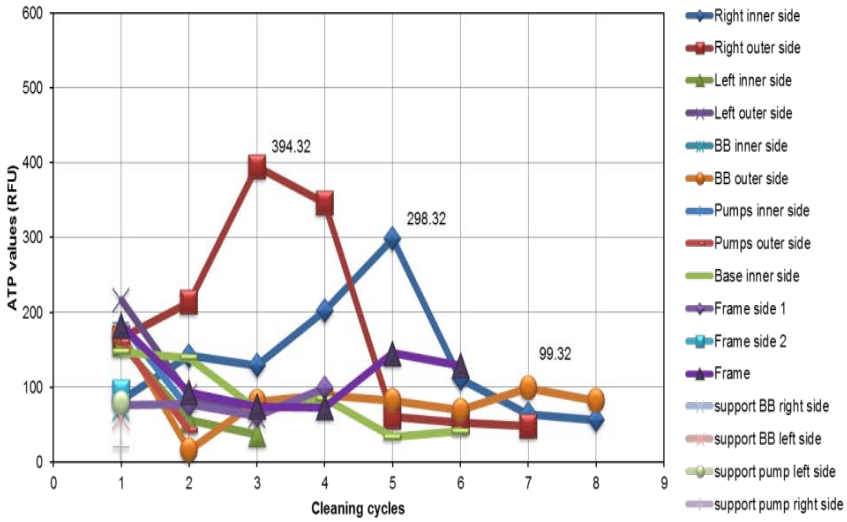


Figure 6-10 UCZ several cleanliness points accessed by ATP detection.

The same UCZ was used for the BEXUS 11 and was denominated UCZ 2. The UCZ 1 was re-cleaned, re-assembled and prepared for the UCZ 2 for the BEXUS 11 within a

shorter period of time (assembling in 37 hours). Because of that the minimum ATP value required was 400 RFU instead of 100 RFU. The obtained ATP values before UCZ 2 sealing were, normalised to the obtained value of a clean ATP swab and the obtained values were considered within the detection limit of the luminometer (data not shown).

- ATP values obtained in experiments located near CASS•E prior lunch of BEXUS flight 10 and flight 11

To understand the level of microbial presence on the surrounding experiments to CASS•E ATP values of untreated areas were measure before and after flight. This was used to verify the difference in microbial content at the launch pad between CASS•E external surface, that had suffered only the minimal re-contamination prevention such as being bagged for transport, and the other experiments and gondola that did not have any type of cleaning implementation or microbial content concerns. Those were made on CASS•E´s exterior, on the canvas of the gondola and on the nearest experiments. These were made to control possible contamination sources, Table 6-5 and Table 6-6.

Table 6-5 ATP values taken on critical areas before flight BEXUS 10, adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

ATP value (RLU) inside house	
Biobarrier left cover flange	851.32
Biobarrier right cover flange	936.32
Biobarrier left Tyvek	239.32
Biobarrier right Tyvek	625.32

At this stage the values of ATP on the outside of the UCZ 1 and on the Tyvek surface of the biobarriers ranged from 239 to 625 RFU and from 851 to 936 RFU for the cover flanges. The canvas of the gondola and the neighbour experiments presented values from 14,344 to 33,353 RFU, Table 6-6.

Table 6-6 ATP values taken on the launch pad of critical and possible contaminants, before BEXUS 10 flight, adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

ATP value (RLU) at launch pad	
Canvas roll above Biobarrier	14344.32
Canvas CASS•E side	33353.32
Canvas right side	27058.32
Canvas Scrat	3016.321

On the second flight a similar detection was made before and after flight and the results obtained showed again the high levels of ATP present in the neighbouring experiments in comparison with CASS•E, Table 6-7.

Table 6-7 ATP values taken on the launch pad of critical and possible contaminants, before BEXUS 11 flight, adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

ATP value at (RLU) Launch pad	
Canvas roll above Biobarrier	1167
Canvas CASS•E side	885
Canvas right side	4054
Scope	3989
Canvas back side	1548
Canvas left side	4977
Perdaix front	1078
Perdaix CASS•E side	1111

In this flight the lowest value obtained at the launch pad was 885 RFU and the highest 4977 RFU. This was less than the observed in the previous flight which was positive for the experiment aim. After the flight BEXUS 11, the ATP values were taken from the same places than before flight, Table 6-8.

Table 6-8 ATP measurements after BEXUS 11, adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

ATP value (RLU) at Launch pad	
Canvas roll above Biobarrier	18319
Canvas CASS•E side	12823
Canvas under CASS•E	2284
Scope	34253
Perdaix front	1068
Perdaix CASS•E side	3881
Biobarrier left cover flange	781
Biobarrier right cover flange	115
Biobarrier left inlet tubing	264
Biobarrier right inlet tubing	562

When comparing the values obtained before and after flight, most of them were lower before the launch than after landing, with the highest range found in the experiment Scope with an increase of ATP value of 30,264.00 RFU. The second increase of ATP value was in the canvas above the biobarriers with 17152.00 RFU raise. The only difference was in Perdaix that presented a value of 10 RFU units between measurements. This value was too low to be considered compared with the magnitude of values obtained at that stage.

- Comparison of ATP values in the air collection pathway before and after flight BEXUS 11

The UCZ 2 was collected and opened inside the cleanroom ISO 8, and the ATP values obtained before and after DHMR and flight compared. The sample line one was not taken because the plastic tube was cut before being possible to perform the ATP detection. Comparing the ATP values obtained in the air pathway after assembling and after flight in the lines 2 and in the control flight was possible to see an increase in the ATP values in the both of the line exposed (line 2) Figure 6-11 and in the control line, (not shown).

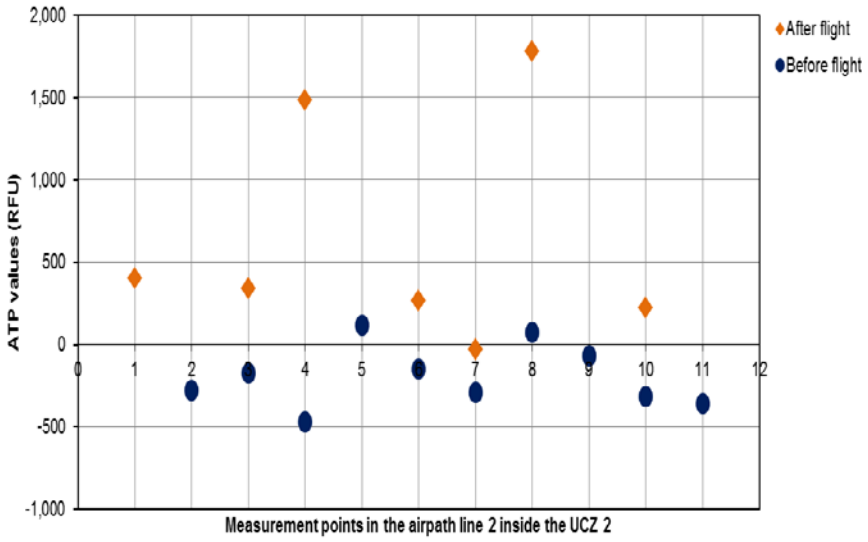


Figure 6-11 ATP values obtained inside the air pathway 2 before and after flight BEXUS 11.

The ATP values increased in all sample lines with the higher value obtained in number 8 with an ATP raise of 2346 %. The ATP values showed that a source of microbial contamination was present in the filters air path. In the ATP values obtained before flight the normalisation made them negative values again this was considered that the obtained values were within the ATP luminometer detection limit, and therefore acceptable.

iv. Dry Heat Microbial Reduction

The components part of the UCZ such as the valves, the filters and filter cases, and the air pathway tubes suffered heating for 156 hours at 110 °C in a normal oven and then their functions were tested. All the tested components survived the functionality tests and were approved to be used inside the UCZ.

The UCZ1 suffered DHMR for 156 hours at 110 °C while due to time constrains the UCZ 2 suffered DHMR only for 32 hours at 110 °C.

c) Discussion

The implementation of an AIV plan was critical to reduce and monitor the presence of bioburden on the UCZ during assembling. The plan included the implementation of cleaning, assessment of cleanliness, preparation of staff, facilities and tools required at

each stage of assembling, in order to keep controlled the level of bioburden. The standard cleaning methods and chosen assessment of cleanliness by ATP detection shown to be efficient an ideal for this instrument because it was fast and easy to use, allowing constant monitoring during critical stages of assembling.

Summary and Conclusion

The AIV plan implementation on CASS•E served to prevent and reduce microbial presence at every stage of development until sample collection and analysis. That was done in order to keep microbial burden at low levels so that possible collected microorganisms from the Stratosphere could be detected and studied. The implementation of the AIV plan showed its complexity and need of an early implementation. It proved that the PP&CC requirements implementation was only possible by the application of an AIV plan in which cleaning and assessment of cleanliness were essential and constant. And also that preparation of working facilities, staff and tools were equally important and required. ATP was used as a monitoring agent which showed to be a good alternative to culturing giving immediate results which were essential for this project that had to be developed on a short period of time when compared with standard space missions.

6.5.4. Tracking contamination pathways with fluorescent beads

Validation procedures were important to confirm that the experiment was occurring as expected and to understand if the obtained data presented or not contamination. In the CASS•E case it was used ATP for validation of the cleaning methods and a novel approach to validate possible collection of stratospheric microorganisms. It was used polystyrene fluorescent beads with 0.2 μM and 1 μM of diameter to validate the experiment and to track possible microbial contamination pathways. The fluorescent beads were sprayed before flight on CASS•E´s external surface and surrounding experiments located on the flight gondola. In the case the beads were found inside the collection filters it would indicate migration of ground contamination from the CASS•E outside and surrounding experiments to the collection filters located inside the UCZ, during sample collection. The UCZ 1 was built for BEXUS flight 10 but because of its failure it was dismantled, re-cleaned re-assembled and re-sterilised, to give origin to UCZ 2 and fly on BEXUS 11. Different sizes of fluorescent beads were used in the flights so that could be differentiated if found inside the collection filters. In Table 6-9 it

was descripted the identification, position and function of the different filters analysed from the UCZ 2 that flew on BEXUS 11.

Table 6-9 CASS•E identification of analysed filters from BEXUS flight 11, adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

Filter Name	Position	Function
Test Line 1	CASS-E line 1 collection filter	Stratospheric sample
Test line 2	CASS-E line 2 collection filter	Stratospheric sample
Flight control	CASS-E line 3 collection filter	Filter flown but not exposed to stratospheric sample
Shipping control1	In shipping case inside Tyvek pouch	Filter shipped, but not flown (negative control)

a) Materials and Methods

i. Materials and Chemicals

Polystyrene green and red fluorescent beads at 0.2 and 1 μM diameter (R200, R0100, G200, G0100) from Thermo Scientific, methanol from Sigma, Axioskop 2 plus *epi*-fluorescent microscope from Zeiss, XL30 environmental scanning electron microscope from FEI/Philips, energy dispersive X-Ray microanalysis instrument (Swift ED) from Oxford Instruments, one air brush, one can of butane/propane propellant, 0.2 μm filters.

ii. Method of fluorescent beads preparation for spray application

100 μl of fluorescent beads were dissolved in 25 ml of ethanol. The solution was sprayed over CASS•E´s external surface and surrounding experiments located on the flight gondola.

Table 6-10 Colours and sizes of fluorescent beads used to track contamination pathways on the BEXUS 10 and 11 flights, adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

Flight	**Bead type and colour**	
	Exterior of CASS•E and biobarriers	Exterior of gondola
BEXUS 10	Green 1 μm	Red 1 μm
BEXUS 11	Green 0.2 μm	Red 0.2 μm

iii. Method of filters visualization under the epi-fluorescent microscope

The samples were observed on the epi-flourescent microscope using specific filter sets and at 40 x resolution. The filter set to visualise green beads had an excitation band pass between 450 and 490 nm and an emission band pass between 515 and 565 nm. The filter set to observe red beads had an excitation band pass from 530 to 585 nm and an emission long pass of 615 nm. The green beads presented at 468 nm their excitation wavelength and emitted at 508 nm, while the red beads excitation wavelength was at 542 nm and emission at 612 nm.

iv. Method of environmental scanning electron microscope (E-SEM) filters analysis

The analysed filter was placed on a vacuum chamber, and an electron beam hit the filter provoking an electronic response on the sample. The sample in response released electrons and X-rays that were collected on a detector. Those electrons gave information about the composition of the sample and also an image. The image was created based on the released electrons that hit on a phosphor located on the detector giving origin to a light flash. The flash was recorder and transformed into an image. The E-SEM was operated by Dr Xian Wei Liu, School of Applied Sciences, Cranfield University, UK.

a) Experimental and results

i. Detection of fluorescent beads presence on the surface of the collection filters from BEXUS flight 11 with epi-fluorescent microscope

The UCZ 2 was opened inside the cleanroom by piercing the top cover biobarrier. The UCZ filters were removed one at a time and marked on the side of collection air path and divided into six parts. Each part was transferred into a sterile petri dish and stored at RT in a dark location until further analysis.

A fragment of each filter was observed under the epi-fluorescent microscope and two filter sets were used to allow the detection of green and red fluorescent beads. Images of beads found in the shipping control line 1 filter and in the collection line 1 filter were observed in Figure 6-12.

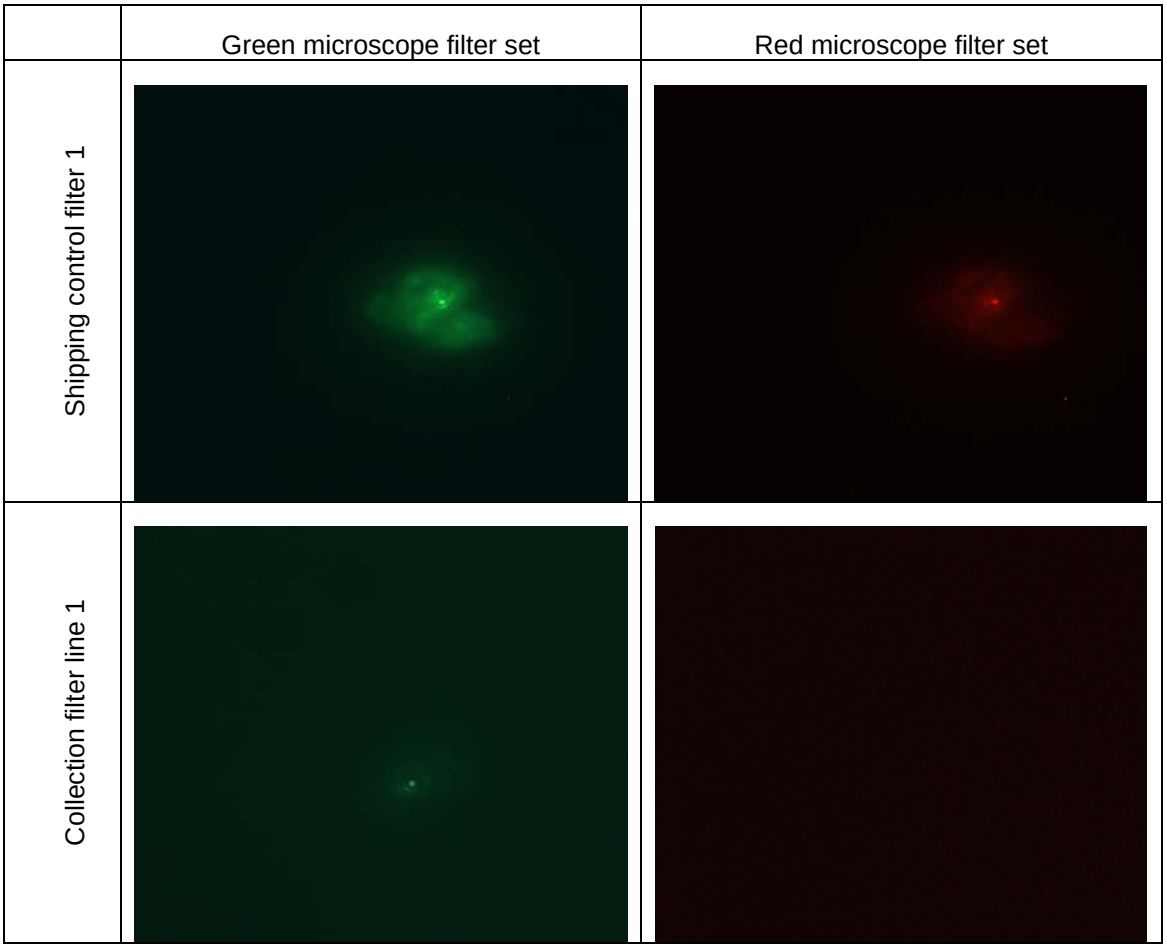


Figure 6-12 Fluorescent beads found on flight 11 filters. Red beads were detected after excitation in both filter sets while the green beads emission wavelength was not captured on the red filter set, adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

The total number of fluorescent beads found on the filter samples was discriminated on Table 6-11. It was found inside the sample collection filters fluorescent beads of both colours. The maximum number of beads was seen on the line 1 collection filter with four green beads. It was seen that the shipping control from line 2 presented the second higher number of beads with three, also green.

Table 6-11 Number of fluorescent beads visualised on CASS•E filters, adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

	Number of green beads	Number of red beads
Shipping control 1	1	1
Shipping control 2	3	0
Line 1 collection filter	4	0
Line 2 collection filter	1	1
Line 3 collection filter (flight control)	1	1

ii. Environmental scanning electron microscope (E-SEM) for filter analysis from BEXUS flight 11

Each filter segment was initially observed generically at a lower resolution and then in 10 positions and at three orders of resolution (500 x, 1500 x and 5000 x), Figure 6-13. For comparison purposes two filters contaminated with 0.2 and 1 μm fluorescent beads were also examined with E-SEM.

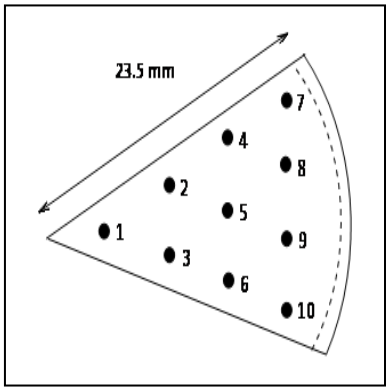
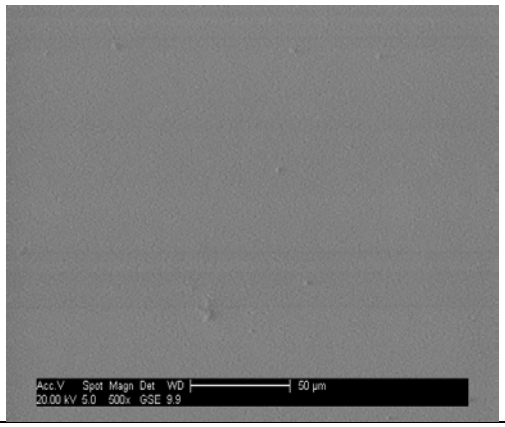
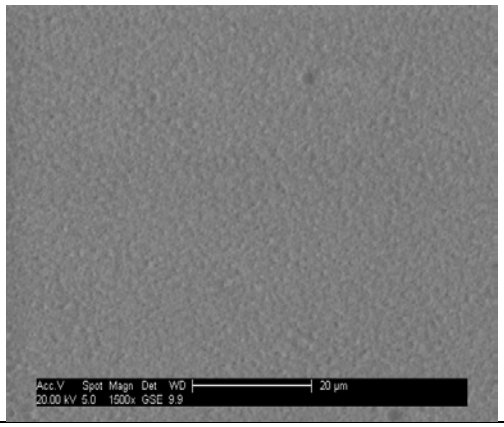
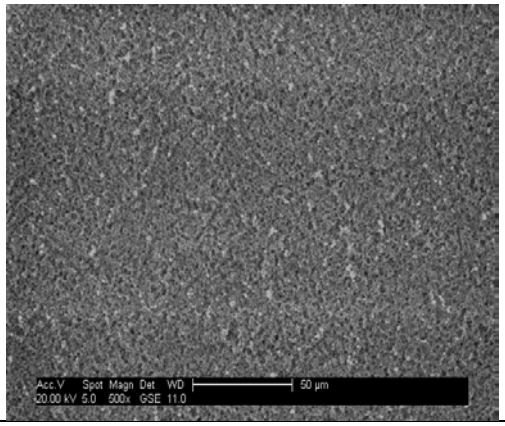
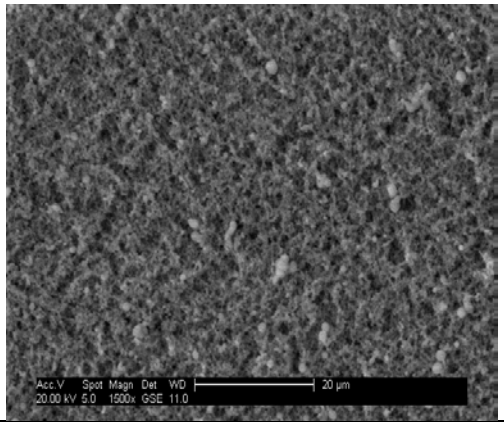
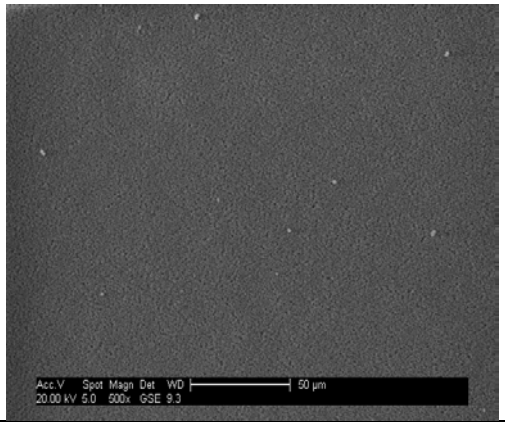
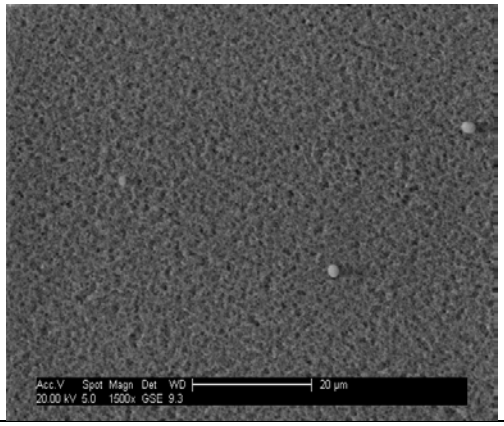


Figure 6-13 Observed areas on the filter segments (dotted line denotes point where filter holder was in contact with filter surface), adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

It was easily visualised the presence of beads in all filter fragments. The presence of beads also on the shipping controls indicated that cross-contamination occurred. The probable reason might have been during the recovery of the filters inside the cleanroom and their preparation for analysis, Figure 6-14.

Filter	Magnification	
	500 x	1500 x
Shipping control 1		
Shipping control 2		
Line 1 collection filter		

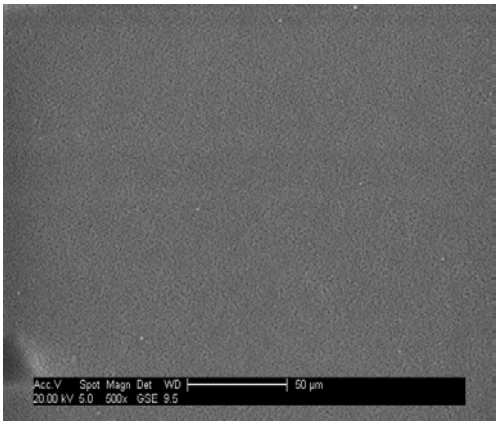
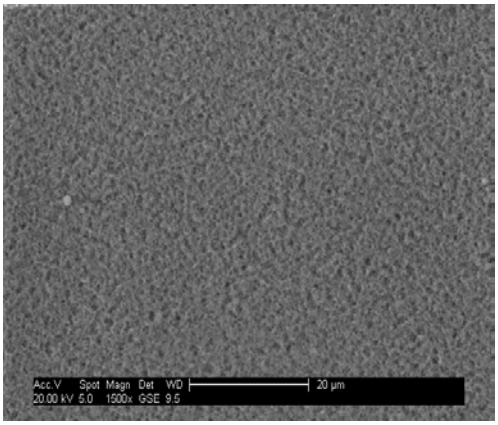
	500 x	1500 x
Line 2 collection filter	Not analysed	Not analysed
Line 3 collection filter (flight control)		

Figure 6-14 E-SEM images of filters at 500 x and 1500 x magnification in position 5 of each filter, adapted from Juanes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

It was seen other particles besides the beads present on the collection filters. Their composition was given by the E-SEM X-ray analysis, and it was seen the high presence of aluminium in one of them (particle B), which indicated the presence of some debris from the UCZ, the presence of some debris was previously observed when opening the UCZ inside the cleanroom. In another particle (D) it was found similar composition to the filter, which was assumed to indicate particle shed from the filter itself, Table 6-12.

Table 6-12 X-ray microanalysis of particles detected (B-E) on flight collection filters, adapted from Juannes-Vallejo et al (2011), (Juanes-Vallejo, Grama et al. 2011).

	Weight % of each element				
	Filter background	Collection filter line 3 (flight control)	Collection filter line 1		
	A	***B***	***C***	***D***	***E***
Carbon	42.55	27.71	44.08	40.42	31.16
Oxygen	56.17	45.03	50.02	55.53	52.41
Magnesium	-	-			6.99
Aluminium	1.28	22.86	0.57	0.42	0.25
Silicon		0.26	0.59	3.69	9.19
Sulphur			0.45		
Cromium			1.19		
Zinc		4.14			

Particles C and E were different from the filter control and presented a more complex composition. It was difficult to know what they were, but could be skin cells or pollen cells, because those were the most probable sources of particles. It was not possible to know if the origin of those particles was stratospheric or from ground origin because beads were found on the filters, which showed the existence of any source of contamination.

The particles described on the previous table, were visualised on Figure 6-15.

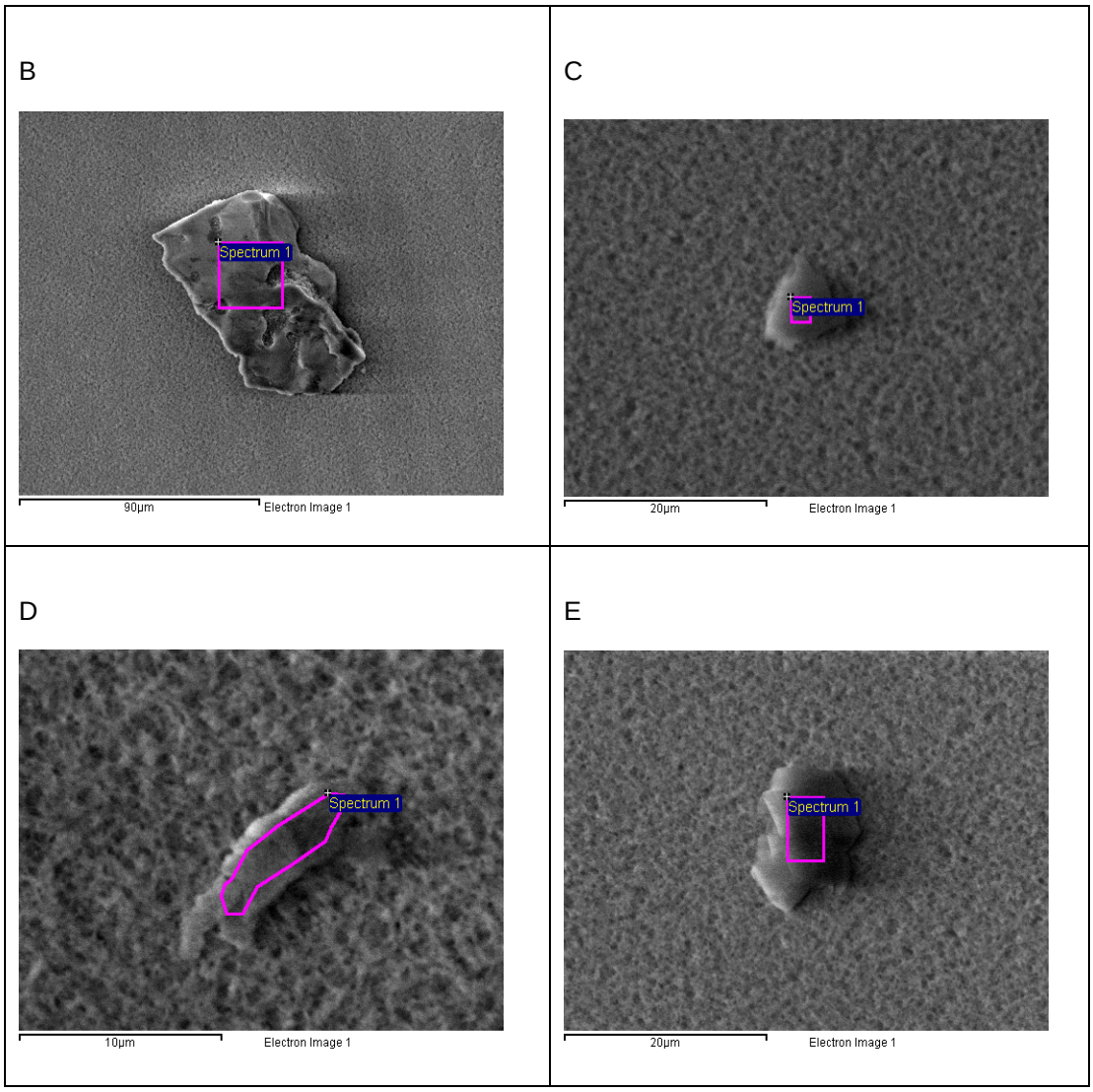


Figure 6-15 Detected particles on flight filters analysed by E-SEM X-ray microanalysis, adapted from Juanes-Vallejo et al (2011),(Juanes-Vallejo, Grama et al. 2011).

b) Discussion

It was found the presence of fluorescent beads on the collection filters, obtained from BEXUS flight 11. It was suggested to be ground microbial contamination because it was also found fluorescent beads on the shipping controls and on the flight control. It was also noticed that the found beads appeared to be the 1 µM size which were used on the first flight. That was explained as a possible cross-contamination of beads during the assembling of UCZ 2 with residual beads from the flight BEXUS 10. In the E-SEM analysis it was easily seen the presence of the fluorescent beads, and it was also considered that the beads contamination could have occurred during the filter removals inside the cleanroom.

Besides the beads it was found other particles on the collection filters. Two of those particles appeared to be from the instrument, while two other particles were different and one presented magnesium while the other presented sulphur and chromium in their composition. Because no similar particles were found on the control filters, it was considered that those particles could have been from the stratosphere. However because fluorescent beads were found on the collection filters, that indicated that contamination had occur either from CASS•E´s exterior or the surrounding experiments. Nevertheless it was expected a higher number of fluorescent beads present on the collection filters than the one observed, which lead to the positive outcome that the applied procedures reduced at some level the collection of ground contamination.

Summary and Conclusion

In summary the novel use of fluorescent beads as proxy for contamination pathway tracking worked well as a validation technique because it indicated that some source of contamination occurred, questioning the origin of the found particles. It was concluded that the use of fluorescent beads improved the knowledge obtained about the collected samples, which could help on validating future experiments, in which ground contamination could be a problem.

6.6. Initial demonstration of Aptamers' resilience to DHMR conditions

The standard PP&CC implementation in the LMC required the bioreceptors survival to DHMR. In this work aptamers were tested as alternatives to antibodies. In this chapter aptamers´ resilience to DHMR was tested. There was no knowledge of similar work developed and of the effects that could have on aptamers with the exception of the standard denaturation and degradation caused by elevated temperatures, if not correctly protected. Several working buffers were taken in consideration because the LMC required a specific working buffer (red cocktail) that had not been used during the aptamer assay development, (Chapter 5). This test was the opportunity to study if the working buffer could have or not preservation properties and protection against the severe tested conditions. Also all the samples were freeze-dried before DHMR treatment. This was the required pre-treatment of the LMC bioreceptors before final integration into the instrument. It was studied the integrity of five biotinylated aptamers and one of the SELEX libraries used for comparison (Chapter 4 and 5). Because it was not developed a working LMC assay with the tested biotinylated aptamers and their targets, the aptamers´ integrity was verified with standard agarose gels and UV quantification.

The aim of this work was to verify the integrity of the tested aptamers and library, and discuss the best conditions obtained to preserve aptamers if integrated in the LMC or similar instruments that required extreme treatments as DHMR. The samples were freeze-dried at different concentrations and in different working buffers and two DHMR standard conditions were tested.

6.6.1. Aptamers tested for 32hr at 110°C and for 52hr at 120°C

As explained in section 6.2.2, in Table 6-2, the microbial reduction of surface and mated microorganisms was made by DHMR for 32 hours at 110 °C. This was the first condition tested for the aptamers and library. The sterilisation method DHMR had different efficiency based on the time and temperature applied,. After the previous test it was decided to test the same aptamers and library solutions in stronger conditions. Applying DHMR for 52 hours at 120 °C would sterilise the LMC instrument of encapsulated microorganisms. The impact of the tested conditions was accessed using UV detection and agarose gels in order to verify aptamers and library integrity. As

mentioned before the oligonucleotides were freeze-dried before suffering the treatment and criopreservatives were added as protective agents.

a) Materials and methods

i. Materials and Chemicals

Oven from Carbolite, freeze-dry machine, Picodrop spectophotometer and UV tips from Picodrop, PCR tubes and electrophoresis apparatus from BioRad, agarose powder (CAS 9012-36-6; A9539), Tris acetate-EDTA buffer (TAE, T6025), PBS, HEPES, Allura red (458848), sucrose (S7903) from Sigma, thiomersal (71230) from Fluka SyBr Green Safer view from abm, liquid nitrogen, tweezers, plastic support, aluminium foil, mercury thermometer up to 300 °C

ii. Method for oven temperature test

The oven was switched on with the convection fan on and the temperature set to 110 °C. The first measurement was taken using a mercury thermometer after two hours working. The oven door was opened and the thermometer put inside, the door closed and 5 min afterwards the thermometer removed and the temperature registered. This was repeated three more times after being started.

iii. Choice of aptamers, library and buffers to test DHMR

The aptamers and library were previously used, they were the OTA aptamer H12 (Table 5-5, Chapter 5), the H12 aptamer truncated version (5'-biotin-CGGGTGTGGGTGCCTTGATCCAGGGAGTCT-3'), the β-estradiol aptamer (Table 5-4, Chapter 5), the ibuprofen aptamer sequence 2 (Table 5-1, Chapter 5), the thrombin 15 oligomer aptamer (Table 5-6, Chapter 5), and the SELEX library 2 (Table 4-16, Chapter 4).

- Buffers tested

The aptamers and library were tested in three different buffers, PBS with 10% (w/v) sucrose pH 7.4, (buffer I), PBS buffer with 5 mM $MgCl_2$, 0.5 % (v/v) Tween 20 and 10% (w/v) sucrose pH 7.4 (buffer II), and red cocktail pH 7.4 used in the LMC (0.5 mg/ml BSA, 10% (w/v) sucrose, 50 µg/ml Allura red, 0.01 % (w/v) Thiomensal, 400 mM HEPES), (buffer III).

- Aptamers concentrations tested

The aptamers and library were tested at three different concentrations, 0.05; 1 and 10 µM.

- Aptamers length tested

The chosen aptamers and library had different lengths, with the smallest being the thrombin 15 oligomer aptamer with a total of 25 nucleotides, and the largest the library with 76 oligonucleotides.

iv. Method for preparation of samples and controls

The samples and controls (kept at RT) were prepared according to their concentrations and buffer, each with a final volume of 100 µl. Each solution was immersed in liquid nitrogen until frozen. Once the samples were frozen, they were freeze-dried until no liquid was visible. On the second test the samples were freeze-dried overnight to ensure the minimum humidity possible in the samples.

v. Methods to measure aptamers integrity with picodrop and agarose gels

- Picodrop measurement

The samples ssDNA concentration was UV measured before and after DHMR treatment. The samples were measured between the 230 and the 335 nm to evaluate their protein contamination content and to allow the study of the ratio 260/280. After the DHMR treatment the samples were re-suspended in the previous buffer in a volume of 100 µl.

- Agarose gel and samples preparation

A 2% agarose gel (200 ml with 10 µl of SyBr Green Safer view) was made and each sample was analysed before and after DHMR in order to verify the oligonucleotides integrity. A total volume of 10 µl of sample was loaded into each well. The sample material was 7, 5 or 3 µl according to their concentration with 3 µl of dye. The gel was run at 100 V for 1 hour in TAE buffer. In the agarose gels the samples integrity was accessed based on the band quality (clear, smeared or inexistent).

b) Experimental and results

i. Oven temperature test

The obtained temperature in the first experiment (32 hr at 110°C) after measurements at different times increased 5 °C more than the desired temperature after 27.5 hours of working, Table 6-13.

Table 6-13 Oven temperature obtained at different times.

Time after starting the oven (hr)	Temperature (°C)
1.0	110
5.5	110
22.5	113
27.5	115

The temperature seemed to be stable during the initial hours and increased in the last ones. Or the fact that the oven door had to be open lead to an increase of temperature during a short period of time, but longer than the tested 5 min to balance the heat lost.

On the second experiment (52hr at 120°C) several temperature values were taken at different times after initiating the oven. It was seen that although the oven was set to 120 °C, the obtained values achieved the 130 °C. It was not understood, once again, if it was a short increase of temperature because the oven door was open or if it was a consistent temperature value, Table 6-14.

Table 6-14 Obtained oven temperatures set at 120 °C over a period of time.

Time after starting the oven (hr)	Temperature (°C)
1	130
1.5	130
1.75	129

ii. Measuring aptamers integrity with picodrop and agarose gels

- Picodrop measurement

The obtained picodrop measurements in the first test were not consistent and for that reason were not used as viable data to verify aptamers and library response to DHMR implementation. On the second experiment the obtained picodrop UV concentration values showed such level of degradation suffered by the aptamers and library that all the samples presented a change of colour and the obtained UV data was inconsistent for interpretation. It was possible to observe that the obtained UV concentration values for the initial solutions and for the RT controls were similar as desired (data not shown). This confirmed that the freeze-dry method in general maintained unaltered the aptamers and library.

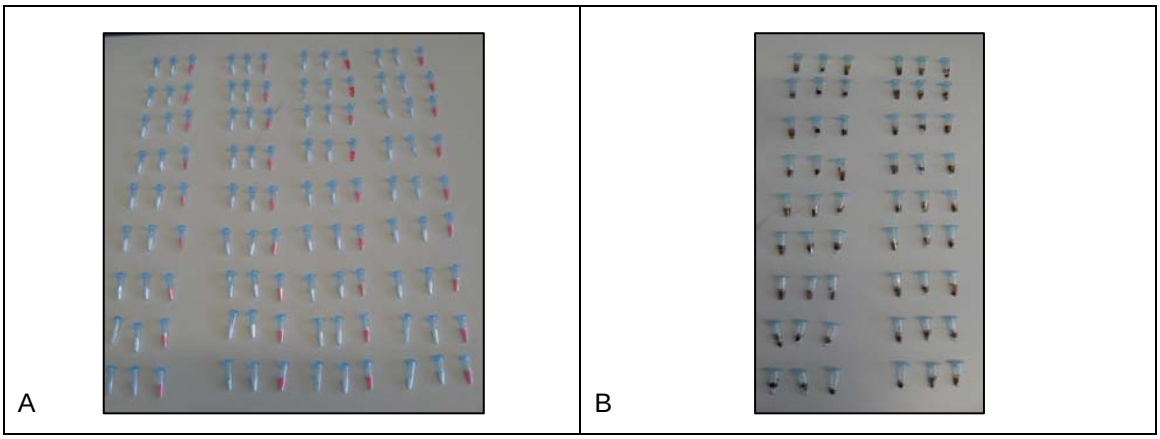


Figure 6-16 Aptamer and library samples, A) freeze-dried and before DHMR implementation and, B) after suffering DHMR.

It was observed that all the samples that suffered DHMR (B) changed colour which showed the excess exposure to high temperature values (120 °C for 52 hours), Figure 6-16.

- Agarose gel

Other way of verifying the oligonucleotides integrity was using electrophoresis. The different tested aptamers and library were run before and after DHMR. The total tested samples were 54 and represented five aptamers and a SELEX library at three different concentrations (0.05, 1 and 10 μM), in three different buffers (buffer I, II and III). The first gel showed the obtained run from sample 1 to 27 before DHMR implementation, Figure 6-17.

Figure 6-17 Agarose gel with i)Thrombin aptamer (samples 1 to 3 were at 0.05 µM; samples 4 to 6 were at 1 µM; and samples 3 to 9 were at 10 µM), ii) H12 aptamer (samples 10 to 12 were at 0.05 µM; samples 13 to 15 were at 1 µM; and samples 16 to 18 were at 10 µM), iii) H12 truncated aptamer (samples 19 to 21 were at 0.05 µM; samples 22 to 24 were at 1 µM; and samples 25 to 27 were at 10 µM). The three buffers were tested in each concentration solution of each aptamer. Samples 1, 4, 7, 10, 13, 16, 19, 22 and 25 were in buffer I, samples 2, 5, 8, 11, 14, 17, 20, 23 and 26 were in buffer II; samples 3, 6, 9, 12, 15, 18, 21, 24 and 27 were in buffer III.

The second gel presented the following analysed aptamers and library before DHMR implementation, from sample 28 to 54, Figure 6-18.

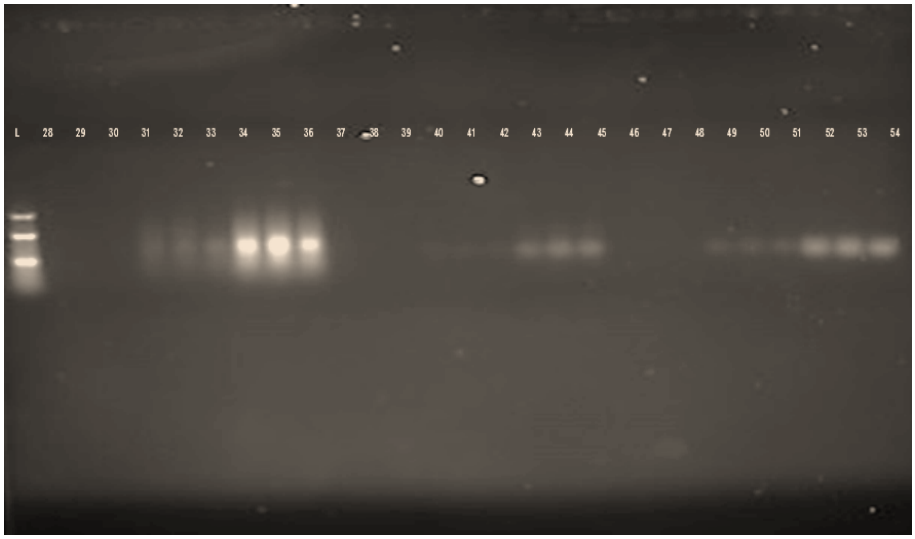


Figure 6-18 Agarose gel with i) estradiol aptamer (samples 28 to 30 were at 0.05 μM; samples 31 to 33 were at 1 μM; and samples 34 to 36 were at 10 μM), ii) ibuprofen aptamer (samples 37 to 39 were at 0.05 μM; samples 40 to 42 were at 1 μM; and samples 43 to 45 were at 10 μM), iii) SELEX library (samples 46 to 48 were at 0.05 μM; samples 49 to 51 were at 1 μM; and samples 52 to 54 were at 10 μM). The three buffers were tested in each concentration solution of each aptamer. Samples 28, 31, 34, 37, 40, 43, 46, 49 and 52 were in buffer I, samples 29, 32, 35, 38, 41, 44, 47, 50 and 53 were in buffer II; samples 30, 33, 36, 39, 42, 45, 48, 51 and 54 were in buffer III.

The solutions at 0.05 μM were not visualised on the gels, this was because their injected concentrations were not enough for visualisation. This happened even after adding a higher volume of sample to the wells in comparison with the higher concentration solutions. It was possible to visualise the aptamers and library at 10 μM, with the exception of thrombin aptamer (samples 7 to 9). At 1 μM it was also possible to visualise all the samples with the exception of the thrombin aptamer and the H12 aptamer (samples 4 to 6 and 13 to 15). It was obtained sharper bands for the library and ibuprofen aptamer than for the estradiol and truncated H12 aptamers. The more faded bands were the ones obtained with the H12 aptamer.

All the samples were freeze-dried and submitted to DHMR for 32 hours at 110 °C. Once treated the samples were re-suspended and run into an agarose gel. Samples from 1 to 27 were run in one gel and only the sample 27 was visualised, which was the H12 truncated aptamer at 10 μM in buffer III (LMC buffer), Figure 6-19.

Figure 6-19 DHMR treated samples run in an agarose gel; i) Thrombin aptamer (samples 1 to 3 were at 0.05 μM; samples 4 to 6 were at 1 μM; and samples 3 to 9 were at 10 μM), ii) H12 aptamer (samples 10 to 12 were at 0.05 μM; samples 13 to 15 at 1 μM; and samples 16 to 18 were at 10 μM), iii) H12 truncated aptamer (samples 19 to 21 were at 0.05 μM; samples 22 to 24 were at 1 μM; and samples 25 to 27 were at 10 μM). The three buffers were tested at each concentration of each aptamer.

The samples 28 to 54 were run on a second gel (estradiol and ibuprofen aptamers and library). It was possible to visualise on that gel the estradiol aptamer at 10 μM in buffer III (sample 36), and the SELEX library at 10 μM in buffer I and III (samples 52 and 54), Figure 6-20.

Figure 6-20 Agarose gel with DHMR treated aptamers and SELEX library; i) estradiol aptamer (samples 28 to 30 were at 0.05 µM; samples 31 to 33 were at 1 µM; and samples 34 to 36 were at 10 µM), ii) ibuprofen aptamer (samples 37 to 39 were at 0.05 µM; samples 40 to 42 were at 1 µM; and samples 43 to 45 were at 10 µM), iii) SELEX library (samples 46 to 48 were at 0.05 µM; samples 49 to 51 were at 1 µM; and samples 52 to 54 were at 10 µM), the three buffers were tested at each concentration of each aptamer and library.

It was observed that during the gel loading sample lost appeared to occurred based on the fact that it was a long gel and time between the initial loads and the last ones seemed to induce diffusion. It was run a new gel only with the samples of ibuprofen aptamer and the SELEX library, and was seen bands for ibuprofen aptamer at 1 µM in buffer III (sample 42) and at 10 µM in buffers I and III (samples 43 and 45) that have not been seen in the previous gel where no bands were obtained for this aptamer. The SELEX library presented the same bands at 10 µM but also an extra one representative of 1 µM in buffer III (sample 51), Figure 6-21.

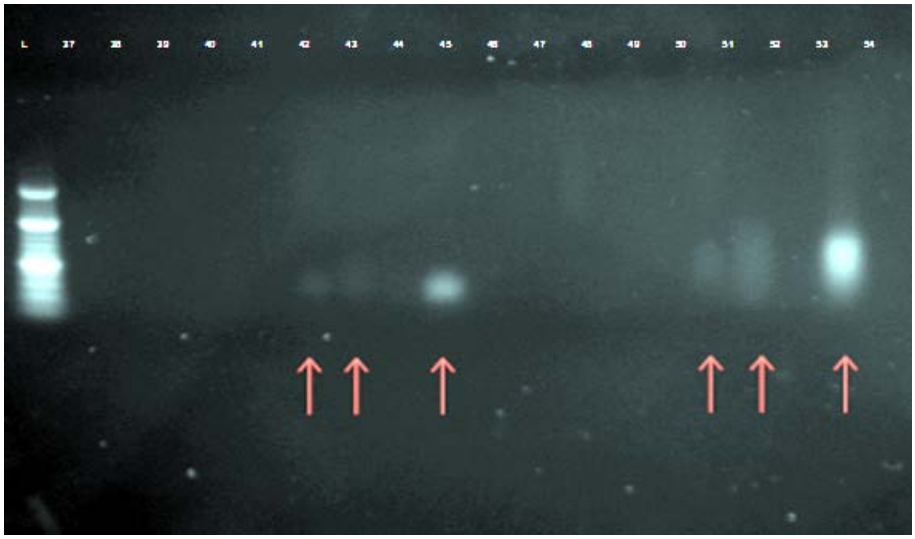


Figure 6-21 Agarose gel with DHMR treated ibuprofen aptamer and SELEX library at different concentrations and buffers, ibuprofen aptamer (samples 37 to 39 were at 0.05 μM; samples 40 to 42 were at 1 μM; and samples 43 to 45 were at 10 μM), SELEX library (samples 46 to 48 were at 0.05 μM; samples 49 to 51 were at 1 μM; and samples 52 to 54 were at 10 μM), the three buffers were tested at each concentration of each aptamer and library.

For control purposes it was also run agarose gels with the RT sample controls. To avoid sample lost by gel diffusion it was loaded three gels instead of two. In the first gel it was run the thrombin and H12 aptamers at their concentrations and buffers. It was visualised only the H12 aptamer at its higher concentrations, Figure 6-22.

Figure 6-22 Agarose gels with RT controls, i) Thrombin aptamer (samples 1 to 3 were at 0.05 μM; samples 4 to 6 were at 1 μM; and samples 3 to 9 were at 10 μM), ii) H12 aptamer (samples 10 to 12 were at 0.05 μM; samples 13 to 15; and samples 16 to 18 were at 10 μM). The three buffers were tested in each concentration solution of each aptamer. Samples 1, 4, 7, 10, 13, and 16 were in buffer I, samples 2, 5, 8, 11, 14 and 17 were in buffer II; samples 3, 6, 9, 12, 15 and 18 were in buffer III.

On the second gel it was seen the H12 truncated and estradiol aptamers. It was only visualised the higher concentrations of H12 truncated aptamer and the 1 and 10 μM solutions of the estradiol. It was verified a degradation in the estradiol samples, Figure 6-23.

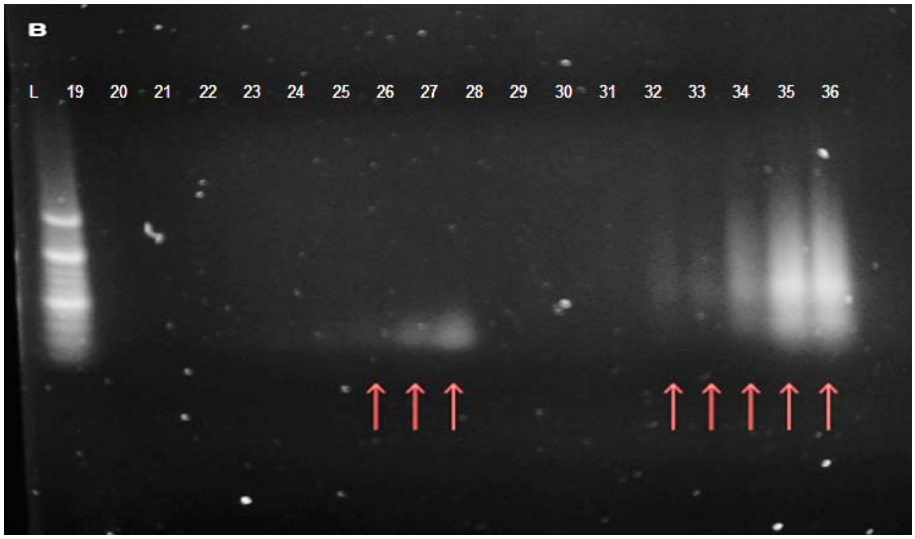


Figure 6-23 Agarose gels with RT controls, i) H12 truncated aptamer (samples 19 to 21 were at 0.05 μM; samples 22 to 24 were at 1 μM; and samples 25 to 27 were at 10 μM), ii) estradiol aptamer (samples 28 to 30 were at 0.05 μM; samples 31 to 33 were at 1 μM; and samples 34 to 36 were at 10 μM). The three buffers were tested in each concentration solution of each aptamer. Samples 19, 22, 25, 28, 31 and 34 were in buffer I, samples 20, 23, 26, 29, 32 and 35 were in buffer II; samples 21, 24, 27, 30, 33 and 36 were in buffer III.

In the last gel it was run the ibuprofen aptamer and the SELEX library. It was observed only the higher concentrations of the ibuprofen aptamer and the 1 μM and 10 μM of the SELEX library, Figure 6-24.

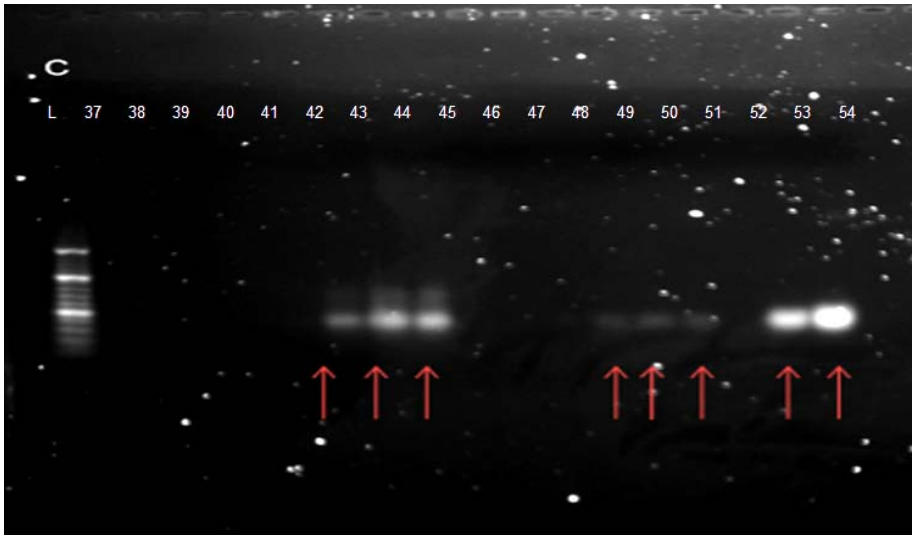


Figure 6-24 Agarose gels of RT controls, i) ibuprofen aptamer (samples 37 to 39 were at 0.05 µM; samples 40 to 42 were at 1 µM; and samples 43 to 45 were at 10 µM), ii) SELEX library (samples 46 to 48 were at 0.05 µM; samples 49 to 51 were at 1 µM; and samples 52 to 54 were at 10 µM). The three buffers were tested in each concentration solution of each aptamer. Samples 37, 40, 43, 46, 49 and 52 were in buffer I, samples 38, 41, 44, 47, 50 and 53 were in buffer II; samples 39, 42, 45, 48, 51 and 54 were in buffer II

On the second experiment the initial agarose gels were considered, (Figure 6-17 and Figure 6-18) for comparison with the DHMR treated samples. After the DHMR treatment it was not seen any band on the agarose gels. The control samples kept at RT showed similar bands to the ones seen in the previous point, in Figure 6-22, Figure 6-23 and in Figure 6-24, (data not shown).

c) Discussion

The picodrop UV readings of the solutions before and after DHMR as well as their RT controls were not consisted, which made impossible to take conclusions from the obtained data.

It was seen in the initial agarose gels that the lower solutions tested at 0.05 µM did not have ssDNA enough concentrated for gel visualisation. Because of that it was not expected to study the DHMR impact on those solutions via the agarose gels. It was possible to see that in three out of five aptamers showed to survive DHMR at 10 µM and in buffer III. This showed that the buffer III, which was the chosen LMC assay buffer and was rich in protective agents that were not present in the other tested buffers, increased aptamer resilience. The higher concentration of aptamer improved the resistance to degradation or at least the rate of affected versus not affected strands

was lower, allowing the band visualisation. It was also seen that the SELEX library resisted better to the treatment, this was expected based on the library length. Based on this it was not expected that the thrombin aptamer and the H12 truncated aptamer resisted DHMR. It was not possible to conclude about the thrombin aptamer as no detection was obtained with the electrophoresis, but the H12 truncated aptamer survided DHMR. At a higher concentration and in buffer III, the small aptamer presented DHMR resilience. It was also seen that initially more degraded aptamers performed poorer than the ones that showed initial better stability. This was verified with the H12 aptamer that presented lower initial bands resolution than the other aptamers, and did not show any band after DHMR. Another observation supporting the importance of initial aptamer integrity for DHMR survival was the estradiol aptamer that presented a certain level of degradation at the initial gel and an increased degradation was seen in the RT control with visible smeared bands. Despite this it was seen estradiol aptamer resilience to DHMR at the higher concentration and in buffer III.

The second implemented DHMR conditions were too violent for the tested aptamers and library solution because the obtained UV values and agarose gels presented in all samples the same negative values. It was also seen a colour change in the samples that suffered DHMR to a dark brown.

Summary and Conclusion

It was decided to test the effects on aptamers of the most common PP&CC sterilisation method; this was made to discuss the aptamers compatibility with required PP&CC application in the LMC instrument.

Five biotinylated aptamers and a SELEX library were submitted to DHMR conditions by being heated at 110 °C for 32 hours. It was tested three different oligonucleotides concentrations and three different buffers. The aptamers and library were freeze-dried prior to DHMR implementation as a protective procedure. The impact of the treatment was studied by comparing UV quantification values and by running agarose gels to verify oligonucleotides integrity before and after the treatment. It was concluded that the tested aptamers seemed to have kept their integrity at the highest concentration and that the standard LMC buffer, in comparison with the other studied buffers, seemed to have kept better the samples integrity. It was also verified that shorter strands seemed to be as resistant as the longer strands. It appeared that the initial aptamer conditions were determinant for resilience, more than aptamers length. It was

also possible to understand that the higher concentration the better to preserve or minimise degradation.

The tested DHMR conditions of 120°C for 52hr caused the loss of integrity of the tested aptamers and library, for that reason it was concluded that those extreme parameters were incompatible with the aptamers and library tested.

6.7. Discussion of PP&CC in the context of the LMC

The PP&CC implementation was a complex process that required the synergy of materials compatibility with clean and sterilisation methods, and design specificities to facilitate cleaning, sterilisation and prevent re-contamination. It was possible to use a small scale experiment as proxy for the LMC. It was implemented on CASS•E the standard cleaning procedures and sterilisation. The cleanliness level was assessed with a fast and easy to use method; the fluorescent ATP detection showed how efficient the previous cleaning step was and if more was required to achieve the chosen cleanliness baseline. ATP detection showed that could be used in PP&CC as a fast and portable detection method.

The implementation of a UCZ was demonstrated to improve PP&CC implementation.. Using an UCZ made AIV simpler, cheaper and reduced the risk of using components that could react negatively to PP&CC implementation leading to failure. The UCZ implementation was certainly a major factor of success because the collection area was defined and located in only one area, allowing better protection and PP&CC implementation and prevention of re-contamination. Another design implementation was the biobarriers that protected the collection inlet tubes until prior sample collection at the stratosphere. Adding the biobarriers also added the problem of opening them at a certain time. In the BEXUS 10 flight one of the biobarriers did not open and the other was destroyed during landing, and because of that it was not possible to analyse the collection filters. On the second flight (BEXUS 11) the biobarriers opened and were intact after landing. Tracking of contamination showed to be useful because it helped validating the collected samples. When opening and analysing the collection filters it was found the presence of fluorescent beads. That showed that probable contamination from the gondola and experiment external surface occurred, or from cross-contamination between UCZ flights assembling. In order to avoid future similar problems it was recommended that the sample collection needed to be made at a longer distance from the gondola, and that should be avoid at all costs to re-use an

UCZ. The implementation of an UCZ and introduction of microbial pathways proxies improved the experiment PP&CC implementation and helped in validating the obtained samples and for that reason should be implemented on the LMC. Also the use of biobarriers protected the experiment from recontamination and showed how critical the opening system could be for the overall mission success.

Regarding the study of aptamers as alternative bio-receptors to LMC, it was tested two DHMR temperature and time conditions in freeze-dried samples. It was confirmed that aptamers, at higher concentrations (10 μM) and in the standard LMC buffer, kept their integrity at 110 °C and for 32 hours. It was also observed that initial aptamer integraty was more important for resilience than aptamers´ structural length. The results showed that aptamers have the ability of being freeze-dried and suffer implementation of DHMR (at least at 110 °C and for 32 hours), as required for the LMC instrument. The obtained data only gave indications of aptamers and library integrity and no functionalization studies were made. The initial results were promising and further studies would be required to fully understand if aptamers were or not suited for LMC implementation regarding PP&CC compatibility.

6.8. Summary and Conclusions

The implementation of PP&CC was a transversal procedure that affected the choice from the aircraft material to its design; and obliged to a group of procedures during AIV that enclosed cleaning, assessment of cleanliness and sterilisation. The LMC as part of a category IV-b mission required a limit of bioburden present on the instrument. In order to do so PP&CC had to be implemented. So far an initial PP&CC plan was made describing the standard cleaning, assessing cleanliness and personnel training required to PP&CC AIV implementation in the LMC.

There was the opportunity to develop and fly a small scale experiment to the stratosphere to collect stratospheric microorganisms and particles. It was implemented the standard PP&CC cleaning and sterilisation methods and assessed cleanliness with a method used traditionally in food industry. CASS•E was a proxy instrument for LMC and allowed to learn lessons regarding PP&CC implementation. Those were that PP&&CC implementation required the use of clean and sterilisation-friendly building materials, that changes in the design such as using a UCZ and biobarriers were essential to reduce time and costs of PP&CC implementation. The biobarriers were also very important to reduce the risk of ground contamination before sampling

collection, protecting the collection area until sample contact. Another interesting procedure taken with CASS•E was the use of fluorescent beads to mimic and track microbial contamination. That showed to work very well, being found inside the UCZ in the collection filters, when were just sprayed on the experiment outside. That showed that even already collecting the sample a few centimetres out of the gondola frame was not sufficient to prevent aspiration of particles from it, and that cross-contamination could occur easily and persistently. The experience with CASS•E reinforced the use of biobarriers (even with found contamination), demonstrated the functionality of having a UCZ, and suggested the implementation of tracking contamination systems that could help to validate the collected samples.

The PP&CC implementation on the LMC would require the compatibility with sterilisation procedures of the integrated bioreceptors. Aptamers were studied as alternative bioreceptors to be implemented on the LMC. It was tested their resilience to DHMR implementation. Two temperatures and period of times were tested, one being known to eliminated surface and mated microorganisms and the other encapsulated microorganisms. Five different aptamers and a SELEX library were tested at different concentrations and in different buffers. The aptamers and the library showed integrity at the highest tested concentration and at the standard LMC assay buffer, but only at the mildest conditions tested. It was concluded that aptamers integrity was compatible with DHMR and that future tests would include aptamers functionality tests.

Chapter 7. Final Discussion, Conclusions and Further work

7.1. Final summary and discussion

The overall aims of this thesis was to understand if aptamers could be implemented in an LMC instrument and development programme, and to consider at an early stage the impact of design and protocols for PP&CC implementation, as well as to understand if aptamers could contribute to improvement in the LMC PP&CC implementation.

In order to address those aims the work was structured in three major areas that were reported in the thesis as three major experimental chapters.

In the first experimental chapter of the three (thesis Chapter four) the possibility of generating aptamers using the FluMag-SELEX method against two LMC targets within the LMC development timeline was tested. The method required target derivatives immobilisation onto the surface of Dynabeads® and iteration steps development prior to aptamer generation. It was decided to confirm surface immobilisation to prevent generation of unspecific aptamers because of the physiochemical nature of the targets and because it was not seen in literature as a common procedure. Due to the LMC targets and Dynabeads® characteristics, it was challenging to find an appropriate analytical technique to verify surface immobilisation. Also the refinement of each iterative step was a time consuming process. Based on those two major setbacks it was not possible to fully test the aptamer generation method to generate aptamers against the LMC targets within the project duration time. But it was possible to preliminary test the method and verify the presence of issues necessary to overcome in order to produce aptamers in the future. A novel change on the standard amplification step, with the use of real-time amplification and monitorisation, making possible to quantify and in one step verify the quality of the amplified material obtained in each iteration was also introduced. The work on this chapter also showed the impact of the LMC targets biochemistry in standard analytical detection methods and the principal issues found in aptamer generation.

On the second experimental piece of work (thesis Chapter five) the implementation of already existing DNA aptamers against small molecules, similar to the LMC targets, into the LMC assay type was tested. This test was made in order to understand the

integration viability of aptamers against small molecules on the LMC assay. It was verified in literature that it was not common to use DNA aptamers against small molecules in the LMC assay format, and only two examples were found. Nevertheless a total of three examples of DNA aptamers against small molecules using the LMC assay format were tested and it was verified that it was not possible to achieve a full working assay within the given project timeline. One of the examples exhibited high background and assay variability and the other two aptamers tested did not present a binding signal. The lack of straightforward results led to the test of the thrombin aptamers and target. It was also observed that the target surface immobilisation seemed to have a general impact on the way aptamers bound to it because it was reported in literature that the thrombin aptamer only bound to free target. Several questions arose from the work about the aptamers compatibility to the current LMC assay format requirements and about their impact on aptamer and target interaction. The implementation of aptamers against small targets in an assay with the current LMC requirements was not consistent and it was not possible to develop a full working inhibition assay for the LMC. It was possible to obtain a better comprehension on aptamers performance and to give input on how future work could be done if aptamers were the chosen bioreceptors for LMC instruments´ type.

In the last piece of experimental work (thesis Chapter six) a small scale experiment to introduce a specific design to improve PP&CC implementation as well as to demonstrate the execution of standard cleaning protocols and sterilisation and validation techniques was used. The experience gained with the small experiment gave origin to early recommendations to the LMC PP&CC implementation plan. The resilience of aptamers to DHMR, a common sterilisation procedure used in space missions was also given a preliminary test. Two conditions were tested and it was verified that aptamers appeared to survive to one of the implemented conditions. It was seen that aptamers presented resilience to DHMR when dissolved in the LMC buffer, which was rich in protective agents, and also when present at higher concentrations. Based on that preliminary data it was possible to conclude that aptamers could be possible candidates to the LMC bioreceptors and that their use would reduce the LMC integration costs and time because it would allow full integration and posterior sterilisation, which is not currently the plan with the chosen bioreceptors that do not survive such extreme treatments.

With this work it was possible to understand the viability of integrating aptamers as alternative bioreceptors in the LMC for the 2018 ExoMars mission. Based on the work developed and described in this thesis it was possible to verify that more time or resources would be necessary to confirm the possibility of generating aptamers against the LMC targets; that the aptamer integration into the LMC assay would need testing of conditions that could prevent or diminish the surface immobilisation and chemical modification impact on the aptamer and target interaction; and that aptamers seemed to survive extreme conditions required for sterilisation by DHMR. Also using CASS•E it was possible to verify and demonstrate the implementation of a UCZ and biobarriers as design changes that aided on the PP&CC implementation, and to introduce a novel validation technique with microbial pathway tracking using fluorescent beads.

7.2. Final conclusions

Based on the previous discussion about this work it was concluded the following:

- The generation of aptamers against small molecules using the FluMag-SELEX method was shown to be a complex method and a complete aptamer generation was not achieved within the allocated timeline and resources, based on this it was not possible to understand if aptamers could be successfully developed against LMC targets or not.
- The integration of the chosen published aptamers against small targets into an inhibition LMC assay format was not achieved but assay variability was verified in one of the cases, which raised questions about the impact of the LMC assay molecular modifications into the aptamer and target interaction
- Using CASS•E as a small scale life-search experiment it was possible to demonstrate the benefits of inserting in the design a UCZ and biobarriers for the PP&CC implementation, and to use a novel validation technique to track microbial contamination. It was extrapolated that those applications could allow an LMC instrument to meet the PP&CC requirements in the development process
- Preliminary tests showed aptamers resilience under specific conditions to implementation of sterilisation by DHMR, which could be helpful in the route to produce an LMC instrument that would meet PP&CC requirements
- It was seen that aptamers might have the potential to be integrated into a LMC instrument type but were not viable for the 2018 ExoMars mission

development programme due to time and resources constraints, and also because of the new questions that arose from this work.

7.3. Future work

For future work within the next six months it would be interesting to test aptamers in the three presented areas of work.

Regarding the aptamer generation, it would be relevant to run a new cycle of iterations, attempting aptamer generation against 5β-cholanic acid, avoiding or at least minimising sample loss between iterations. In parallel the aptamer generation against lysine could also be tested. Lysine which was successfully surface immobilised could represent the amino acids core target and the fact that was hydrophilic instead of hydrophobic could improve ssDNA chemical interaction and promote aptamer generation.

In the aptamers integration into the LMC assay format it would be necessary to understand the impact on aptamer and target interaction of surface immobilisation and other chemical modifications. The implementation of longer spacers as a poly T tail on the aptamer as well as the use of a spacer, or a longer spacer than the PEG_2 on the targets would be studied. Also, if possible, different atomic positions of the targets to perform the chemical modifications and see its impact on aptamer recognition would be compared.

For the PP&CC implementation it would be beneficial to perform aptamer integrity studies, running binding assays before and after DHMR implementation.

In a medium, long-term, it would be necessary to study affinities levels of selected strands against 5β-cholanic acid or lysine. That could be done with SPR instead of equilibrium binding in solution, in order to select an aptamer that would bind to a surface immobilised target, different from the initial matrix Dynabeads®.

In the aptamer integration into the LMC assay, once binding was achieved different ionic buffer composition should be tested in more detail to obtain the optimal running assay conditions, and only then perform inhibition assays. This could be critical because of the implementation of the LMC running buffer that could need adjustments to aptamers requirements.

Once the aptamers integrity was confirmed after DHMR it would be interesting to perform radiation tests that could mimic the exposure of aptamers during the mission

flight. It would be done by also studying structure and integrity before and after implementing radiation.

If a new approach would be taken from the previous aptamer work line, it would be the generation of aptamers against 5β-cholanic acid and lysine applying the structure-switch method and perform the selection in solution, Figure 7-1.

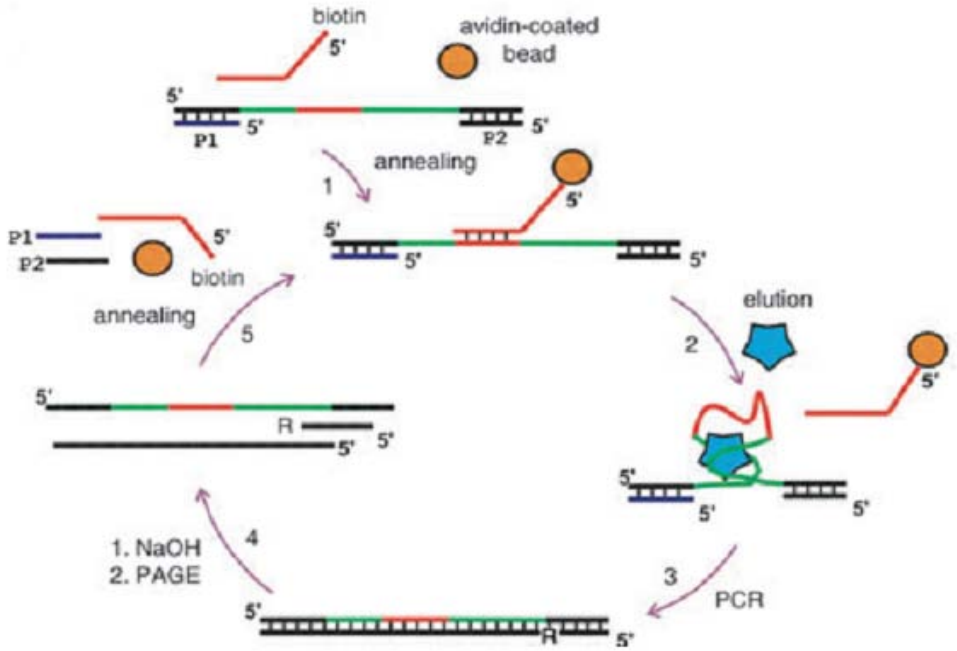


Figure 7-1 Strucure-switch aptamer generation method, adapted from Nitiu, et al (2005), (Nutiu, Li 2005).

Also the detection assays would be fully in solution and the integration of molecular beacons in the aptamer sequence would signal when the target binding occurred. That would eliminate any effect of surface immobilisation, (Nutiu, Li 2005). Also affinities and equilibrium studies would be done in solution, simplifying the protocols and virtually eliminating the problems found in this work.

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APPENDICES

Appendix A

A.1 Biomarkers list considered for life detection on Mars

Table A-1 Biomarkers list considered for life detection on Mars, adapted from Cullen and Sims (2007), (Cullen, Mark 2007).

LMC Targets		
Extant	Extinct	Contaminants
Phosphoenolpyruvate Phosphate	Generic isoprenoid	
Acetyl phosphate Phosphate	Pristane	Generic fungal
Cyclic AMP Phosphate	Phytane	Teichoic Acid
Generic pyrimidine base Nucleobase	carotane	phosphate
Generic purine base Nucleobase	carotenoids	polymer
DNA Nucleobase	Tetramethyl benzenes	LPS
Nicotinamide Vitamin	Tetramethyl cyclohexanes	Staphylococcus Bacterium
Flavin Vitamin	Squalane	Streptococcus Bacterium
Fe-S centers Redox center	Generic ABC terpane Hydrocarbon	Bacillus Bacterium
Quinones	Generic hopane Hydrocarbon	Micrococcus Bacterium
Generic carotenoid	Gammacerane Hydrocarbon	Pseudomonas Bacterium
Phycocyanin Pigment	Diasterane	Dipicolinic acid
Thioesters Ester	Porphyrin	Carboxylic acid
Generic extant porphyrin Porphyrin	**Meteoric:**	Hydrazine
Chaperones Protein	Napthalene	
ATP Synthase	Coronene	
Phytane		
Fatty acids (1 or 2)		

Teichoic acid Amino acid Phosphate wall polymer LPS Macromolecule Ectoine Trehalose Squalene Diploptene Hopanoid Melanoidins **Sediment/cell extracts:** Acid mine drainage Methanogens Cyanobacteria Extract/abiotic mix	Pyrene Dimethylbenzene Isovaline Isobutyric acid Generic aromatic Carboxylic acid Carboxylic acid	

A.2 Elemental analysis of L-cysteine Dynabeads® immobilised

Table A-2 Elemental analysis results from immobilised Dynabeads® with L-cysteine via EMCS spacer.

	Sample 1 (Dynabeads®)				Sample 2 (Dynabeads® with immobilised L-cysteine via EMCS spacer)				Sample 3 (Dynabeads® with immobilised EMCS spacer)			
Element	C	H	N	S	C	H	N	S	C	H	N	S
Average (%)	44.28	5.00	3.80	<0.10	44.24	5.09	3.78	<0.10	44.15	5.14	3.74	<0.10
SD	±0.02	±0.00	±0.04		±0.04	±0.07	±0.01		±0.08	±0.00	±0.06	

A.3 XPS peaks and subpeaks for immobilised species

- 5β-cholanic acid immobilisation via spacer cadaverine on carboxylic Dynabeads®.

Figure A-1 XPS sprectra of 5β-cholanic acid immobilised via cadaverine on carboxylic Dynabeads® surface (replicate 1).

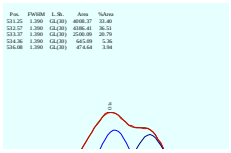

Figure A-2 Carboxylic Dynabeads® Oxygen subpeaks - O1s (replicate 1).

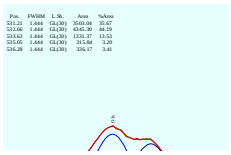

Figure A-3 Carboxylic Dynabeads® with immobilised cadaverine, Oxygen subpeaks - O1s (replicate 1).

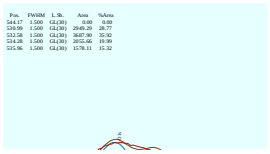

Figure A-4 Carboxylic Dynabeads® with immobilised 5β-cholanic acid via cadaverine, Oxygen subpeaks - O1s (replicate 1).

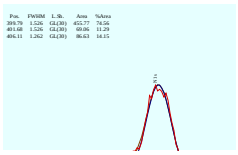

Figure A-5 Carboxylic Dynabeads® Nitrogen subpeaks - N1s (replicate 1).

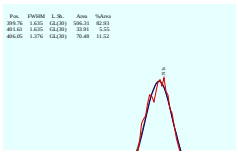

Figure A-6 Carboxylic Dynabeads® with immobilised cadaverine, Nitrogen subpeaks - N1s (replicate 1).

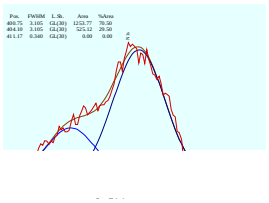

Figure A-7 Carboxylic Dynabeads® with immobilised cadaverine and 5β-cholanic acid, Nitrogen subpeaks - N1s (replicate 1).

- 5β-cholanic acid direct immobilisation on amino Dynabeads®.

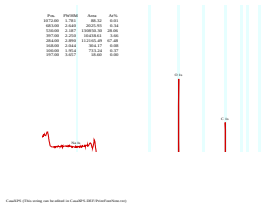

Figure A-8 Amino Dynabeads® with immobilised 5β-cholanic acid full XPS spectra.

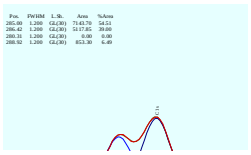

Figure A-9 Amino Dynabeads in DMSO buffer, Carbon subpeaks – C1s (replicate 1).

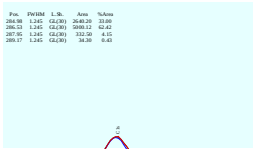

Figure A-10 Amino Dynabeads® with immobilised 5β-cholanic acid, Carbon subpeaks - C1s (replicate 1).

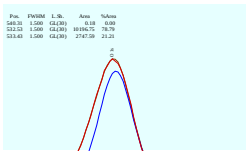

Figure A-11 Amino Dynabeads® in DMSO buffer, Oxygen subpeaks - O1s (replicate 1).

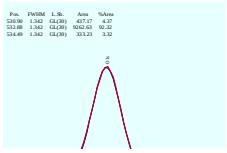

Figure A-12 Amino Dynabeads® with immobilised 5β-cholanic acid, Oxygen subpeaks - O1s (replicate 1).

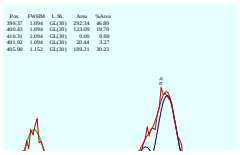

Figure A-13 Amino Dynabeads® in DMSO buffer, Nitrogen subpeaks - N1s (replicate 1).

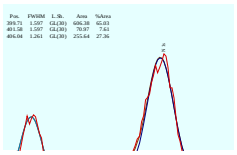

Figure A-14 Amino Dynabeads® with immobilised 5β-cholanic acid, Nitrogen subpeaks - N1s (replicate 1).

- Lysine immobilisation on amino Dynabeads®.

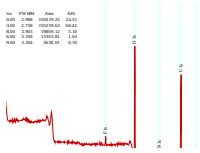

Figure A-15 XPS spectra of carboxylic Dynabeads® immobilised with lysine (replicate 1).

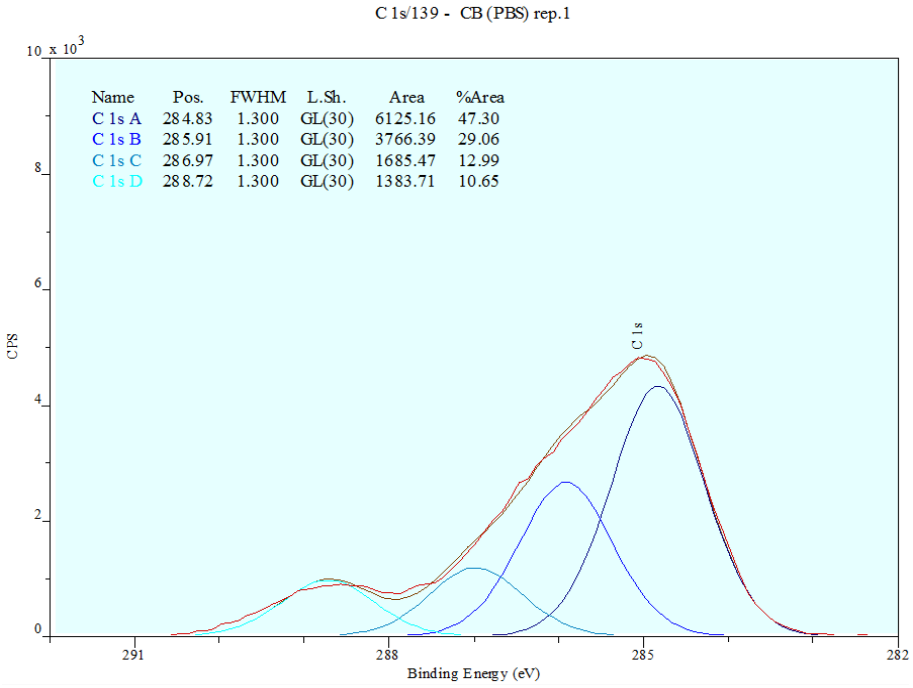

Figure A-16 Carboxylic Dynabeads® in PBS buffer, Carbon subpeaks - C1s (replicate 1).

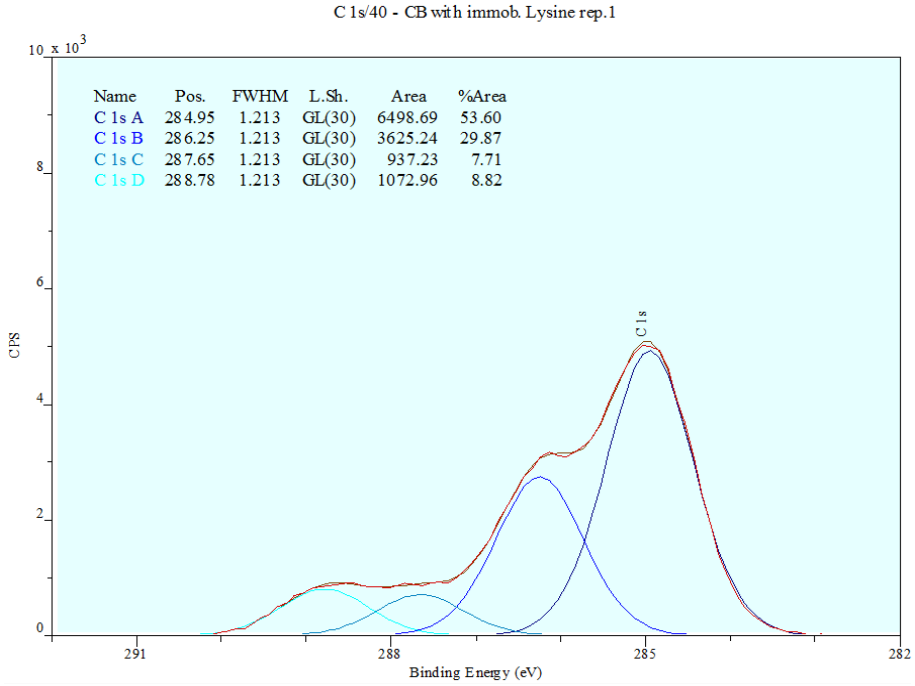

Figure A-17 Carboxylic Dynabeads® with immobilise lysine, Carbon subpeaks - C1s (replicate 1).

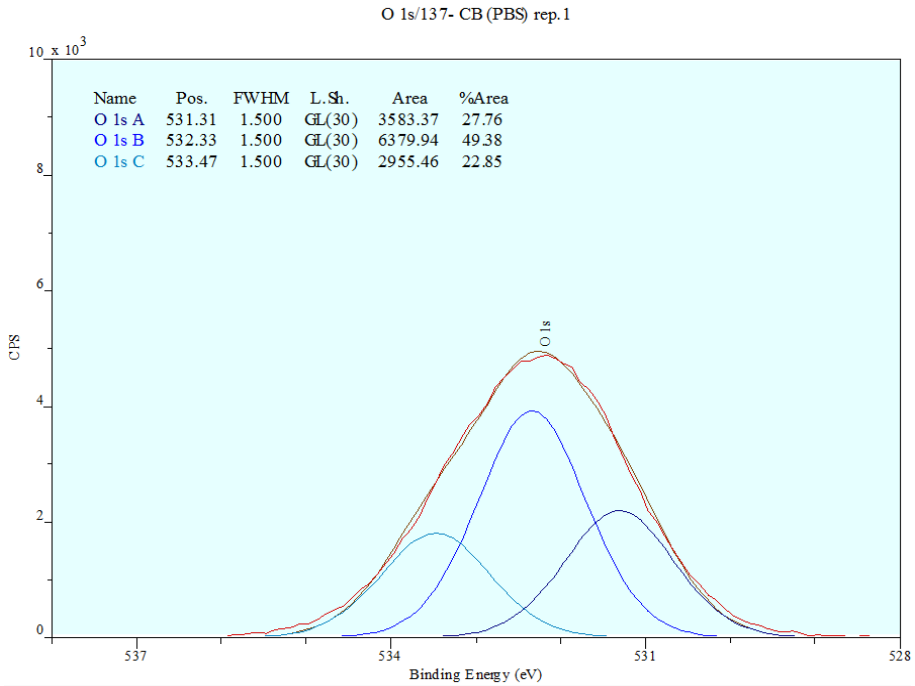

Figure A-18 Carboxylic Dynabeads in PBS buffer, Oxygen subpeaks - O1s (replicate 1).

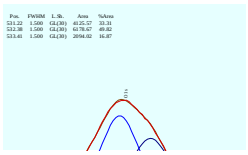

Figure A-19 Carboxylic Dynabeads® with immobilise lysine, Oxygen subpeaks - O1s (replicate 1).

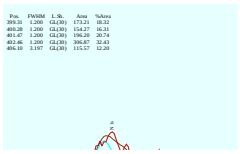

Figure A-20 Carboxylic Dynabeads® in PBS buffer, Nitrogen subpeaks - N1s (replicate 1).

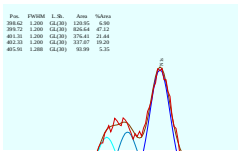

Figure A-21 Carboxylic Dynabeads® with immobilise lysine, Nitrogen subpeaks - N1s (replicate 1).

A.4 Microbial reduction based on time and temperature

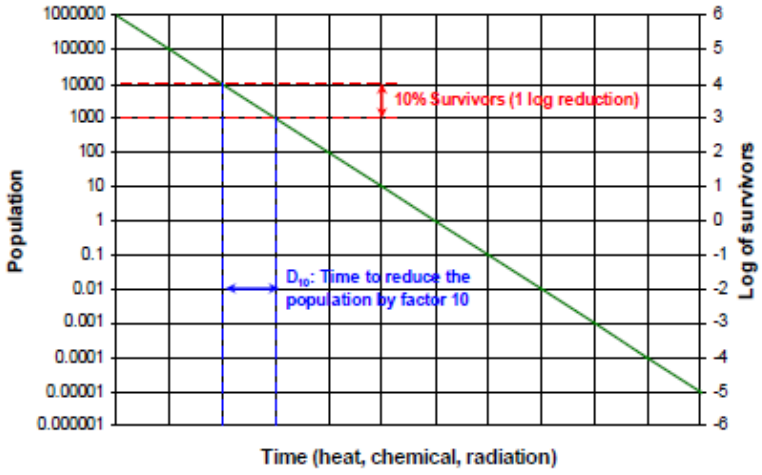

Figure A-22 Graph illustrating the effect of time and heat in the microorganism reduction, adapted from ECSS –q-st-70-53c, (ECSS 2012).

A.5 Operation list for PP&CC implementation on CASS•E

Table A-3 Operation to perform to implement PP&CC before and after flight, adapted from SED v5.0, (Juanes-Vallejo, Grama et al. 2011).

O2	The experiment shall only be handled by operators wearing nitrile gloves during integration and launch.
O3	The part of CASS•E exposed to the exterior of the Gondola shall be protected with a *remove before flight cover.*
O4	The *remove before flight cover* shall be removed before flight.
O5	The UCZ shall open once the balloon has reached the Stratosphere (at 20km above sea level) and not before.
O6	The UCZ shall be re-sealed prior to the descent phase of the balloon.

Appendix B

B.1 Article in Journal:

Planetary and Space Science 72 (2012) 129–137

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journal homepage: www.elsevier.com/locate/pss

Development status of the life marker chip instrument for ExoMars

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ABSTRACT

The Life Marker Chip (LMC) is one of the instruments being developed for possible flight on the 2018 ExoMars mission. The instrument uses solvents to extract organic compounds from samples of martian regolith and to transfer the extracts to dedicated detectors based around the use of antibodies. The scientific aims of the instrument are to detect organics in the form of biomarkers that might be associated with extinct life, extant life or abiotic sources of organics. The instrument relies on a novel surfactant-based solvent system and bespoke, commercial and research-developed antibodies against a number of distinct biomarkers or molecular types. The LMC comprises of a number of subsystems designed to accept up to four discrete samples of martian regolith or crushed rock, implement the solvent extraction, perform microfluidic-based multiplexed antibody-assays for biomarkers and other targets, optically detect the fluorescent output of the assays, control the internal instrument pressure and temperature, in addition to the associated instrument control electronics and software. The principle of operation, the design and the instrument development status as of December 2011 are reported here. The instrument principle can be extended to other configurations and missions as needed.

1. Introduction

Recent exploration of Mars has revealed abundant evidence for environmental conditions capable of supporting life in the past, such as the discovery of subsurface ice in the Vastitas Borealis by NASA's Phoenix lander (Smith et al., 2009), and the abundant geological evidence for interaction with water (Ehlmann et al., 2011). There is even the possibility of extant life given detection of methane in the atmosphere of Mars (Formisano et al., 2004), suggesting the possibility of methanogenic bacteria in the subsurface (Krasnopolsky et al., 2004) although many abiotic sources of methane (e.g., hydrothermal, volcanic, clathrates) are also plausible. The "follow the water" strategy is now being overtaken by the "follow the carbon" strategy and the next key objective is to detect organic compounds on Mars and to assess the habitability of past and present day Mars, a key aim of the Mars Science Laboratory mission. Once carbon is detected the identification of its source

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B.2 Conference Paper:

CRANFIELD ASTROBIOLOGICAL STRATOSPHERIC SAMPLING EXPERIMENT (CASS•E): OVERVIEW OF FLIGHT HARDWARE CONFIGURATION, IMPLEMENTED PLANETARY PROTECTION AND CONTAMINATION CONTROL PROCEDURES AND PRELIMINARY POST-FLIGHT RESULTS

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ABSTRACT

CASS•E is an experiment that is the initial part of a program aimed at collecting direct evidence of microorganisms in the Earth's stratosphere. The experiment was launched on-board a stratospheric balloon in collaboration with Eurolaunch through the BEXUS (Balloon-borne EXperiments for University Students) program from Esrange, Sweden, in October and November 2010. It consisted of a pump which drew air from the stratosphere through inlet tubes and collected samples on a particle collection filter located inside an Ultra Clean Zone. Due to the low density of microbes in the stratosphere compared to the known levels of contamination present during ground handling, the experiment incorporated Planetary Protection and Contamination Control (PP&CC) protocols in its design and construction in order to minimise the chance that any microbes detected were ground derived contamination. Space community derived cleaning and sterilisation techniques were employed throughout Assembly Integration and Testing (AIT) as well as bio-barrier mechanisms which were designed to open only in the stratosphere to prevent re-contamination of the instrument after sterilisation. The material presented in the paper covers the design, AIT and preliminary post-flight results of CASS•E. The objective of this initial implementation and the first flights was to better understand the issues of incorporating PP&CC and additional features to control and understand contamination into a balloon platform and thereby inform future experiments rather than perform a definitive collection of stratospheric samples for life detection.

1. INTRODUCTION

The study of life in extreme environments on Earth, including the stratosphere, contributes to our understanding of the possibility of life elsewhere in the Universe. Previous balloon experiments to attempt collection of microbial life in the stratosphere have addressed, to varying levels, the issue of contamination with non-stratospheric microorganisms that may occur pre- and post-stratospheric flight phases [2, 3]. However, it has proven difficult to convince the wider scientific community that resultant claims of stratospheric life collection are not simply ground or other tropospheric derived contamination.

A similar concern about Earth-derived microbial contamination in life detection experiments and spacecraft for planetary exploration missions has lead to the development of Planetary Protection and Contamination Control (PP&CC) protocols to address these concerns; *i.e.* to minimise the potential for contamination during build, assembly, verification and handling of instrumentation and spacecraft. Examples of protocols include thorough cleaning to minimise the level of contamination with viable microorganisms (termed 'bio-burden'), measurement of the achieved level of bio-burden and then followed by the use of Dry Heat Microbial Reduction (DHMR) by heating items under controlled humidity (< 1.2 g/m^3 water) for a given length of time. The time and temperature required in order to achieve a x10^4 to x10^6 reduction in bio-burden vary depending on the nature of the item to be sterilised. Therefore, such protocols are intended to achieve a final level of bio-burden that is compatible with a given acceptable risk of contamination [4, 5].

A critical aspect of the use of PP&CC protocols are approaches to maintain, after treatment, the achieved levels of bio-burden whilst performing subsequent pre-flight handling and the flight of the experiment or spacecraft. Physical barrier approaches to stop re-

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CRANFIELD ASTROBIOLOGICAL STRATOSPHERIC SAMPLING EXPERIMENT (CASS•E): OVERALL PERFORMANCE OF THE EXPERIMENT DURING FLIGHT AND PARTICLE COLLECTION FILTER ANALYSIS

Abstract

The Cranfield Astrobiological Stratospheric Sampling Experiment (CASS•E) was an experiment which used two pumps to draw air from the Earth's Stratosphere through collection filters in order to perform post-flight detection and characterisation of any collected microorganisms. It was flown on-board the BEXUS-10 and BEXUS-11 stratospheric balloon flights in October and November 2010 from Esrange, Sweden, as part of the Balloon-borne EXperiments for University Students (BEXUS) program. Due to the low density of microorganisms expected in the Stratosphere compared to the levels of microorganisms present during pre- and post-flight ground handling and tropospheric ascent and descent, the design implementation included multiple steps to minimise contamination of the samples. Therefore, the experiment incorporated Planetary Protection and Contamination Control (PPCC) protocols which included Space-qualified cleaning and sterilisation techniques employed throughout the experiment Assembly, Integration and Testing (AIT). Most importantly, the experiment's inlet tubes were protected by bio-barrier mechanisms that would only open upon arrival to the Stratosphere, thus ensuring that any particles collected would be truly stratospheric. During the BEXUS-10 flight a malfunction in the bio-barrier mechanisms impeded pumping stratospheric air; however, the BEXUS-11 flight was successful and particles were collected. This report presents the overall performance of the experiment during flight and the preliminary results of the analysis of the material collected on the particle filters.

Prague; 27 September -1 October 2010

IAC-10.A1.5.3

CASS•E : CRANFIELD ASTROBIOLOGICAL STRATOSPHERIC SAMPLING EXPERIMENT

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CASS•E is a life detection experiment that aims to be capable of collecting microorganisms in Earth's Stratosphere. The experiment will be launched on a stratospheric balloon in collaboration with Eurolaunch through the BEXUS (Balloon-borne EXperiments for University Students) program from Esrange Sweden in October 2010. It essentially consists of a pump which draws air from the Stratosphere through a collection filter mechanism. Due to the low number density of microbes in the Stratosphere compared to the known levels of contamination at ground level, the experiment incorporated Planetary Protection and Contamination Control (PP&CC) protocols in its design and construction in order to confirm that any microbes detected are truly Stratospheric in origin. Space qualified cleaning and sterilisation techniques were employed throughout Assembly Integration and Testing (AIT) as well as bio-barriers which were designed to open only in the stratosphere and so prevent recontamination of the instrument after sterilisation. The material presented here covers the design and AIT of CASS•E.

I. Introduction

The Earth's Stratosphere is an extreme environment with near vacuum pressure, high levels of ultraviolet radiation and temperatures as low as –90°C[1]. The study of life in extreme environments on Earth is furthering understanding for the possibility of life elsewhere in the Universe [2].

Historically, balloon experiments to detect life in the Stratosphere have not fully addressed the issue of ground contamination that may occur both before and after landing of Stratospheric balloon platforms used, in accordance with PP&CC protocols developed for space missions[3,4,5,6]. The concern is that ground levels of microbes will dominate over levels expected in the Stratosphere making detection and origin determination difficult.

PP&CC protocols have therefore been implemented. These involve the use of cleaning and sterilisation techniques to reduce bio-burden to acceptable levels. These were then followed by the verification of sterility levels using standard microbiological assays as well as the implementation of methods to prevent re-contamination of the cleaned and sterilised instrument during AIT [7].

Besides the implementation of these protocols which ensure no contamination occurs to the critical areas that collect microbes, the experiment was designed to function at the low temperatures and pressures expected during the flight and sustain the forces expected during landing.

The mission is guided by category IVb COSPAR (Committee on Space Research)[8] requirements.

The overall aims of CASS•E can be summarised as follows:

1. To design, build and fly an experiment for use on a Stratospheric balloon platform that is capable of collecting microorganisms in the Earth's Stratosphere.

2. To ensure, through the implementation and assessment of appropriate PP&CC protocols, that microorganisms collected are truly Stratospheric rather than contamination introduced during AIT, ground handling, launch, flight and recovery.

II. Experiment Overview

The experiment, mounted to the gondola of a Stratospheric balloon, takes approximately 1 hour to reach an altitude of between 25 to 30 km[1]. It floats at this altitude for approximately 4 hours, after which the tether holding the gondola to the balloon is severed. The gondola then falls back to ground using a parachute.

In order to collect Stratospheric microorganisms CASS•E will draw a volume of Stratospheric atmosphere through a collection filter. The flow will be achieved using a vacuum pump once the BEXUS balloon reaches the Stratosphere.

Due to the flow rates achievable with pumps compatible with the size and mass requirements of the BEXUS platform, and the expected number densities of microbes in the Stratosphere[3,4] it is difficult for any Stratospheric microorganisms to be collected. The intention is for scientifically rigorous collection of Stratospheric organisms to occur in a future larger ver-

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