CRANFIELD UNIVERSITY

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Development of antibodies for Life-detection experiments in extreme environments; Implications for Astrobiology

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PhD Thesis

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ACADEMIC YEAR 2010 – 2011

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Submitted November 2010

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

Thesis Abstract

The Life Marker Chip (LMC) instrument is an antibody assay-based system which will attempt to detect molecular signatures of Life in the Martian subsurface as part of the payload on board the European Space Agency (ESA) ExoMars mission rover, currently scheduled for launch in 2018. The LMC will have the ability to detect up to 25 different molecular targets of different origins that are associated with meteoritic in-fall, extinct or extant Life, prebiotic chemistry and spacecraft contamination. Regolith / crushed rock samples will be collected for the LMC by the rover and subjected to solvent extraction to extract organic molecules for analysis by the immunoassays.

One of the key stages in the development of the LMC is the selection of antibodies to be used in the flight instrument. The challenge lies in the nature of the molecules or classes of molecules that are LMC targets and the need for antibodies that remain functional in the extreme conditions during a planetary exploration mission, especially the radiation environments.

The work described within focuses on two main aspects of the search for LMC-relevant antibodies; the effect of space radiation on antibody performance [in the form of both ground-based and Low Earth Orbit (LEO)-set studies] and the development of "customised" antibodies against some of the molecules that are being investigated as potential LMC targets.

The need to study the effects of space radiation on antibodies arose due to lack of any heritage of their use in interplanetary missions. For all the antibodies in the LMC, the ability to resist inactivation due to space radiation seen during a Mars mission will be a prerequisite. The objective of the ground-based radiation studies was to expose a number of LMC-relevant antibodies to simulated Mars mission radiation in the form of proton and neutron radiation which are the components of the mission radiation environment that are expected to have the dominant effect on the operation of the LMC.

Various combinations and multiples of the nominal mission fluence values that had been calculated via modelling of the radiation environment for a mission to Mars were used to demonstrate the effects of radiation on antibody activity, both at the actual radiation levels envisaged for the ExoMars mission and at much higher levels. Five

antibodies were freeze-dried in a variety of protective molecular matrices and exposed to various radiation conditions. After exposure, the antibodies' ability to bind to their respective antigens was assessed. At radiation levels equal to 1x times the mission dose, four antibodies out of the five maintained the majority of their binding activity, while at 10x and 250x times the mission dose, the same four out of five antibodies maintained considerable amounts of their binding activity. The preparation and treatment (lyophilisation/rehydration) of the antibody samples were found to have a detrimental impact on their performance. These experiments indicate that the expected radiation environment of a Mars mission does not pose a significant risk to antibodies packaged in the form anticipated for the LMC instrument.

The LEO radiation studies that are described were part of ESA's 2007 BIOPAN-6 Low-Earth Orbit space exposure platform. Two representative LMC antibodies were used in the form of lyophilised samples integrated in glass fibre pads, loaded into a custom-made sample holder unit which was mounted on the BIOPAN-6 platform. BIOPAN-6 went into orbit for 12 days, after which all samples were recovered and their binding performance was measured via ELISA. The factors expected to affect antibody performance were the physical conditions of a space mission and the exposure to space conditions (i.e. the radiation environment in LEO). Both antibodies survived complete inactivation due to these factors.

Phage display technology offers the possibility to rapidly generate high affinity recombinant antibodies against small molecules with poor immunogenic potential (haptens), like a number of prospective LMC target molecules. In the present work, a naive and an immunised phage antibody library have been employed in the production of recombinant antibodies against phytane and β-carotane. The naive library gave extremely poor results for both targets. The immunised library had been constructed after animal immunisation with a mixture of LMC targets, and resulted to the identification of phage clones that appeared able to recognise target conjugated to a carrier protein, without presenting cross-reactivity with the carrier proteins alone. Therefore phage display technology with the use of immunised libraries was considered an appropriate method to pursue for obtaining antibodies for the Life Marker Chip instrument.

Acknowledgments

This research has been funded by the Engineering and Physical Sciences Research Council (EPSRC) (Year 1) and the Science and Technology Facilities Council (STFC).

The modelling of the radiation environment for the ExoMars mission has been carried out by Alexander Hands (QinetiQ, UK), who also organised the irradiations that took place at the Theodor Svedberg Laboratory (TSL) in Uppsala, Sweden.

The phage antibody libraries that were used in the present studies have been constructed by Grampian Bioconsultants Ltd. (Aberdeen, Scotland).

I would like to thank my supervisor, Prof. David C. Cullen, for the opportunity he gave me by taking me on and for all the great adventures that came because of that; also for his infinite support and patience in the past four years.

A huge thank you to Dr. Bernard Derek Annan and Dr. Manisha Sathe, for being my laboratory mentors and the greatest of friends.

Many thanks to the LMC group in Cranfield University: Dr. Paul K. Wilson, Dr. Alan Buckley, Dr. Megan Barnett, Catherine Rix and Marjorie Allen, for all their help with everything and for making life in the lab super enjoyable. Also, to the staff, students and friends at Cranfield University for four unforgettable years.

I would like to thank the LMC group in the University of Leicester, especially Prof. Mark R. Sims and Daniel Thompson for his contribution in the ground-based radiation studies.

Another massive thank you to the phage experts in Grampian Bioconsultants Ltd.: Professor Andrew Porter, Dr. Gillian Broadbent, Dr. Keith Charlton, Dr. Soumya Palliyil, Benjamin Arnold and Bindu Ravi, for sharing their knowledge and never giving up in helping to make sense out of insanely difficult things.

I would also like to extend an immense thank you to Alex Hands from QinetiQ for his invaluable input in the ground-based radiation studies, all his help and guidance during the irradiations at the TSL in Uppsala and for his insightful comments on the relevant chapter of the present thesis.

To Prof. Loukas Vlahos, from the Aristotle University of Thessaloniki, a big thank you for all the support and encouragement.

Finally and crucially, I would like to thank my family for their endless love and support, and, most importantly, for never teaching me how to be a realist.

Table of Contents

List of Figures

List of Tables

1 .

General Introduction and Thesis Structure

Thesis Preamble – The Life Marker Chip Instrument

The driving force behind the experiments that make up the present work has been the undertaking of the development of an instrument called the Life Marker Chip (LMC) by an international scientific consortium, lead by the University of Leicester and Cranfield University. The LMC is an antibody-based biosensor that will be part of the ExoMars mission, a joint initiative between the European Space Agency (ESA) and NASA, currently scheduled for launch in 2018. The mission belongs to a greater framework for Mars planetary exploration that has recently been announced by ESA and NASA.

The LMC will be part of the Pasteur payload on board the ExoMars rover and it will have the ability to detect up to 25 different molecular targets of different origins that are associated with meteoritic in-fall, extinct or extant Life, prebiotic chemistry and spacecraft contamination. Regolith / crushed rock samples from up to four different locations can be collected for the LMC, which is designed to integrate sample processing and analysis into what is essentially an analytical laboratory in miniature.

The Life Marker Chip reflects a rise in the interest in the use of immunoassays in Life detection experiments in extreme environments, with antibodies being considered very promising tracers of molecular signatures of Life (biomarkers) in the field of Astrobiology (Steele *et al*., 2001, Parro *et al.*, 2005; 2011). The target selection for the LMC instrument was the result of an international biomarker workshop held in 2006 (Parnell *et al*., 2007), where an attempt was made to compose a list of molecules that could constitute proof of the presence of Life, past or present, in light of the ExoMars mission. This list of potential targets included markers of present (extant) Life, markers of fossil (extinct) Life, molecules of meteoritic origin and contamination markers. For a more comprehensive description of some candidate LMC targets (including their chemical structures), see Appendix 1.

One of the key challenges that had to be addressed in the LMC development process has been the selection of antibodies that have a combination of characteristics (sensitivity, specificity, resilience, etc.) that make them appropriate for use in the final (flight) model of the instrument. In contrast to antibodies that are developed for laboratory experiments, the LMC antibodies must not only detect their respective targets even at extremely low amounts in a sample, they must also maintain their performance quality

in the array of conditions envisaged for a planetary exploration mission, primarily in terms of space radiation.

Within the LMC development framework, and given the factors that must be taken into account in order to acquire antibodies that can be used for the instrument, the present work focused on two main areas; the effect of space radiation on LMC candidate antibodies and the production of bespoke antibodies against LMC targets using phage display technology. The radiation studies were further separated in ground-based and Low Earth Orbit (LEO)-set experiments, the latter in the form of BIOPAN-6, an ESA-sponsored experimental platform for the exposure of biological material to LEO conditions.

Experimental Work Overview and Thesis Structure

1.1 Ground-based Radiation studies (Chapter 2)

During the course of these studies, the radiation environment envisaged for a mission to Mars was simulated using a cyclotron facility in Sweden. The proton and neutron components of the mission radiation environment are those that are expected to have the dominant effect on the operation of the LMC, and for this reason they were the radiation environment components that were used in these irradiations. A total of five LMC candidate antibodies were freeze-dried in a variety of protective molecular matrices and were exposed to the various combinations and doses of proton and neutron radiation. After exposure, the antibodies' ability to bind to their respective antigens was assessed using enzyme-linked immunosorbent assays (ELISA).

1.2 Radiation studies in Low Earth Orbit (LEO) – BIOPAN-6 (Chapter 3)

In September of 2007, various components of the LMC instrument, including two representative antibodies in lyophilised format, were put in LEO for a period of 12 days, as part of ESA's BIOPAN-6 platform for direct exposure of biological material to space conditions. This was an opportunity to test the antibodies' performance after exposure to a natural (non-simulated) space environment, as well as the combination of conditions that compose an actual space mission (launch, operation, re-entry where

applicable). After recovery of the samples, their ability to recognise and bind to their respective targets was assessed via ELISA.

The BIOPAN experiments, though not directly comparable to the ground-based studies for a number of reasons (including the heterogeneity of a natural radiation environment, the doses of radiation the samples were exposed to and the addition of all "actual mission factors"), helped form a more complete view of the behavior of antibodies in a hypothetical planetary exploration mission setting.

1.3 Development of recombinant antibodies using phage display technology (Chapter 4)

Phage display was used to explore its potential to produce recombinant antibody molecules against two of the "difficult" LMC targets (phytane and carotane). This method offers the possibility to generate high affinity recombinant antibodies against small molecules with poor immunogenic potential (haptens). As a number of potential LMC targets are haptens, there is a need to have a standard method of anti-hapten antibody generation, and the traditional routes proved insufficient from an early stage of the LMC development.

Two phage antibody libraries were used in these experiments; a naive library (which had not been constructed following animal immunisation with our particular targets) and an immunised library which had been specifically constructed against a mixture of LMC targets. The screening of the naive library for binders against phytane and carotane was performed initially, followed by screening of the immunised library. The quality of the results of the screening process was assessed using ELISA.

1.4 Additional Notes on the Thesis Structure

A final "Discussion" section (*Chapter 5*) at the end of chapters 1-4 summarises the findings of the research work, along with conclusions that were drawn after consideration of these results.

It is important to note that instead of a separate chapter presenting a review of the literature and a standalone "Materials and Methods" chapter, these sections for each of the three sets of experimental work were incorporated into the individual chapters (2-4), aiming to give the reader a better understanding of each cluster of experiments, their theoretical background and the details of their execution, before bringing all the data together in Chapter 5.

Thesis Aims and Objectives

1.5 Aims

The main aim of the thesis was to consider aspects of antibodies within the early stage developments of the LMC. In particular, to contribute to the development of the LMC via (i) de-risking the concern that the radiation environment to be encountered by the LMC instrument would degrade the antibodies at the heart of the LMC or require additional radiation shielding mass that would make the overall LMC unviable and (ii) exploring the possibility of phage display recombinant antibody technology as a viable tool to produce antibodies against potential targets within the development schedule context of the LMC.

1.6 Objectives

Given the limited amount of data concerning the effects of particle radiation on proteins (antibodies), there was a need to study the effects of simulated space radiation on representative LMC candidate antibodies in order to confirm that they would be able to survive radiation conditions relevant to a Mars mission, provided they are appropriately packaged.

The main objective of the ground-based studies was to expose a number of lyophilised antibodies to various combinations and doses of proton and neutron radiation, produced by a cyclotron source, at levels that would closely simulate those calculated via

modelling of the radiation levels expected during a mission to Mars. A further objective was to assess a number of lyophilisation matrices for their ability to retain antibody function during lyophilisation, storage, radiation exposure and rehydration. The antigen binding functionality of the antibodies was measured post-radiation exposure using a simple immunoassay approach and compared to a number of control samples that allowed correction for any effects of shipping (from Cranfield University to the Swedish cyclotron facility) and/or storage conditions on the exposed samples.

The LMC experiment on BIOPAN-6 was complementary to the series of ground-based irradiations and offered additional information about the ability of antibodies to recognise and bind to their respective antigens after exposure to space radiation. By exposing antibodies to a heterogeneous (in composition and particle energy levels) radiation environment, the objective was to obtain a more accurate representation of the radiation situation of an actual mission to Mars. In addition to the primary radiation (protons, electrons, heavy ions), an additional LMC on BIOPAN objective was to evaluate the effect (if any) of secondary radiation and any spacecraft radiation sources on antibody performance.

Given that the LMC antibodies had no history of space flight, another objective of the BIOPAN experiment was to assess the importance of the physical aspects of a space mission. These included the conditions during launch and re-entry (shock loadings, acoustic energy during launch etc.) and the general ground handling of samples, from the point of sample preparation all the way to recovery and analysis.

In order to determine whether phage display could be the main method of acquiring antibodies against high priority LMC target haptens within the LMC development programme, the final cluster of work aimed to demonstrate the generation of recombinant antibodies against two potential LMC targets (phytane and β-carotane) with the employment of phage display technology.

By using both a naïve and an immunised phage antibody library, their potential could be directly compared; the use of naïve libraries does not involve the construction cost and time delay of obtaining an immunised library, and could therefore prove more resource-efficient if successful. Additionally, the naïve library which was used had been

constructed as an immunised library against another mixture of small molecules (lactone-type haptens), and this was seen as a chance to examine whether there would be any kind of positive bias in acquiring binders against our own haptens.

Given that this was the first time that phage display technology was to be used in the context of the LMC development, the present work was also an opportunity to adjust and optimise the protocols related to the screening of the libraries to fit the requirements of specific targets, in order to isolate high-quality binders against them.

2 .

Effects of Simulated Space Radiation on Immunoassay Components for Life Detection Experiments in Future Planetary Exploration Missions

Introduction

2.1 Use of immunoassays in Life detection experiments on the surface of Mars

The driving force behind the radiation studies described in the present chapter is the on-going development of the Life Marker Chip (LMC) instrument, which is part of the Pasteur instrument payload of the European Space Agency's (ESA) ExoMars rover mission. The ExoMars rover mission's primary goal is the search for evidence of past and/or present life on Mars and is currently scheduled to launch to Mars in 2018. The mission belongs to a greater framework for Mars planetary exploration that has recently (2009) been announced by ESA and NASA (Brown, 2009).

The LMC is frequently described in the general media as a "pregnancy test for Mars", a term which hails from the fact that both the LMC and commercial off-the-shelf pregnancy tests are based on lateral flow immunodiagnostic technology (Wilson and Howell, 2002). Thus the LMC uses (i) multiple dried-down fluorescently-labelled antibodies that (ii) dissolve into an aqueous-based liquid extract of a Martian regolith / crushed rock sample containing extracted organic molecules, and (iii) this initiates a multiplexed inhibition immunoassay before (iv) being flowed over a microarray composed of immobilised copies of the various multiplexed immunoassay targets. For a given antibody, (v) if the corresponding target molecule is present in the liquid extract, the antibody binding site is occupied and thus inhibited from binding to the corresponding microarray spot which (vi) subsequently does not fluoresce. Conversely, if the relevant target molecule is not present in the liquid extract, the antibody binding site is left vacant and therefore able to bind to the corresponding microarray spot which fluoresces. The immunoassay target molecules that are being chosen as LMC targets are those representative of ancient, preserved biomarkers of extinct life, biomarkers of extant life, those representing meteoritic input and other abiotic organic molecules as well as organic molecular markers of rover / instrument borne Earth contamination (Parnell *et al.*, 2007).

2.2 Radiation environment during a mission to Mars

All space missions inside and outside the Earth's magnetosphere are subject to a radiation environment harsher than that found at sea-level on Earth. Space radiation is therefore of concern as it could have a detrimental effect on antibodies and other molecular assay reagents which form part of instruments like the LMC; with this in mind, the radiation environment during a mission to Mars has been recently modelled using Monte Carlo simulations (Le Postollec *et al*., 2009a). A vital stage in the development of the LMC has been the study of the effects of space radiation on the biological, and chemical, assay components.

The radiation field within the Earth's magnetosphere, despite being far from negligible for missions in Low Earth Orbit (LEO), is not significant in the context of a planetary exploration mission, as the payload will only be exposed to it for a very short period of time. The key radiation environments are limited to the radiation en route to a planet (cruise phase) and the radiation environment on the planet's surface (surface phase).

Over a two-year cruise period to Mars, which was the transit duration for the original ExoMars mission circa 2006 (cruise phase duration is expected to be nine-months for the current mission design), the radiation environment to which all mission instruments are exposed is mainly due to Galactic Cosmic Rays (GCR) and Solar Particle Events (SPE). GCR exposure is expected to be approximately constant, while radiation associated to SPE will be more sporadic. (Hands and Rodgers, 2006). Modelling of the radiation environment during the transit phase using standard tools (SPENVIS and CREME96), determined that the dominant radiation threat would come from the SPE protons, followed by protons and high energy ions present in GCR (Hands and Rodgers, 2006).

On the surface of Mars, the radiation environment is composed of four types of directly ionising radiation; ultraviolet (UV), solar energetic particles (SEP) composed mainly of protons, galactic cosmic rays (GCR) mainly composed of stripped ions from hydrogen to uranium, and mineral radiation (MR). Estimations have been made for the surface radiation environment, as well as the one en route to Mars, and the findings suggest that

the proton dose received on the surface is very small compared to the dose while in transit, due to attenuation from the Martian atmosphere.

An additional component of the Martian radiation environment, in the context of the operation of the LMC instrument, is the neutron environment. The neutron flux at the Martian surface is considerably higher than the proton flux and dominates over the previously mentioned radiation types (Hands, 2008). The Martian surface neutrons are produced as cosmic rays penetrate the thin Martian atmosphere, a process exacerbated by the lack of a magnetosphere like the Earth's, and reach the surface thereby interacting with elements in the upper layer of regolith and scattering secondary neutrons back towards space.

2.3 Previous studies on the effects of radiation on biological systems

The majority of recent studies on irradiation-induced alterations in biomolecules focus on the effects of gamma irradiation (usually from a ^{60}Co source), since gamma ray treatment has applications in the sterilisation of medical supplies (Grieb *et. al*, 2002, Caballero *et. al*, 2004), the immobilisation of bioactive materials, food irradiation and a number of other uses, like the modification of antigenicity of certain proteins (Kume and Matsuda, 1995, Caproni *et. al*, 2007). The latter studies showed that radiation was able to affect the antigenicity of certain proteins as it resulted to conformational (denaturation) and physicochemical (aggregation) changes to the molecules. Additionally, the studies on the use of gamma irradiation for sterilisation purposes highlight the need for the use of radioprotective excipients, *i.e*. compounds that can increase stability prior, during and after irradiation (*e.g*. ascorbate – Grieb *et. al*, 2002).

On a structural level, the effects of gamma irradiation on proteins have been extensively studied (Kempner, 2001), and the mechanism of damage can be either direct via rupture of covalent bonds in the protein molecules as a result of a photon depositing energy into the molecule, or indirect, with the participation of water molecules. In the second case, the production of free radicals and other non-radical reactive oxygen species (ROS) is responsible for the majority (99.9%) of the protein damage. Gamma irradiation of aqueous solutions in the presence of oxygen results in formation of hydrated electrons,

hydrogen atoms, hydrogen peroxides, and the most damaging hydroxyl radicals (SOH). Exposure of proteins to such generated ROS can alter the physical and chemical structure of the target causing consequent oxidation of side-chain groups, protein scission, backbone fragmentation, cross-linking, unfolding, and formation of new reactive groups (Zbikowska *et al*., 2006).

A number of studies on the effects of ionising radiation on various proteins, carried out mainly in the 1980's, were reviewed by Kempner in 1993. Some of the main conclusions from this review were that exposure to high energy electrons or gamma rays results in covalent bond ruptures within proteins and that there is observable loss of protein activity after irradiation, regardless of the location of primary ionisation. Another issue was that irradiation itself may not directly disrupt the conformational relationships of complex protein structures, which are only affected when chemical or physical stress is applied to the system. In the context of the LMC development, where antibodies are always flown and stored in a lyophilised condition, this effect could manifest itself after they have been rehydrated.

High-energy and high atomic number ions (HZE particles) have also been a subject of interest with regards to their effects on biological systems in light of future manned space missions. HZE particles as part of the GCR have been used in studies to assess the potential dangers to manned space missions (Hada *et al*, 2007, also reviewed by Held, 2009). Their potential carcinogenic role has been investigated (Alpen *et. al*, 1994), as well as the ways they affect gene expression and cause DNA damage. The studies on the effects of HZE on the molecular level imply the presence of unique patterns of damage, depending on the identity and energy of the particles involved (Nelson, 2003), and there seem to be no data on the effect of high energy ions on the functionality of antibodies.

A review of publications on irradiation of isolated / purified biomolecules, especially antibodies, exposed a general lack of research on the effects of particle irradiation especially when considering protons and neutrons, given their importance in the composition of the space radiation environment. We attribute this to the lack of medical and/or environmental applications driving the implementation of such studies and

therefore their current sole relevance appears to be driven by astrobiological questions. This topic has recently been addressed in the form of research on the effects of particle radiation on biological materials, given their potential importance to planetary exploration missions (Le Postollec *et al*., 2009b). This study examined the effect of neutron radiation on polyclonal antibodies and fluorescent dyes, and the conclusion was that this type of radiation does not result to loss of antibody functionality and has no effects on the intensity of the dyes. An additional step in the collection of information about the effects of particle radiation is being attempted in light of future manned planetary missions in the form of publications on potential cell damage due to space radiation (Durante and Manti, 2008, Hellweg *et al*., 2008, Held, 2009).

2.4 Biological material integration in the context of the LMC

For the LMC, two classes of molecular materials are of interest within the context of their stability to the cumulative radiation environment of a Mars mission. One class, fluorescent dyes, has been studied initially elsewhere (Thompson *et al*., 2006). The second class, antibodies, is the focus for the current work. For a number of reasons, an early decision within the design of the LMC was to integrate the core molecular materials of the immunoassays in dried or lyophilised (freeze-dried) state.

In terms of molecular stability, antibodies are not considered extremely sensitive, as the disulfide bonds and intimate domain-domain interactions within the molecule make them more stable (e.g. to thermal stress) than other proteins They are, however, prone to a variety of physicochemical instabilities like aggregation, denaturation, deamidation, oxidation etc. (reviewed by Wang *et al*., 2007). For this reason, lyophilisation has been widely employed in antibody technology, in combination with a variety of protective excipients that are expected to increase antibody stability and resistance to denaturation. Among the protective additives, sucrose and NaCl are very common, with sucrose being considered preferable due to its tendency to form intimate H-bonds with the antibody and increase the glass transition temperature of formulation, making lyophilisation more effective (Wang *et al*., 2007).

Lyophilisation is expected to increase storage life, especially where storage temperatures can include transient exposure to temperatures up to and above +40°C.

Furthermore, increased radiation resistance when compared to antibodies stored in hydrated formats is expected due to the elimination of radiolytic products of water molecules that can chemically degrade proteins.

2.5 Study objectives

Given the limited amount of previous experimental work on particle radiation effects on proteins, we were faced with the need to study the effects of simulated space radiation on the key biological components of the LMC (antibodies) in order to confirm that these materials would be able to survive radiation conditions relevant to a Mars mission, provided they are appropriately packaged.

Thus, the primary objective of the present work has been to expose a number of lyophilised antibodies to various combinations and doses of proton and neutron radiation, produced by a cyclotron source, at levels that would closely simulate those calculated via modelling of the radiation levels expected during a mission to Mars. A further objective was to assess a number of lyophilisation matrices for their ability to assist in the preservation of antibody function during lyophilisation, storage, radiation exposure and rehydration. As a significant over test, a number of samples were irradiated at ten and 250 times the estimated mission doses with the objective to assess their ability to survive extreme radiation stress. The antigen binding functionality of the antibodies was measured post-radiation exposure using a simple immunoassay approach and compared to a number of control samples that allowed correction for any effects of shipping (from Cranfield University to the Swedish cyclotron facility) and storage conditions on the exposed samples.

In summary, this study is aiming to provide experimental data that support the assumption that when suitably packaged, antibodies can survive exposure to the radiation environment expected to be encountered during a Mars mission and therefore de-risk this aspect of the LMC development.

Experimental Design, Materials and Methodology

2.6 Experimental Design

2.6.1.1 Estimation of radiation exposure during a Mars Mission

The initial stage in the design of the experimental approach has been the calculation of radiation doses that would simulate the radiation environment en route to and on the surface of Mars. The calculated radiation doses for a Martian mission arose during a set of initial exposure experiments, carried out at the Paul Scherrer Institute (PSI) in Switzerland in 2007 (data not shown).

As described previously, the dominant radiation threat to the LMC molecular reagents during a mission to Mars will be high energy protons during the mission cruise phase and neutron radiation during surface operations. Using standard modelling tools (SPENVIS and CREME96), the proton fluence for a two-year transfer phase was calculated with a 95% upper confidence limit at 2×10^{10} protons/cm^2 (taking into consideration the proton attenuation due to a 4mm aluminium shield thickness which corresponds to the estimated shielding level within the ExoMars structure.) This fluence is polyenergetic; the protons have a variety of different energies and therefore in order to simulate this fluence with available cyclotron resources, normalisation of the energy spectrum to a single energy equivalent is required. Therefore the equivalent 60 MeV monoenergetic fluence was calculated at 5×10^{10} protons/cm^2 (Hands, 2006). At approximately 50 MeV (which was the proton energy that would be used at the facility for the proton irradiations) this fluence is reduced [due to higher Linear Energy Transfer (LET) of the lower energy protons] to 4×10^{10} protons/cm^2. This figure was doubled to provide a suitable minimum "overtest" fluence of 8×10^{10} protons/cm^2, corresponding to a total ionising dose of approximately 14 Krads (140 Gy) (Hands, 2008a).

The neutron irradiation of the biological samples was introduced after modelling of the Martian radiation environment raised the issue of the effect of surface radiation (where the total secondary neutron flux is substantially greater than the proton flux) on the biological components of ExoMars. Modelling of the radiation environment on the surface of Mars was performed with the use of a Monte Carlo simulation. For a six-month long operation on the Martian surface, the neutron dose was calculated, after

normalisation, at ~20 milliSieverts (mSv), which is equivalent to a fluence of 1.8 x 10^7 peak neutrons/cm^2 (at the test facility used in these experiments), rounded at 2 x 10^7 neutrons/cm^2 and doubled to give an experimental minimum test fluence of 4 x 10^7 neutrons/cm^2 (equivalent to a one-year exposure period) (Hands, 2008a).

In conclusion, the choice for the radiation doses that would be used in the present studies to simulate the relevant space radiation environments was based on modelling of the Martian radiation environment and a set of preliminary experiments, and in the case of proton irradiation the nominal mission dose was set at 8 x 10^{10} protons/cm^2, while for neutron irradiation the nominal mission dose was set at 4 x 10^7 neutrons/cm^2. Wherever "mission dose" is mentioned from this point onwards, it corresponds to these values for proton and neutron irradiation respectively.

2.6.1.2 Phased Ground-based Radiation Exposure Campaign

The simulated Mars mission radiation studies were planned to take place in two stages, Phase I (February 2008) and Phase II (September 2008). The overall design for both phases was, as mentioned previously, based on a preliminary radiation study that took place at the PSI in 2007. The reasons for having two rounds of irradiations included the availability of antibodies against biomarkers of interest (availability of sample antibodies), the need for additional exposure time at the host facility and an increasing volume of information on improved lyoprotection and radioprotection methods for the antibodies.

2.6.1.3 Choice of Radiation Levels

The antibodies were to be exposed to various multiples of mission doses and combinations of proton and neutron radiation. The radiation levels that were chosen aimed to duplicate the doses calculated for the ExoMars mission and determine whether proton or neutron radiation would have the most significant effects on antibody functionality.

In Phase I, the irradiations focused on investigating the effects of combined proton and neutron irradiation, both in doses simulating the "mission dose" for ExoMars, and at 10

times these values, as an overtest aiming to test the antibodies' survival limits. Some samples would also receive the "mission dose" of protons or neutrons in isolation to clarify, as mentioned, if either type of radiation would dominate the damage caused to the samples.

Phase II was designed to provide further information about irradiation damage to a number of antibodies that were not available during Phase I, in addition to confirming the findings of the previous round for all antibodies that were used in Phase I. A further test during Phase II was the irradiation of antibody samples at 250 times the mission dose for protons, an extreme dose that would allow us to determine the higher threshold of exposure for antibody inactivation. Neutron irradiation at 250 times the ExoMars dose were not performed at this stage, as the effect of neutron radiation had been deemed negligible, after analysis of the results from Phase I.

2.6.1.4 Selection of Antibodies for Study

The decision on which antibodies would be used in the radiation tests was based on two main criteria; they had to be representative of the broad range of types of antibodies that could be included in the final format of the LMC and they had to be part of existing working assays within the lead authors' group. Consequently, priority was given to those antibodies that had been proven to function in immunoassay format, recognising and binding to their respective antigens with considerable sensitivity. Availability was another factor that affected the choice of antibodies for each round of experiments. Table 2-1 provides a list of the antibodies used in the present study and represent antibodies against a broad range of antigens – whole cell, protein and small molecule – as well as IgG antibody types – polyclonal, monoclonal and recombinant fragments and derived from a range of animal species.

Table 2-1: Antibodies used in the ground-based radiation experiments (details given in Materials and Methodology section)

Antibody	Abbreviation	Antigen/Target	Production Method
Rabbit IgG antibody against Chaperonin 60 (GroEL)	Anti-GroEL	Chaperonin 60 (GroEL)	Polyclonal
Mouse IgG antibody against *Staphylococcus aureus*	Anti-*S. aureus*	Peptidoglycan from *S. aureus*	Monoclonal
Rabbit IgG antibody against Fluorescein Isothiocyanate (FITC)	Anti- FITC	Fluorescein Isothiocyanate (FITC)	Polyclonal
Rabbit IgG antibody against Phytanic Acid	Anti-phytanic acid	Phytanic Acid/BSA conjugate	Polyclonal
Sheep IgG derived antibody against Atrazine	Anti-atrazine	Atrazine	Recombinant single-chain antibody fragment (scAb)

2.6.1.5 Strategy for Sample Preparation

From the initial stages of the LMC instrument development, it was decided that the antibodies would be integrated into the instrument as freeze-dried preparations and freeze-dried directly into laser-cut glass fibre pads. This format, in addition to the stability and radioresistance advantages it was expected to offer (mentioned earlier), facilitated antibody handling and integration during LMC assembly – *i.e.* assembly involved only handling a dry glass fibre pad containing pre-dosed and quality assured antibodies. This format was maintained during the radiation studies as it eased antibody handling and more importantly allowed radiation exposure in a flight-model representative format. Each antibody sample was freeze-dried into a separate pad and for each set of experimental conditions, three replicates of each antibody pad were used to confirm the reproducibility of the results.

In order to increase the resistance of the antibodies to inactivation prior, during or after exposure to radiation, three protective lyophilisation matrices (solute mixtures) were used in the freeze-drying process, in addition to samples that were freeze-dried in the buffer solutions they were provided in by their manufacturers. These matrices were mixtures of sugars, proteins and free radical scavengers, which allowed us to assess the effectiveness of these additives on the stability and post-rehydration activity of the

antibody samples. The disaccharide (sucrose) and polymer (Blue Dextran) used in these mixtures were added as lyoprotectants, bovine serum albumin (BSA) served as an anti-aggregation agent and Tween 80 is considered protective during freezing and thawing, and has been suggested to prevent protein adsorption to ice/water interfaces during freezing and assist protein refolding during thawing. Ascorbate is an effective radical scavenger (Niki, 1991). A detailed list of the reagents used in each matrix can be found in Table 2-2: The lyophilisation matrices used in the ground-based radiation studies and their respective components in detail.

To ensure a uniform exposure of the pads to the radiation source, and further facilitate handling and shipping, the pads were loaded into 384-well microtitre plates, over an area that did not exceed the few cm diameter of the particle beam. Figure 2-1 shows a microtitre plate loaded with antibody sample pads.

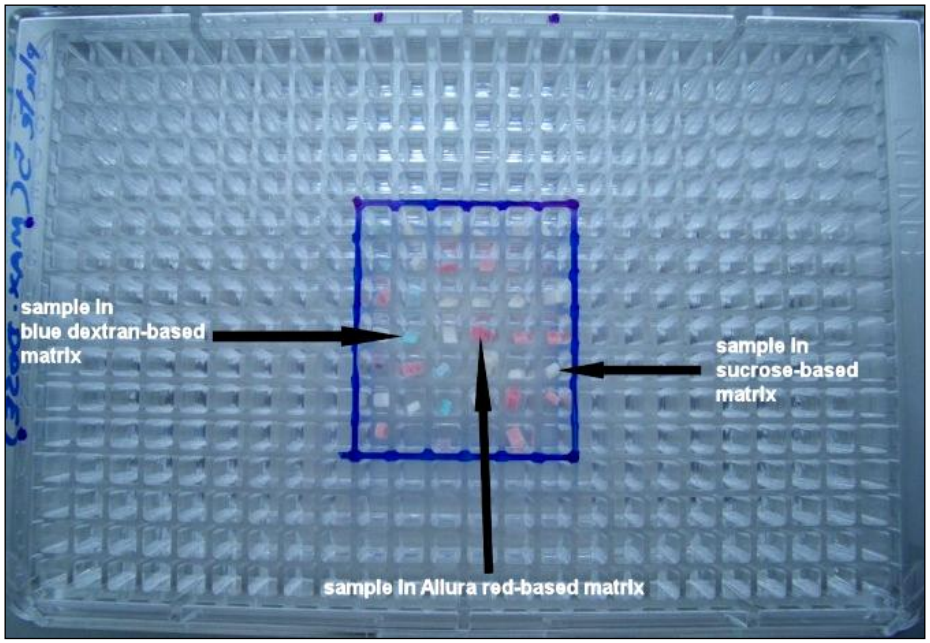


Figure 2-1: 384-well microtitre plate with antibody sample pads loaded in the central square area of 32mm x 32mm (plate area is 12.5cm x 8cm)

The antibody sample pads were stored in ambient atmosphere at ambient temperature. After being loaded, the microtitre plates were covered by a layer of aluminium foil. The plates' plastic lids were placed over the layer of aluminium foil and the lids sealed in place using masking tape.

In addition to the microtitre plates with samples for exposure to radiation, a number of control antibody pads were prepared and packed identically to the samples for exposure, *i.e.* antibodies freeze-dried into pads, loaded in microtitre plates. One set of controls was kept at Cranfield University, in ambient temperature and atmosphere, for the duration of the study prior to final analysis ("storage" controls). A second set of control pads were prepared and packed identically to the samples for exposure, and were shipped alongside them to the radiation facility, but not irradiated ("shipping" controls). Both sets of control pads were analysed in parallel with the radiation-exposed samples to determine differences in antibody functionality caused solely by exposure to radiation, after subtraction of the effects of the storage and shipping process on the samples.

Materials and Methodology

2.7 Antibodies and Lyophilisation Matrix Components

The anti-GroEL antibody used in the radiation studies was purchased from Sigma-Aldrich (cat. No G6532) at a stock concentration of 10 mg/ml and the single-chain antibody fragment against atrazine was provided by Immunosolv Ltd. (Aberdeen, Scotland) at a stock solution of 0.3 mg/ml. The mouse monoclonal antibody to peptidoglycan from *Staphylococcus aureus* was purchased from Abcam (cat. No ab20002) at a stock concentration of 1 mg/ml; as was the rabbit polyclonal antibody against phytanic acid (ab51309), at a stock concentration of 1 mg/ml. The rabbit-raised polyclonal anti-FITC antibody was purchased from Abcam (cat. No ab19491-100), at a stock concentration of 1 mg/ml

The lyophilisation matrices were an important component of the radiation tests. Two matrices were used in Phase I; one of the mixtures included the dye Blue Dextran ('Blue Dextran-based matrix") and the second mixture included sucrose ("Sucrose-based matrix") (Table 2-2). A third matrix was added during Phase II, a mixture including the dye Allura Red ("Allura Red-based matrix"). The Allura Red matrix was included due to co-development of the LMC instrument, where Blue Dextran was found to interfere optically with the fluorescence-based immunoassay format used and the high molecular weight nature of Blue Dextran affected the viscosity of the resulting assay solution.

Hence, Allura Red was used as a visual low molecular weight tracer dye for microfludic development reasons and did not interfere with the fluorescence-based immunoassay format used. The list of components for the lyophilisation matrices included the bovine serum albumin (BSA) which was used in all three lyophilisation matrices and was purchased from Sigma-Aldrich (cat. No A4503), the HEPES buffer was also from Sigma-Aldrich (cat. No H7523), as was the sucrose (cat. No S7903), the Blue Dextran (cat. No D5751), Allura Red (cat. No 458848) and the ascorbate (Fluka - cat. No 95210). Tween 80 was purchased from SAFC (cat. No W29, 170-6-K).

Table 2-2 shows the components of each of the three lyophilisation matrices used in these studies.

Table 2-2: The lyophilisation matrices used in the ground-based radiation studies and their respective components in detail

Component Function	***Blue Dextran*-based Lyophilisation Matrix**	***Sucrose*-based Lyophilisation Matrix**	***Allura Red*-based Lyophilisation Matrix**
Anti-aggregation	1% w/v BSA	1% w/v BSA	0.05% w/v BSA
Lyoprotectant	0.1% w/v Blue Dextran	1% w/v sucrose	10% w/v sucrose
Free radical	0.34 % w/v ascorbate	0.34% w/v ascorbate	-
Cryoprotectant	-	1% v/v Tween 80	-
Dye	-	-	0.005% w/v Allura Red
Preservative	-	-	2% v/v Thiomersal
Buffer solution	100 mM HEPES	100 mM HEPES	100 Mm HEPES

For the ELISA reagents, the majority came from Sigma-Aldrich, including chaperonin 60 (GroEL) (cat. No C7688), an inactivated *S.. aureus* cell suspension (cat. No S2014), an anti-mouse secondary antibody for the *S.. aureus* assay (cat. No A6782), phosphate buffered saline (PBS) tablets (cat. No P4417), Tween 20 (cat. No P7949), anti-rabbit, HRP-labelled polyclonal IgG (cat. No A6154), citrate-phosphate buffer with sodium perborate (cat. No P4922) and 3,3′,5,5′-Tetramethylbenzidine dihydrochloride (TMB) tablets (cat. No T3405). A phytanic acid/BSA conjugate was acquired from Abcam (cat. No ab51309). The FITC/BSA conjugate used in the FITC assay was from Sigma-Aldrich (cat. No A9771), and the streptavidin/HRP conjugate used at the end of this assay was from Zymed Laboratories (cat. No 43-4323) at a stock concentration 0f 1.25

mg/ml; the secondary antibody used in this assay was a goat anti-rabbit Fc-specific biotinylated antibody from QED Biosciences (cat. No. 84400). An anti-rabbit, Fc region specific, HRP-labelled secondary antibody which was used in the phytanic acid assays was purchased from Novus Biologicals (cat. No NB7179). The blocking buffer for the ELISA was prepared with the use of skimmed milk from Marvel, while the 1M sulphuric acid was purchased from Fisher (cat. No J/8420/17).

2.8 Preparation of glass fibre pads and sample integration

The material for the glass-fibre pads was GF/D glass microfibre filters (47 mm ø) from Whatman. The 2 x 4 x 0.6 mm^3 rectangular pads were laser-cut using a Fenix CO_2 Laser Marker from SYNRAD. 6 µl of each antibody in buffer or in the lyophilisation matrices were allowed to fill each glass fibre pads by ejecting a drop from a pipette and allowing this to wick into a pad. This volume appeared to fully saturate a pad, as assessed by visual inspection. All the antibodies were used at the stock concentrations they were provided at by their manufacturers in the buffer-only pads, and at 50% dilution of the stock concentration in the pads where added matrices were present (3 µl stock antibody solution & 3 µl protective mixture). After the sample solution was added to each pad, the pads were immediately frozen by immersion into liquid nitrogen. Due to the pads' small size and little thermal inertia, it was observed that removing them from liquid nitrogen prior to the freeze-drying step of the process resulted in rapid thawing. The problem was overcome by keeping the frozen pads in liquid nitrogen, inside 20 ml plastic beakers which were then placed in the vacuum chamber of a bench top freeze-drier (Coolsafe 55-4 bench-top freeze drier from ScanVac). Upon establishment of the vacuum inside the chamber, the liquid nitrogen quickly boiled away. The samples were dried overnight (approx. 12 hours). The lyophilised samples were then loaded into 384-well microtitre plates from Nunc (cat. No 242765) within a central square area of 32mm x 32mm.

2.9 Radiation Exposure

2.9.1.1 Exposure Facilities and Sample Transportation

The two radiation test campaigns took place at the Theodor Svedberg Laboratory (TSL) in Uppsala, Sweden. The accelerator of the laboratory is the Gustaf Werner cyclotron. In addition to a mono-energetic proton beam which is capable of reaching energies up to nearly 200 MeV, the Blue Hall of the TSL has a neutron facility, devoted to component testing performed by customers from the private sector and departments of Uppsala University. The neutron beams are generated by the $^{7}Li + p \rightarrow {}^{7}Be + n$ reaction, from which neutron beams are obtained that have particle energy spectra with a narrow-band high-energy flux-peak region, and a significant wide-band flux-tail region that extends from the peak region towards zero MeV (Prokofiev et al, 2007).

The proton beam is collimated and the diameter is stable at 8 cm, with a top hat radial distribution. The neutron beam, which originates from a source point (a lithium target) is best described as cone-shaped, with a diameter of approximately 10 cm at the samples' distance from the source. With this data in mind, the samples' exposure to both radiation beams was considered uniform (in the case of the neutron beam, exposure is not uniform in theory, but given the samples' dimensions and distance from the source, this was considered a reasonable approximation).

Given the calculated mission fluence of $4x10^{10}$ protons cm^{-2}, which was doubled to $8x10^{10}$ protons cm^{-2} to provide the minimum sample fluence, the minimum irradiation time would only be half a minute, given the fluence capacities of the TSL facility. This was viewed as impractical for this type of facility – where the beam control was indirect and remote – so during the experiments the flux was reduced by at least an order of magnitude to give a more manageable irradiation time per sample. For the neutron irradiations, the minimum exposure time was three minutes, taking into consideration the facility's capacity, but once again, the irradiation time was prolonged by decreasing the beam intensity.

Phase I experiments were carried out between the 20th and the 22nd of February 2008 and Phase II experiments between the 3rd and 6th of September 2008. The microtitre plates with the antibody samples were hand-carried (in passenger aircraft carry-on luggage; also subjected to security X-ray scanning) to TSL and returned the same way,

in order to avoid any physical or chemical alterations to the samples due to unknown storage conditions during transport that could occur with checked-in luggage or via couriers or other freighting methods.

2.9.1.2 Details of radiation exposure

In Phase I four sample loaded microtitre plates were exposed to various combinations and doses of radiation; “Test Plate 1” received the combined “mission dose” for protons and neutrons, “Test Plate 2” received the proton mission dose only, and “Test Plate 3” was irradiated at the mission dose for neutrons only. Finally, “Test Plate 4” received 10 times the combined proton and neutron mission dose.

In Phase II, three radiation test plates were prepared and once again irradiated with different combinations and levels of radiation; “Test Plate 1” receiving the combined mission dose for protons and neutrons, “Test Plate 2” received 20 times the combined proton and neutron mission dose, and “Test Plate 3” was irradiated at approximately 250 times the calculated ExoMars mission dose for protons only. Table 2-3 and Table 2-4 show a detailed description of the samples used in each Phase and each level/combination of radiation exposure.

Table 2-3: Outline of antibody samples used in Phase I of the ground-based radiation studies and the doses/combination of radiation each sample was exposed to

Phase I: Plate Description and Radiation Dose	Antibody Sample		
	Anti-atrazine scAb	Anti-GroEL ab	Ab to *S. aureus*
Control Plate 1 Cranfield University Storage Control Plate	3 pad replicates	3 pad replicates	3 pad replicates
Control Plate 2 Shipping Control Plate	3 pad replicates	3 pad replicates	3 pad replicates
Radiation Test Plate 1 combined 2x ExoMars proton dose & 2x ExoMars neutron dose	3 pad replicates	3 pad replicates	3 pad replicates
Radiation Test Plate 2 2x ExoMars proton dose	-	3 pad replicates	-
Radiation Test Plate 3 2x ExoMars neutron dose only	-	3 pad replicates	-
Radiation Test Plate 4 Combined 10x ExoMars proton dose &10x ExoMars neutron dose	-	3 pad replicates	-

During both phases of the radiation studies, in the case of the proton irradiations, the energy range of the beam extended a few MeV around its peak value of 50 MeV.

Table 2-4: Outline of antibody samples used in Phase II of the ground-based radiation studies and the doses/combination of radiation each sample was exposed to

Phase II: Plate Description and Radiation Dose	Antibody Sample			
	Anti-GroEL ab	Ab to *S. aureus*	Ab to Phytanic acid	Anti-FITC ab
Control Plate Storage Control Plate	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates
Control Plate Shipping Control Plate	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates
Radiation Test Plate 1 combined 2x ExoMars proton dose & 2x ExoMars	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates
Radiation Test Plate 2 combined 20x ExoMars proton dose & 20x ExoMars	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates
Radiation Test Plate 3 250x ExoMars proton dose	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates	3 Pad Replicates

In the consideration of the possible errors during irradiation of the samples, their plastic casing, within the microtitre plates, should be taken into consideration, as it is thought to have a small degrading effect on the incident energy. Other potential errors included beam uniformity, beam control dosimetry, scattering effects and background radiation, although these factors were considered to be of negligible effect (Hands, 2008). Figure 2-2 shows a sample-loaded microtitre plate about to be exposed to the neutron beam, in the TSL's Blue Hall.

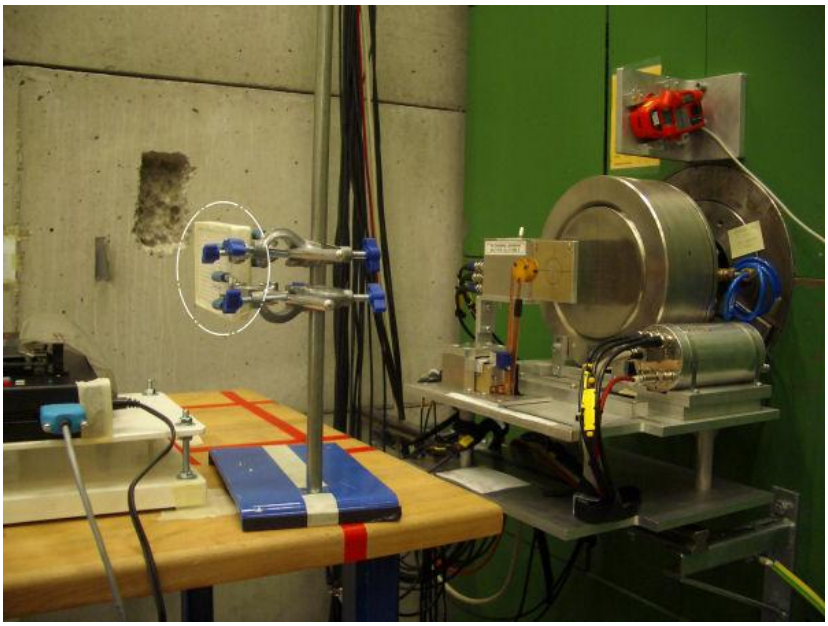


Figure 2-2: Sample-loaded microtitre plate (circled), clamped in place, about to be exposed to neutron radiation. Beam enters from the right side of the image.

2.10 Radiation dose measurement

In order to calculate the actual radiation dose that corresponds to each sample-loaded pad (2 x 4 mm^2), the data used were those provided by the TSL facility and comprised the "reference fluence" (which is the particle fluence at the beam exit point) and the "sample fluence" (which is the particle fluence at the mounted sample's distance from the beam exit point). Table 2-5 and Table 2-6 give the total doses per pad for the proton and neutron irradiations in Phase I and II.

Table 2-5: Proton and neutron fluences recorded for each radiation test plate during Phase I and calculated sample doses (*reference fluence*: measured at the beam's exit point, *sample fluence* measured at approximately the test plate's distance from the beam source, *total number of particles per pad* calculated using the pad's dimensions 2x4 mm^2)

Phase I	Proton Reference Fluence ($p.cm^{-2}$)	Proton Sample Fluence ($p.cm^{-2}$)	Total Protons per Pad	Neutron Reference Fluence ($n.cm^{-2}$)	Neutron Sample Fluence ($n.cm^{-2}$)	Total Neutrons per Pad	Relation to "mission dose"
TEST PLATE 1	8.01E+10	8.01E+10	6.40E+09	6.11E+07	4.02E+07	3.20E+06	2x
TEST PLATE 2	8.04E+10	8.04E+10	6.40E+09	-	-	-	2x
TEST PLATE 3	-	-	-	6.11E+07	4.02E+07	3.20E+06	2x
TEST PLATE 4	4.01E+11	4.01E+11	3.20E+09	3.03E+08	2.00E+08	1.60E+07	10x

Table 2-6: Proton and neutron fluences recorded for each radiation test plate during Phase II and calculated sample doses (*reference fluence*: measured at the beam's exit point, *sample fluence* measured at approximately the test plate's distance from the beam source, *total number of particles per pad* calculated using the pad's dimensions 2x4 mm^2)

Phase II	Proton Reference Fluence ($p.cm^{-2}$)	Proton Sample Fluence ($p.cm^{-2}$)	Total Protons per Pad	Neutron Reference Fluence ($n.cm^{-2}$)	Neutron Sample Fluence ($n.cm^{-2}$)	Total Neutrons per Pad	Relation to "mission dose"
TEST PLATE 1	8.01E+10	8.01E+10	6.40E+09	5.37E+07	3.63E+07	2.90E+06	2x
TEST PLATE 2	8.07E+11	8.07E+11	6.40E+09	5.74E+08	3.89E+08	3.11E+07	20x
TEST PLATE 3	1.00E+13	1.00E+13	8.00E+11	-	-	-	250x

2.11 Assessment of Remaining Antibody Binding Activity Post Radiation Exposure

Once the irradiations were completed, the radiation test plates were hand-carried back to Cranfield University, where they were stored in the dark, at room temperature until their rehydration and ELISA testing, which for all samples took place within a three-month period after irradiation.

2.12 Antibody Recovery from glass fibre pads

For both Phase I and II the antibodies in the pads were rehydrated by being initially removed from the microtitre plate wells and each pad placed inside a 10 µl plastic pipette tip. This step provided a stable position for the pad (inside the internal taper of the tip) during the rehydration and wash-out process and prevented the pad from coming in contact with any pooled wash-out liquid. The 10 µl tips were then put inside 1.5 ml Eppendorf tubes. This facilitated the centrifugation step that followed and provided a means to collect the rehydrated material. The rehydration protocol involved placing 6 µl of PBS solution on the surface of each pad which wicked into the pad, followed by a centrifugation step using an MSE MicroCentaur centrifuge from DJB Labcare to allow collection of the rehydrated material from the pad. The procedure consisted of ten centrifuge runs of approximately one minute each, using 6 µl of PBS in each run, yielding a ~60 µl solution with an expected antibody dilution factor of 10, assuming 100% wash-out efficiency.

2.13 ELISA for assessment of remaining antibody binding activity

The rehydrated solutions were used immediately in an ELISA, since preliminary experiments had shown they are unstable, even after a 24-hour storage period at +4°C. The enzyme-linked immunosorbent assays (ELISA) applied in the analysis of samples, followed the same protocol in all cases, the only variable being the antibodies and antigens used in each assay. The ELISAs were all binding assays, aiming to test the antibodies' ability to recognise and bind to their respective targets – no competition or

inhibition assays with free antigens was attempted. Table 2-7 shows the reagents used in each of the five different immunoassays performed at this stage.

Table 2-7: Analytic overview of the reagents used in each of the immunoassays used to assess the antibodies' post-exposure performance

	Immunoassays				
	GroEL	***S. aureus***	**Phytanic**	**Atrazine**	**FITC**
Coating Antigen	GroEL	Inactivated *S. aureus* cells	Phytanic acid/BSA conjugate	Atrazine/BSA conjugate	FITC/BSA conjugate
Coating Concentration	10 μg/ml	1 mg/ml	10 μg/ml	10 μg/ml	0.5 μg/ml
Primary Antibody Dilution	Dilution range of 1:20 to $1:2x10^5$	Dilution range of 1:100 to $1:1x10^6$	Dilution range of 1:200 to $1:2x10^6$	Dilution range of 1:100 to $1:1x10^6$	Dilution range of 1:400 to $1:4x10^6$
Secondary Antibody	anti-rabbit HRP-labeled	anti-mouse HRP-labelled	anti-rabbit F_c region-specific HRP-labelled	anti-human kappa light chain, HRP-labelled	goat anti-mouse biotinylated

More specifically, ninety six-well polystyrene microtitre plates from Nunc were coated with antigen and incubated for one hour at room temperature. This comprised 100 μl of antigen solution added to each well and after the coating step the plates were emptied and tap-dried and unbound sites at the walls of the wells were blocked using a 1% (or 5%) blocking buffer of Marvel skimmed milk in phosphate buffer saline (PBS). This comprised 200 μl of blocking buffer added to each well and the plates incubated for one hour at room temperature. Once the blocking was complete, the plates were emptied and tap-dried. The sample antibody solutions were then split in triplicate wells, serially diluted in accordance to each assay's working range, and a control assay was run in parallel using stock antibody solutions. This comprised 100 μl of sample/control antibody solution added to each well and the plates incubated for one hour at room temperature. Upon completion of this step, the plates were washed using a BW 50 plate washer from BioHit, and the washes consisted of three 200-μl 0.05% Tween 20 in PBS (PBST) washes. Then the enzyme (HRP) labeled secondary antibody was added, in an appropriate dilution, at 100 μl per well and the plates were incubated for one hour at room temperature. Another washing step of three 200 μl PBST washes followed. The

final stage of the ELISA consisted of the colour development reaction, using an HRP substrate of TMB in phosphate-citrate buffer. One hundred μl of this solution were added to all wells and colour was allowed to develop over 5-7 minutes (according to colour intensity). The reaction was stopped using 50 μl of 1M sulfuric acid, and the optical density of the samples was measured at a wavelength of 450 nm, using a VarioSkan Flash reader from ThermoScientific.

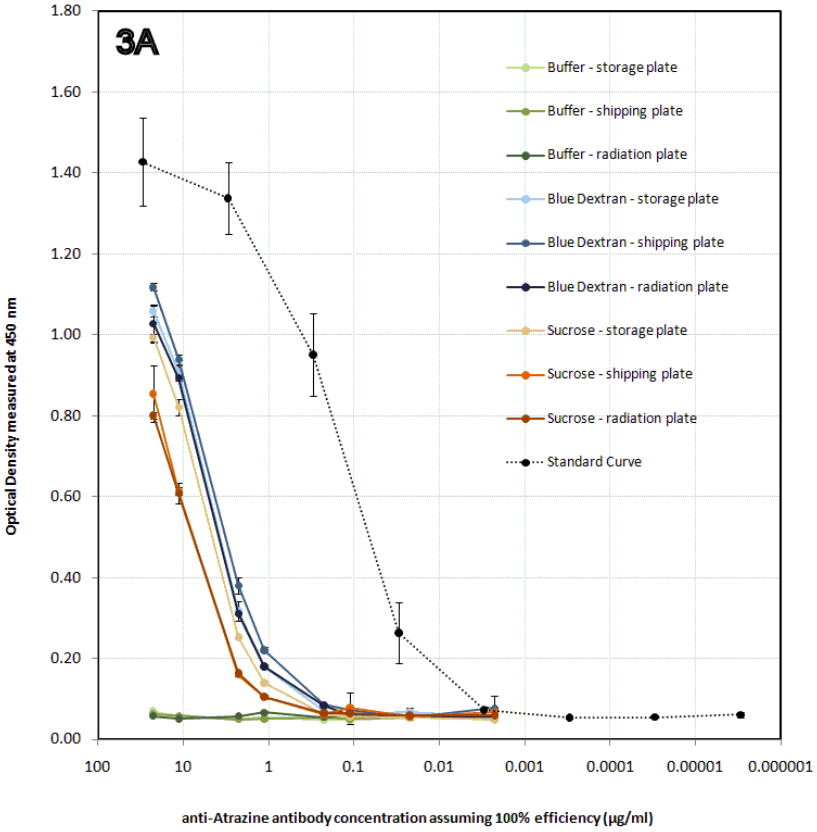
Experimental Results

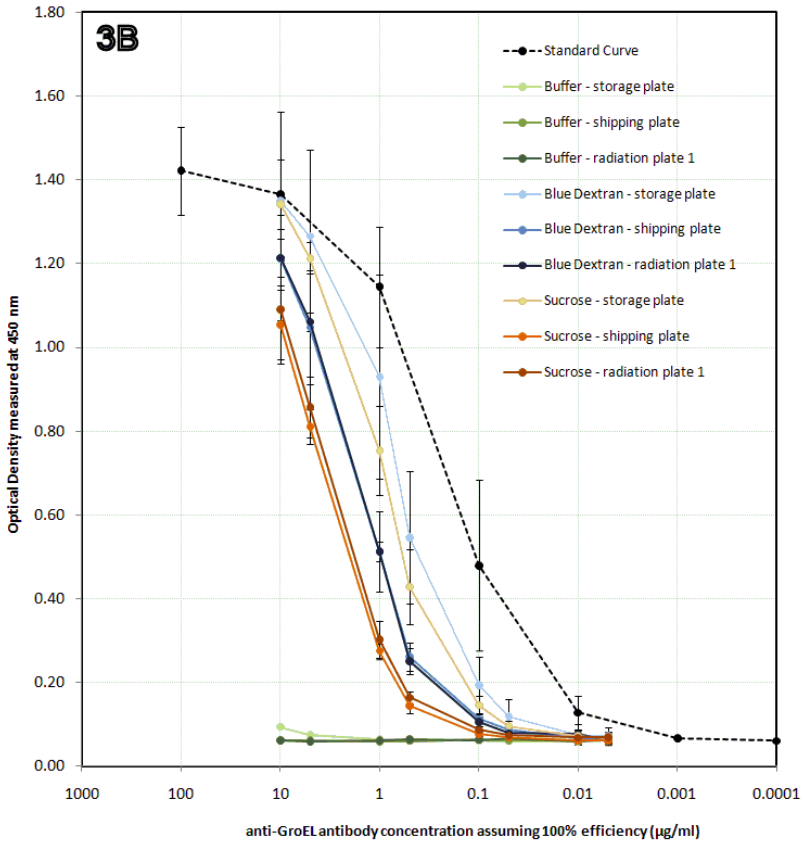
Upon completion of the two radiation test campaigns, the ELISA results were collected and plotted with standard curves that were obtained from ELISAs using fresh stock solutions of all antibodies, *i.e.* in addition to the various sample sets including shipping and storage controls, in order to determine the degree of activity loss in each case. Each sample was represented in triplicate throughout the experiments; in the following figures, each data point corresponds to the average value of the three replicates.

2.14 Phase I Results

2.14.1.1 Effect of lyophilisation matrix on retention of antibody binding activity

In Phase I of the radiation studies, the antibodies available for testing were the polyclonal antibody against the protein GroEL, the monoclonal antibody against *S. aureus* and the recombinant antibody against atrazine. All three antibodies were freeze-dried in three formats: in buffer solution, in the Blue Dextran-based lyophilisation matrix and in the sucrose-based lyophilisation matrix. Due to availability limitations, only the anti-GroEL antibody was used in radiation plates 2 to 4. Figure 2-3 shows the ELISA results for all three antibodies, with the varying factor being the lyophilisation matrix used in each case (3A: anti-atrazine, 3B: anti-GroEL, 3C: anti-*S. aureus*).


3A
Optical Density measured at 450 nm
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
anti-Atrazine antibody concentration assuming 100% efficiency (µg/ml)
Buffer - storage plate
Buffer - shipping plate
Buffer - radiation plate
Blue Dextran - storage plate
Blue Dextran - shipping plate
Blue Dextran - radiation plate
Sucrose - storage plate
Sucrose - shipping plate
Sucrose - radiation plate
Standard Curve


3B
Optical Density measured at 450 nm
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1000
100
10
1
0.1
0.01
0.001
0.0001
anti-GroEL antibody concentration assuming 100% efficiency (µg/ml)
Standard Curve
Buffer - storage plate
Buffer - shipping plate
Buffer - radiation plate 1
Blue Dextran - storage plate
Blue Dextran - shipping plate
Blue Dextran - radiation plate 1
Sucrose - storage plate
Sucrose - shipping plate
Sucrose - radiation plate 1

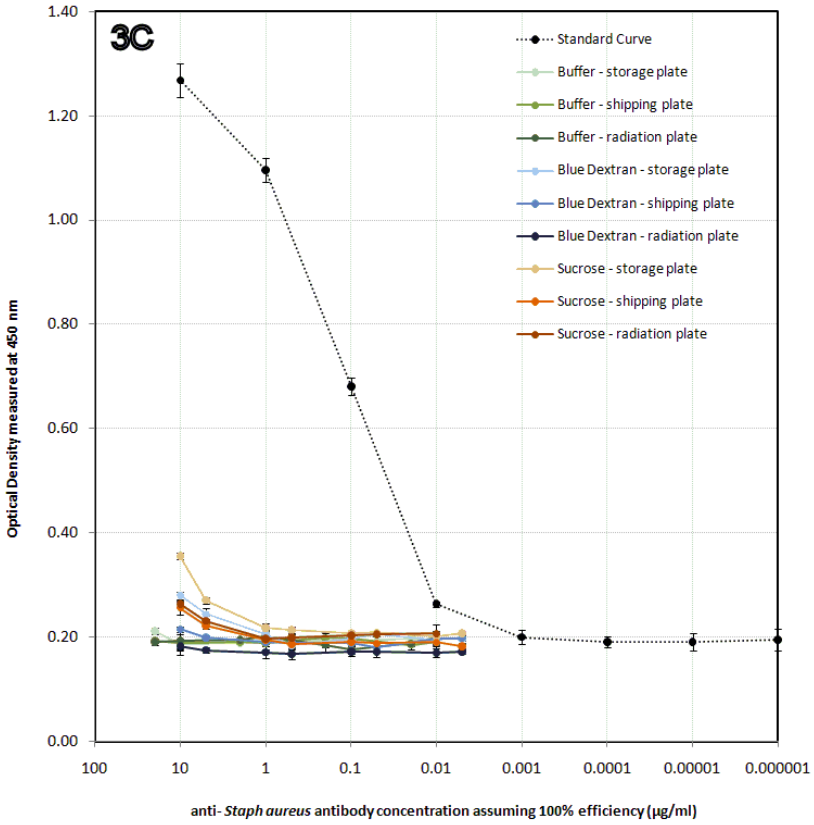


Figure 2-3: Binding performance of the anti-atrazine (3A), anti-GroEL (3B) and anti-*S. aureus* (3C) antibody in various lyophilisation matrices measured at λ_{450} ("100% efficiency" refers to wash-out efficiency)

From Figure 2-3 it becomes obvious that the monoclonal antibody to *S. aureus* (Figure 3C) had the poorest overall performance, regardless of the lyophilisation matrix used. In the case of the anti-atrazine antibody (Figure 3A), there appears to be no significant differences between the controls and radiation-exposed samples, while the anti-GroEL antibody (Figure 3B) presents a clearer distinction of binding activity between controls and irradiated samples.

2.14.1.2 Effect of varying radiation dose on retention of antibody binding activity

As far as the effects of radiation are concerned, Figure 2-4 shows the performance of the anti-GroEL antibody in the sucrose-based matrix, after being exposed to various doses and combinations of proton and neutron radiation (for details, see Table 2-3).

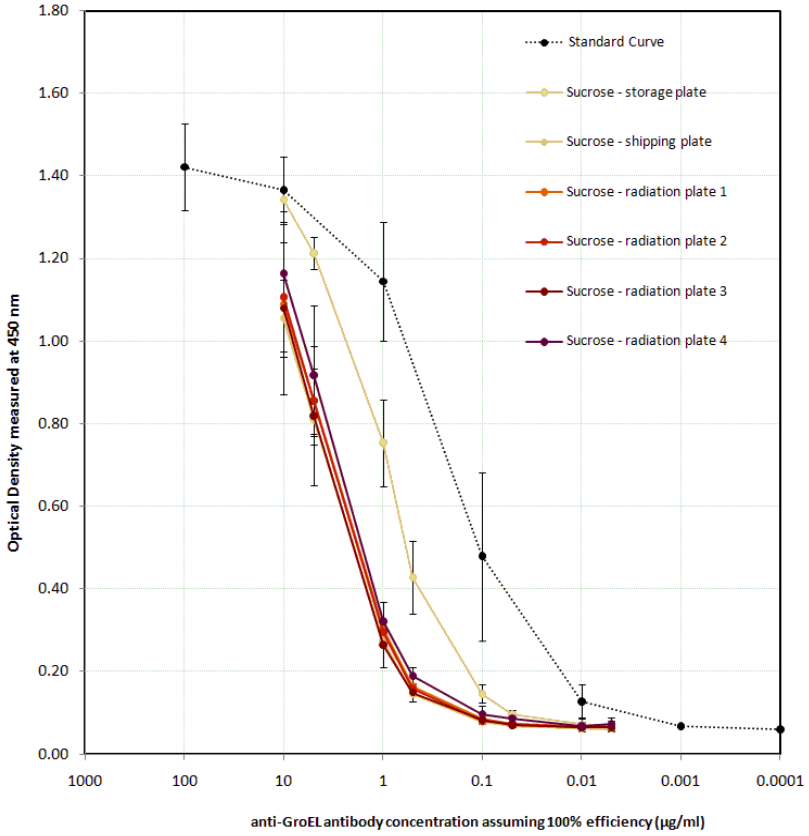


Figure 2-4: Binding performance of the anti-GroEL antibody after exposure to various doses/combinations of radiation measured at λ_{450} ("100% efficiency" refers to wash-out efficiency)

In Figure 2-4, the storage controls seem to be more active than the shipping controls and irradiated samples, a fact that could be attributed to transit X-ray scanners (airport scanners) and/or cosmic rays; another possible explanation would be any drastic changes in temperature during transportation that could result to decreased activity of all samples except the storage controls.

2.14.1.3 Summary of antibody binding activity for Phase I

In order to quantify the differences in each antibody's binding activity due to the use of specific protective matrices and/or irradiation, the data is summarised in Table 2-8.

Table 2-8: Binding activity retention (%) for all antibodies used in Phase I of the ground-based radiation studies (n/a: not available)

	Anti - Atrazine Antibody		Anti - GroEL Antibody		Anti - *S. aureus* Antibody	
	Assay mid-point (µg/ml)	Retained Antibody Activity (%)	Assay mid-point (µg/ml)	Retained Antibody Activity (%)	Assay mid-point (µg/ml)	Retained Antibody Activity (%)
standard curve	0.16	100.0%	0.23	100.0%	0.12	100.0%
buffer - storage plate	n/a	0%	n/a	0%	n/a	0%
buffer - shipping plate	n/a	0%	n/a	0%	n/a	0%
buffer - radiation plate 1	n/a	0%	n/a	0%	n/a	0%
buffer - radiation plate 2	n/a	0%	n/a	0%	n/a	0%
buffer - radiation plate 3	n/a	0%	n/a	0%	n/a	0%
buffer - radiation plate 4	n/a	0%	n/a	0%	n/a	0%
Blue Dextran - storage plate	8.65	1.9%	0.75	30.8%	n/a	0%
Blue Dextran - shipping	7.53	2.1%	2.33	9.9%	n/a	0%
Blue Dextran - radiation	8.38	1.9%	2.36	9.7%	n/a	n/a
Blue Dextran - radiation	n/a	n/a	3.69	6.2%	n/a	n/a
Blue Dextran - radiation	n/a	n/a	3.91	5.9%	n/a	n/a
Blue Dextran - radiation	n/a	n/a	4.47	5.1%	n/a	n/a
sucrose - storage plate	11.11	1.4%	1.20	19.1%	n/a	0%
sucrose - shipping plate	24.81	0.6%	5.99	3.8%	n/a	0%
sucrose - radiation plate 1	18.69	0.9%	5.31	4.3%	n/a	n/a
sucrose - radiation plate 2	n/a	n/a	5.68	4.0%	n/a	n/a
sucrose - radiation plate 3	n/a	n/a	6.61	3.5%	n/a	n/a
sucrose - radiation plate 4	n/a	n/a	5.20	4.4%	n/a	n/a

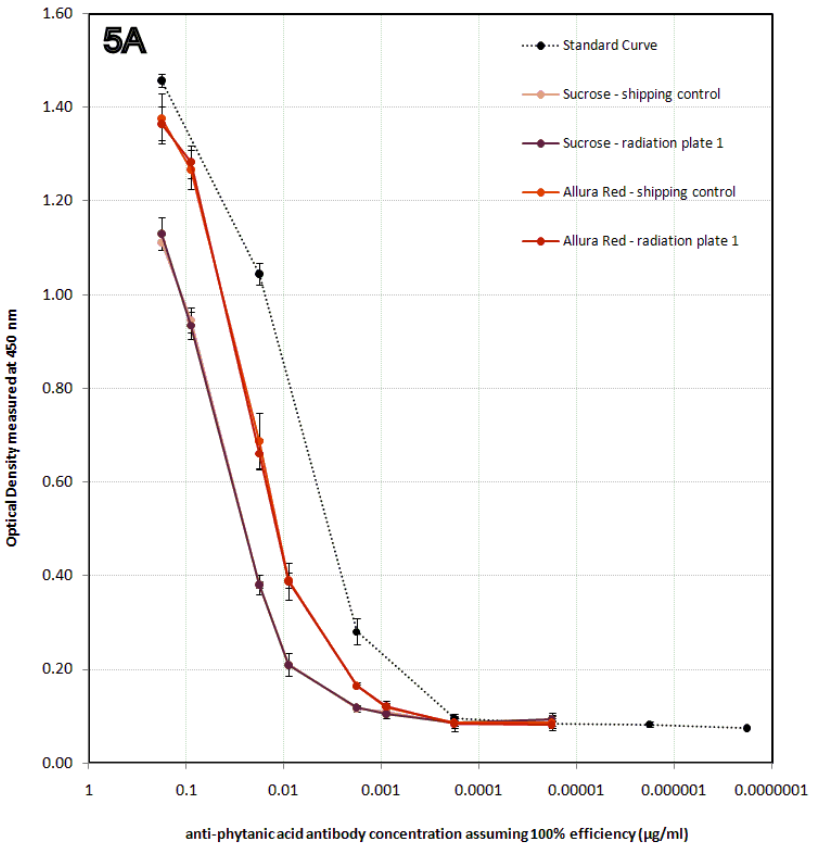
2.15 Phase II Results

An important factor in the choice and preparation of samples for Phase II experiments was the availability of the various antibodies that would be used. The anti-GroEL antibody, which was available in considerable amounts and high concentration stocks, was used in Phase II in order to confirm the results obtained from Phase I, in addition to testing the protective effect of the Allura Red-based lyophilisation matrix which was being used in other aspects of the LMC development at the time. The monoclonal antibody to *S. aureus* was also used, albeit only lyophilised in the sucrose-based matrix (allowing further result comparison between Phase I and II). Two antibodies that were not used in Phase I were added to the sample set during Phase II; a polyclonal antibody against phytanic acid and a polyclonal antibody against FITC. Both antibodies were available in limited amounts, and priority was given to samples lyophilised in the sucrose-based matrix, which was considered the most efficient at the time of studies. An

error in the handling of the storage control samples rendered them unusable[1]; for this reason they are not included in the results of Phase II. Given the difficulty in repeating the experiments to replace the storage controls, and judging by the results in Phase I were the storage and shipping controls gave similar results, the shipping controls were considered adequate as variation control sample set.

2.15.1.1 Effect of lyophilisation matrix on retention of antibody binding activity

Figure 2-5 shows the ELISA results for the anti-GroEL (5B) and anti-phytanic acid (5A) antibodies; the former was freeze-dried in three different matrices (Blue Dextran-based, sucrose-based and Allura Red-based), while the latter was lyophilised in the sucrose and Allura Red-based matrices only.



[1] The storage controls were accidentally transported to Sweden with the shipping control samples.

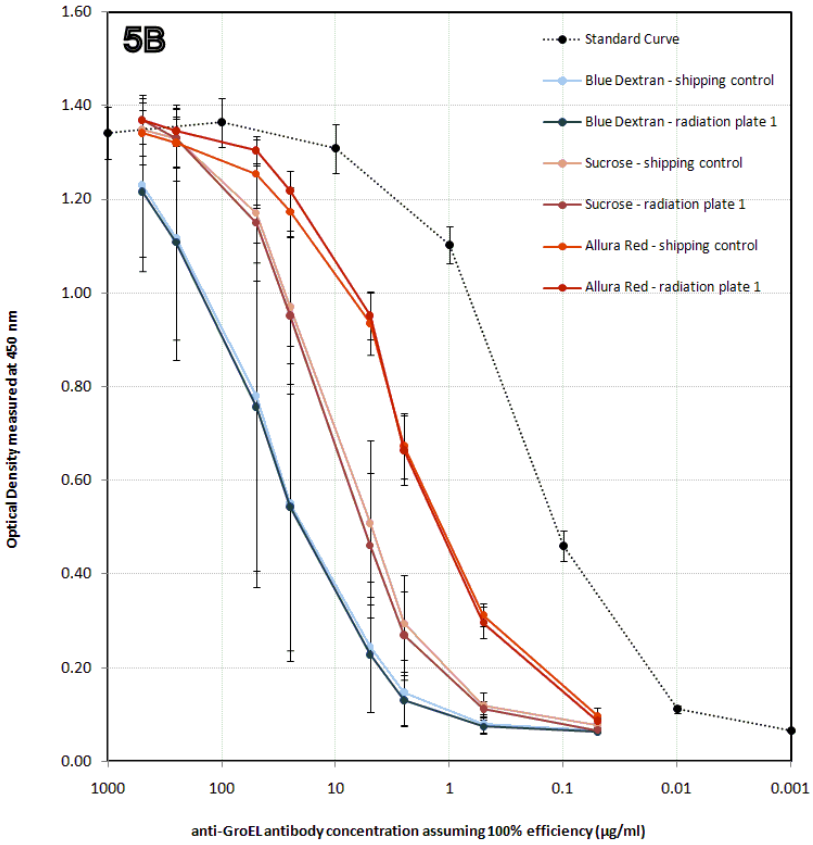
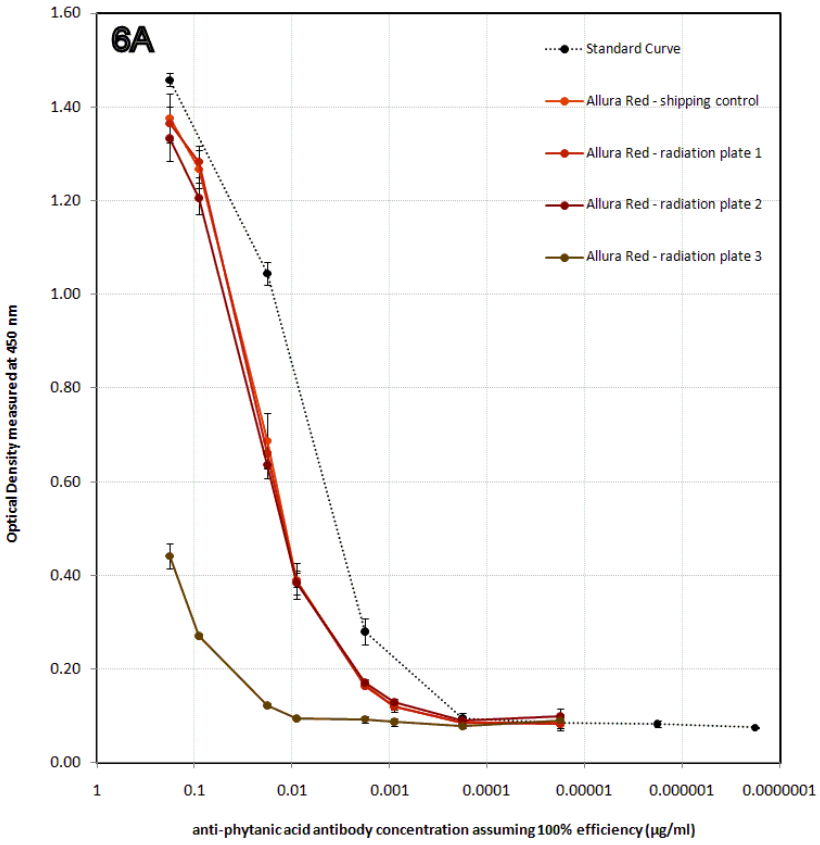


Figure 2-5: Binding performance of the anti-phytanic acid (5A) and anti-GroEL (5B) antibody after lyophilisation in various protective matrices measured at λ_{450} ("100% efficiency" refers to wash-out efficiency)

From Figure 2-5 it is obvious that the use of the Allura Red-based matrix resulted to higher signals than those obtained from the use of the sucrose-based matrix, while the Blue Dextran-based matrix appeared to result to the lowest signals out of the three matrices.

2.15.1.2 Effect of varying radiation dose on retention of antibody binding activity

Figure 2-6 shows the ELISA results for the same two polyclonal antibodies, anti-GroEl (6B) and anti-phytanic acid (6A), but this time the varying factor is the dose and combination of radiation that each sample was exposed to.


6A
Standard Curve
Allura Red - shipping control
Allura Red - radiation plate 1
Allura Red - radiation plate 2
Allura Red - radiation plate 3
Optical Density measured at 450 nm
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
anti-phytanic acid antibody concentration assuming 100% efficiency (µg/ml)

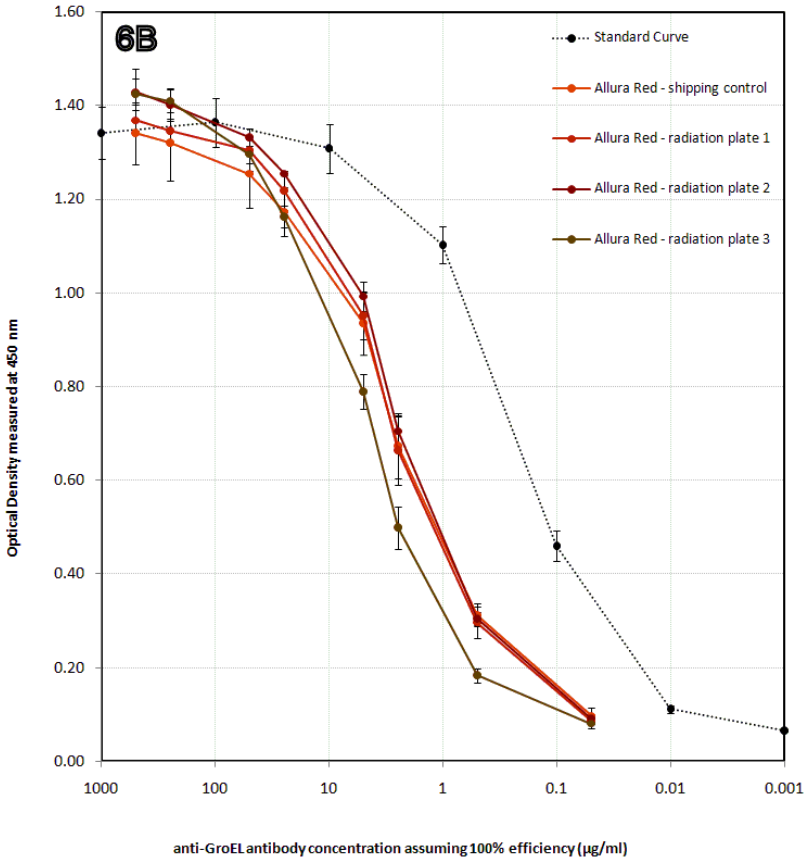
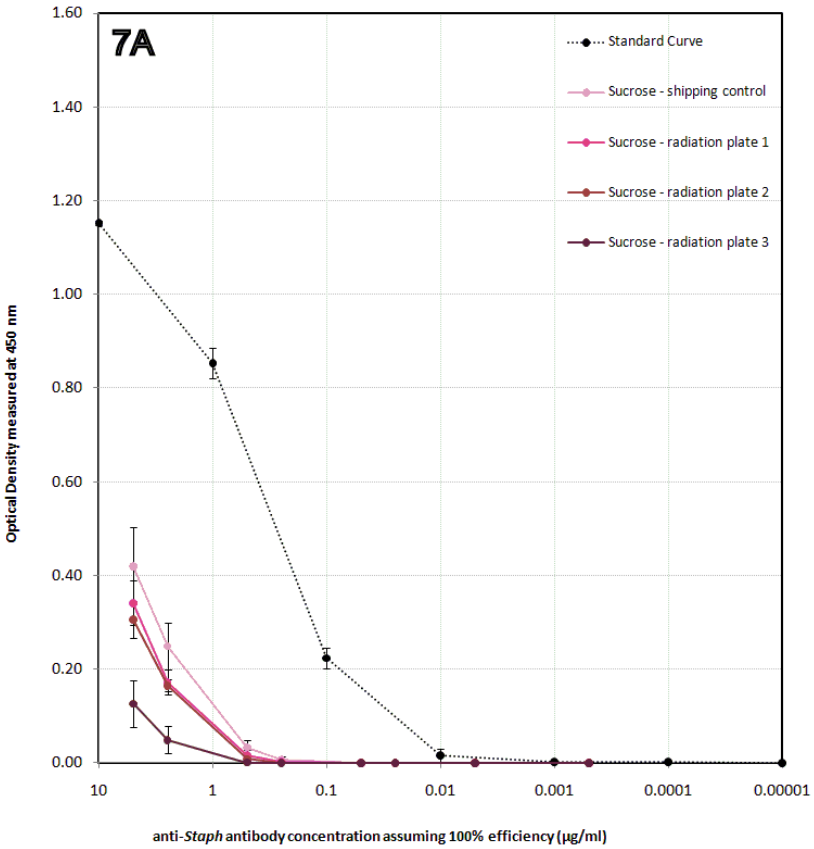


Figure 2-6: Binding performance of the anti-phytanic acid (6A) and anti-GroEL (6B) antibody after exposure to various doses/combinations of radiation measured at λ_{450} ("100% efficiency" refers to wash-out efficiency)

The different reaction of antibodies to radiation-related stress is obvious in Figure 2-6, where the anti-GroEL antibody seems to maintain its binding activity even after exposure to 250 times the ExoMars envisaged proton radiation dose, while the anti-phytanic acid antibody shows visibly reduced activity after being exposed to the same dose of radiation.

Figure 2-7 shows the ELISA results obtained for the anti-*S. aureus* and the anti-FITC antibodies. Both of them were only freeze-dried in the sucrose-based matrix, so the varying factor in Figure 2-7 is the level of radiation to which each sample was exposed to.


7A
Standard Curve
Sucrose - shipping control
Sucrose - radiation plate 1
Sucrose - radiation plate 2
Sucrose - radiation plate 3
Optical Density measured at 450 nm
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
10
1
0.1
0.01
0.001
0.0001
0.00001
anti-Staph antibody concentration assuming 100% efficiency (µg/ml)

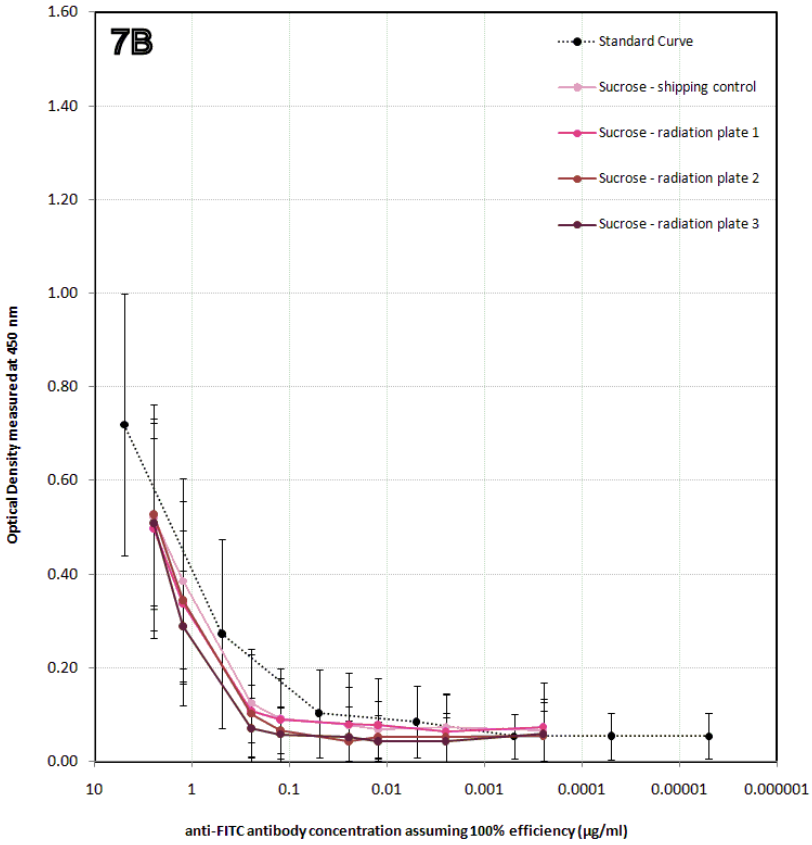


Figure 2-7: Binding performance of the anti-*S. aureus* (7A) and anti-FITC (7B) antibody after exposure to various doses/combinations of radiation measured at λ_{450} ("100% efficiency" refers to wash-out efficiency)

From Figure 2-7 it is clear that the two antibodies showed different levels of activity retention (compared with the standard curves which serve as an indication of the "maximum" antibody activity), with the anti-*S. aureus* antibody being heavily affected, similarly to Phase I.

2.15.1.3 Summary of antibody binding activity for Phase II

In order to quantify the loss of activity for all antibodies used in Phase II, the data was summarised in Table 2-9.

Table 2-9: Binding activity retention (%) for all antibodies used in Phase II of the ground-based radiation studies (n/a: not available)

	Anti - Phytanic Acid Antibody		Anti - GroEL Antibody		Anti - *S. aureus* Antibody		Anti - FITC Antibody	
	Assay mid-point (µg/ml)	Retained Antibody Activity (%)	Assay mid-point (µg/ml)	Retained Antibody Activity (%)	Assay mid-point (µg/ml)	Retained Antibody Activity (%)	Assay mid-point (µg/ml)	Retained Antibody Activity (%)
standard curve	0.01	100.0%	0.23	100.0%	0.42	0.0%	1.49	100.0%
Blue Dextran - shipping plate	n/a	n/a	37.37	0.6%	n/a	n/a	n/a	n/a
Blue Dextran - radiation plate 1	n/a	n/a	38.38	0.6%	n/a	n/a	n/a	n/a
Blue Dextran - radiation plate 2	n/a	n/a	52.18	0.4%	n/a	n/a	n/a	n/a
Blue Dextran - radiation plate 3	n/a	n/a	80.81	0.3%	n/a	n/a	n/a	n/a
sucrose - shipping plate	0.07	14.5%	10.61	2.2%	n/a	0.0%	2.68	55.6%
sucrose - radiation plate 1	0.07	13.5%	12.16	1.9%	n/a	0.0%	5.19	28.7%
sucrose - radiation plate 2	0.06	15.6%	12.22	1.9%	n/a	0.0%	4.95	30.1%
sucrose - radiation plate 3	0.64	1.5%	22.86	1.0%	n/a	0.0%	76.70	1.9%
Allura Red - shipping plate	0.03	33.2%	2.56	8.9%	n/a	n/a	n/a	n/a
Allura Red - radiation plate 1	0.03	32.0%	2.66	8.6%	n/a	n/a	n/a	n/a
Allura Red - radiation plate 2	0.03	30.4%	2.58	8.9%	n/a	n/a	n/a	n/a
Allura Red - radiation plate 3	2.53	0.4%	4.95	4.6%	n/a	n/a	n/a	n/a

Discussion

2.16 Limitations of chosen conditions to simulate a Mars mission

The simulated conditions of a Martian mission, in this case the radiation environment, are subject to a number of limitations and approximations and can never be an entirely accurate representation of the events during a real mission.

Thus in the present study, it is assumed that SPE-related proton radiation is the dominant threat en route to Mars, ignoring the radiation hazard by GCR ions, despite the fact that the heavy ion bombardment that is related to GCR is expected to be constant, while the presence of SPE protons will be more sporadic – the modelling assumed a worst case scenario with the "worst week" case in the CREME96 model used. Similarly, the composition of the radiation environment on the surface of Mars was simplified to include only the dominant secondary neutrons, not taking into consideration the other types of radiation which are present (UV, mineral radiation, SPE-related protons, GCR). Some of these other types of radiation are blocked by the thin Martian atmosphere and others by the rover's outer shielding, making them secondary to the effect of neutron radiation.

Mission duration is another factor to consider; for the purpose of modelling of the radiation environment is was assumed that the Earth-Mars transit phase would take place over a 2-year period, with an additional 6-month period of surface operation. This estimation was based on the early ExoMars mission design and is a significant over test of the current (circa 2010) ExoMars mission design which has a 9-month transit period.

2.17 Assumptions and limitations of the estimated mission dose

The present experiments were designed around estimated radiation doses during a mission to Mars that were the result of modelling of the radiation environment based on "worst case scenarios" for every type of radiation that forms part of this environment. Consequently, they do not necessarily offer a representation of the actual levels of radiation that will be encountered by the ExoMars mission. The advantage of this simulated environment is that it takes into account the maximum doses of radiation that

could be deposited on the mission instruments – it is therefore a suitable over test of the expected conditions.

Dose deposition rates should also be mentioned, since in terms of an actual mission the total dose of radiation would be accumulated over the mission's duration period, while in the present experiments the samples were exposed to the same dose of radiation in a period ranging over some minutes, rather than months or years. The assumption is that the difference in the rate of deposition will not affect the outcome of the experiments. It is however possible that a longer exposure time (for the same fluence) would result in reduced effects due to annealing of any radiation damage.

2.18 Limitations of realisation of estimated mission dose via cyclotron facility

The use of the Gustav Werner cyclotron at the TSL facility allowed the generation of high-energy particles that can be used in simulated space radiation experiments. However, the use of these facilities introduces additional limitations to the exposure conditions; space radiation is composed of particles of various energies (polyenergetic) and this cannot be duplicated easily with the use of the cyclotron, as the resulting beam is monoenergetic (or quasi-monoenergetic in the case of neutrons). For this reason, the entire spectrum of energies is narrowed down to a single representative value and the exposure time/runs are set based on this figure. This was a necessary compromise for both the proton and neutron irradiations, and was deemed a satisfactory way to simulate the radiation environment in question.

Other issues with the use of the cyclotron had to do with a level of uncertainty concerning beam intensity, beam uniformity, beam control dosimetry, back-scattered effects and background radiation (Hands, 2008). These errors were considered negligible, compared to uncertainties related to calculation of mission doses and the safety margin which was introduced by doubling the modelled dose as an over test.

2.19 Choice of antibody integration method

As mentioned in the Introduction and Experimental Design sections, antibodies were used throughout the radiation test campaigns in a lyophilised format. This decision was based on extensive data in the literature about the increase in shelf life and stability of antibodies when freeze-dried, compared to molecules in solution and was in accordance with the general strategy for antibody integration to the LMC instrument. Lyophilisation was also expected to increase resistance to inactivation due to radiation exposure, by eliminating, for instance the presence of radiolytic products due to the presence of water in a sample. The importance of an optimised procedure for lyophilisation, taking into account the physical and chemical degradations that samples are exposed to during and after freeze-drying, became obvious from preliminary studies with simulated space radiation (Paul Scherrer Institute, 2007 – results not shown), prompting significant changes in the freeze-drying protocols, and the addition of protective matrices.

2.20 Lyophilisation matrix and glass fibre pads

The antibodies were lyophilised into glass fibre pads in accordance to the envisaged final design format in the LMC instrument and in order to facilitate handling and exposure to the radiation source

As far as the matrix that the antibodies were freeze-dried in, in addition to samples freeze-dried into the buffer solutions they were originally in (*i.e.* from their manufacturers), samples were lyophilised in various protective matrices, the components of which were chosen to offer both lyo- and radioprotection.

It became obvious that samples that were lyophilised in buffer solutions handled the drying and recovery process very poorly, showing practically zero binding activity in subsequent ELISA (Figure 2-3). This data lead to a better understanding of the importance of the use of protective matrices, which have since then been integrated to all levels of sample preparation for the LMC instrument.

2.21 Sample rehydration and pad washout

The rehydration of antibodies in order to assess the retention of binding activity after lyophilisation and radiation exposure was a process that was optimised in parallel to the design of the ground-based radiation studies. The importance of an efficient method for sample recovery lies in differentiating the effects of handling/freeze-drying from the standalone effect of radiation in many cases. Out of all lyophilised samples, the storage controls provided the most immediate measure of comparison to non-lyophilised antibody activity (standard curves), and the fact that an efficient wash-out protocol (pad washout studies demonstrated an efficiency of at least 40% - data not shown) was in place allowed us to assess the effect of the lyophilisation process alone, which, as can be seen in Table 2-8 and Table 2-9 is by no means negligible (the overall maximum activity retention was observed in the case of the anti-FITC antibody shipping control in the sucrose-based matrix at 55.6% of the activity of the non-lyophilised antibody). For the remaining antibodies, the anti-phytanic acid shipping control in Allura Red-based mixture showed the maximum retention of activity at 33.2%, the maximum activity for the anti-GroEL antibody was at 30.8% (Blue Dextran – storage control), for the anti-atrazine antibody the maximum observed activity was at 2.1% (Allura Red matrix – shipping control) and finally the anti-*S. aureus* antibody appeared not to be active after recovery. A number of factors affecting wash-out efficiency may be associated with these results, *e.g*. basic instability of the proteins to freeze-drying and non-specific binding of antibodies to the various surfaces they interact with during handling. The dominant factor among the possible causes for these observations has not been clarified in the present study.

2.22 Lyophilisation matrix effects

Of the three protective lyophilisation matrices used in the present studies, the Allura-Red based mixture was considered the most successful protectant (Figure 2-5) wherever used, based on the ELISA results obtained in each case, followed by the sucrose-based mixture. The Blue Dextran-based matrix gave poorer results than the other two matrices when it was used with the anti-GroEL antibody in Phase II (Figure 2-5A), but appeared

to offer more protection than the sucrose-based mixture in samples from Phase I (Figure 2-3). This discrepancy could be due to slight differences in the preparation of the samples for the two different test campaigns (*e.g*. the use of different batches of matrix components and anti-GroEL antibody). The high efficiency of the Allura Red matrix could be attributed to the combination of compounds used in the mixture, *e.g.* the presence of Thiomersal as an antibiotic agent (not present in the Sucrose-based and Blue Dextran-based matrices). The absence of ascorbate from the mixture did not lead to any problems, implying that it is not essential for the preservation of antibody functionality.

2.23 Radiation

Without taking the effects of handling/recovery into consideration, the standalone effect of radiation exposure can be seen in Figure 2-4 (anti-GroEL antibody), Figures 2-6A and 2-6B (anti-phytanic acid antibody and anti-GroEL antibody) and Figure 2-7B (anti-FITC antibody). For the anti-GroEL antibody, which was the one sample which was used in all combinations of experimental conditions, what can be said is that radiation exposure alone does not seem to affect its ability to bind to its respective antigen, provided a lyophilisation matrix was used in the sample preparation. Even at 250 times the proton radiation dose envisaged for the ExoMars mission, the majority of the antibody binding activity remains, compared to samples exposed to 2x and 20x ExoMars proton dose (>50% activity retention). Exposure to proton or neutron radiation (not combined) revealed that one type of radiation does not seem to have a more observable effect than the other; this is primarily because no significant effects were observed following any of these independent exposures and it confirms the conclusions of Le Postollec *et al*. (2009) about the effects of neutron radiation on antibody performance.

In the case of the anti-phytanic acid antibody, significant loss of activity was observed at 250 times the proton dose, but no significant reduction of signal for the 20 times mission dose of protons. The behaviour of the anti-FITC antibody was very similar,

suggesting that the selected “maximum” experimental proton dose (250 times) was close to the most intense radiation stress that these antibodies can handle.

2.24 Antibodies

The five antibodies that were used in the present experiments were both of polyclonal and monoclonal origin and also a recombinant single chain antibody fragment, as far as the production method was concerned. One of the initial questions raised was whether antibodies of the same type would react similarly to radiation exposure.

Excluding activity loss due to sample preparation and handling, the three polyclonal antibodies (anti-GroEL, anti-FITC and anti-phytanic acid) proved radioresistant, with deactivation appearing at very high levels of radiation (250 times the nominal mission dose for protons). This was seen both for the anti-phytanic acid antibody (Figures 2-5A, 2-6A) and the anti-GroEL antibody (Figures 2-5B, 2-6B). The anti-FITC antibody was not especially sensitive to the lyophilisation process (the ELISA results from the shipping controls showed less than 50% loss of binding activity) and maintained high levels of binding activity for 1x and 10x times the ExoMars combined dose of radiation, with activity loss observed at 250x times the ExoMars levels of protons (Figure 2-7B).

For the monoclonal antibodies (anti-*S. aureus* and anti-atrazine scAb), the monoclonal antibody to *S. aureus* was very sensitive to lyophilisation and/or recovery from the glass fibre pads, and this concealed any standalone effects of radiation (Figure 2-7A). In the case of the anti-atrazine recombinant antibody fragment (Figure 2-3A), the lyophilisation process resulted to over 90% loss of activity, judging from the ELISA results for the storage controls. 1x combined ExoMars mission dose of radiation did not result to further loss of binding activity, suggesting that this single-chain antibody fragment is able to survive irradiation.

From the ELISA results it is obvious that there is no stable pattern or trend in the way antibodies react to radiation exposure, even among antibodies of the same type (monoclonal/polyclonal). Despite these differences in performance, it was observed that all antibodies suffered various degrees of activity loss at extremely high doses of proton

radiation; the exact mechanism of this effect is not fully understood at this point, but a number of speculations can be made based on the combination of the design and outcome of the experiments. It is, for instance, believed that the lyophilisation of the antibodies prior to irradiation resulted to the elimination of water in the proximity of the proteins and reduced potential for secondary effects of radiation via the radiolysis of water molecules. Therefore it can be concluded that the major radiation effects on the antibodies were direct ones, after energy deposition on the molecule, either in the form of bond breakage or by the production of various reactive radical species. The loss of activity that was observed after irradiation with high doses of proton radiation can consequently be attributed to the higher probability of bond breakage in the presence of higher numbers of energetic particles (protons).

2.25 Future studies

The evolution of the present study will lead to a better understanding of the effects of space radiation on immunoassay components for future planetary exploration missions. The number of antibodies used in these radiation campaigns was a sub-set of the total library planned for the flight model of the LMC instrument. New antibody assays are under development and further representative antibodies should undergo similar testing to determine the effects of space radiation on their ability to bind their targets.

Another set of experiments to consider for future testing could be ion irradiations of immunoassay components, in an attempt to simulate the effect of GCR ions on the operation of the LMC. The dose levels would be much lower than those for protons, but the damage coefficient is expected to be much higher.

An area that requires further optimisation is the lyophilisation process. It became obvious from the results obtained from the irradiation studies that the majority of antibody activity is lost due to freeze-drying and/or recovering functional protein (>80% activity loss in most cases). Overcoming these issues will not only be beneficial for the following rounds of irradiation studies, but the overall development of the LMC instrument.

The ground-based radiation campaigns took place in parallel to an experiment to assess the antibodies' activity to survive exposure to actual space conditions in Low Earth Orbit (LEO). BIOPAN-6 was an experimental platform onboard ESA's Foton M3 mission (2007), which went into LEO for 12 days and allowed direct exposure of biological material to space conditions. Two of the antibodies used in the present studies (anti-GroEL and anti-atrazine) were also used in the BIOPAN experiment and neither was deactivated due to exposure to these conditions – estimated to be one fifteenth of a total Mars mission dose for the BIOPAN-6 flight (see Chapter 3).

Summary and Conclusions

Overall, the ground-based irradiations of immunoassay reagents - specifically antibodies - at simulated radiation levels envisaged for the ExoMars mission to Mars, and also at much higher levels, have given valuable information and data input for the LMC development. It is apparent that the dominant effect on the loss of activity of five representative antibodies studied was the processing and packaging of the antibodies as freeze-dried preparations in glass fibre pads and their subsequent retrieval from the pads for use in ELISAs. The subsequent steps of international shipping of the samples to an irradiation facility and their irradiation typically contribute far less to the loss of antibody activity. Therefore, it is apparent that further studies of the freeze-drying, packaging and sample recovery process are required. Initial studies of varying the solutes co-freeze dried with the antibodies have displayed promise in improving the recovery efficiency. For the radiation effects, the important observation is that at a representative Mars mission dose, none of the antibodies studied exhibit any evidence of loss of activity due to the radiation. This therefore achieves a key de-risking step for the LMC instrument development process of demonstrating that the core antibody reagents will survive the radiation environment to be encountered on the ExoMars mission. Whilst this study de-risks the use of antibody-based assays in the exploration of the Red Planet, the noticeable loss of antibody activity at higher radiation levels (x250 Mars mission dose) does suggest that further shielding or alterative radiation protection approaches will need to be considered for some other astrobiology targets such as future Europa missions.

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Survivability of immunoassay reagents exposed to the radiation environment of Low Earth Orbit using the ESA BIOPAN-6 experimental platform as a simulated Mars planetary exploration mission

Introduction

3.1 Preamble and Relationship to Ground-Based Radiation Studies

The Life Marker Chip (LMC) instrument is an antibody assay-based sensor which will attempt to trace molecular signatures of Life in the Martian subsurface as part of the payload on board the ExoMars mission rover. The ExoMars mission, a collaboration between the European Space Agency (ESA) and NASA, is ESA's flagship mission to Mars, currently scheduled for launch in 2018. The LMC will be part of the Pasteur payload on board the ExoMars rover and it will have the ability to detect up to 25 different molecular targets of different origins that are associated with meteoritic in-fall, extinct or extant Life, prebiotic chemistry and spacecraft contamination. Regolith / crushed rock samples from up to four different locations can be collected for the LMC, which is designed to integrate sample processing and analysis into what is essentially an analytical laboratory in miniature.

The molecular reagents at the core of instruments such as the LMC have no heritage of interplanetary mission use. Therefore, the design of such instruments for space missions must take into account a number of risk factors, among which the intense radiation environment that will be encountered en route to, and on the surface of, planets. In order to study the effects of space radiation on lyophilised immunoassay reagents, primarily antibodies, a number of ground-based and space studies were carried out, the latter in the form of ESA's 2007 BIOPAN-6 Low-Earth Orbit (LEO) space exposure platform.

The ground-based studies confirmed that representative LMC antibodies, pre-treated and packaged in the LMC-intended format, remain functional after exposure to levels of proton and neutron radiation that closely simulate those envisaged for a mission to Mars and even up to 250 times the ExoMars dose for proton radiation (Derveni *et al.*, unpublished data – see chapter 2). Nevertheless, ground-based simulations of space radiation have their limitations. In an actual space mission, a body is exposed to a low flux of particles of a broad spectrum of atomic mass and energy, moving in various directions. In ground-based studies where accelerators are used, high fluxes of a single, monoenergetic source of particles are produced in a narrow, unidirectional beam (Horneck, 1992). In addition to the issues related to the composition of the simulated

radiation environment, ground-based studies offered no information concerning the performance of antibodies after exposure to the physical aspects of a space mission in terms of ground handling, launch, flight and possible atmosphere re-entry/recovery. This means that in order to study the effect of the full range of conditions that comprise a planetary exploration mission, ground-based simulations should ideally be combined with actual exposure to the environment in space.

3.2 Biological Experiments in Low Earth Orbit

Low Earth Orbit (LEO), which can generally be defined as the orbit between 0 and 2000 km from the Earth's surface, is characterised by extreme environmental conditions (significant radiation field, high vacuum and extreme temperatures), which, aided by the advances in space technology, render it an important location for *in situ* space studies. Chemical and biological experiments in Earth Orbit have been taking place for the past 40 years (reviewed by Horneck, 1998b & 1999), aiming to answer questions related to the molecular evolution of the interstellar medium, the possibility of interplanetary transfer of life and the importance of solar UV radiation in terms of pre-biotic and biological evolution. The majority of biological studies in LEO have focused on the physiological, genetic and biochemical response of a variety of microorganisms after or during exposure to space conditions. In the past, biological experiments in Earth Orbit have been part of a number of missions, including Gemini, Spacelab, LDEF, MIR and EURECA. The focal points of the European activities in this field include the experimental platforms BIOPAN (for short-term exposure to space conditions) and EXPOSE (for long-term exposure to space conditions on the ISS) (Schulte *et al*. 2007).

One of the most recent reviews of microbiology in LEO points out that presently representatives of all three domains of life have been exposed to the environment in Earth Orbit (Olsson-Francis and Cockell, 2010) as part of experiments with a wide range of applications in future space exploration, including life support systems, energy sources and planetary protection. The materials for these experiments were whole active cells or spores, and even individual cellular constituents (plasmid DNA, amino acids, urea and purple membranes) (Dose *et al.*, 1995), depending on the nature of the

experiment. With new planetary exploration missions underway, like ExoMars, new instruments are being developed, with components that have no history of exposure to space conditions, like the antibodies in the core of the LMC. The performance of lyophilised antibodies after exposure to a combination or even individual elements of the space environment has not been studied before and no data existed on their subsequent performance as molecular tracers. Given that the LMC antibodies will be effectively shielded from all aspects of a planetary mission (temperature extremes, space vacuum etc.) except for ionising radiation, it was considered an important early development issue to investigate their response to the radiation environment in LEO. This was to complement a series of ground-based studies of simulated ExoMars levels of proton and neutron radiation (Derveni *et al*., unpublished data – see chapter 2).

3.3 The Radiation Environment in Low Earth Orbit

The natural radiation environment in LEO is composed of charged particles of cosmic and solar origin and particles in the radiation belts trapped by the Earth's geomagnetic field (Stassinopoulos, 1988). Additionally, secondary radiation is produced from the interaction of cosmic rays with spacecraft shielding material, in the form of proton recoils, neutrons and other by-products (Wilson *et al*., 1993). The composition of the radiation environment is not constant; spatial and temporal variations are observed (Reitz, 2008). The intensity of the radiation exposure is also variable and closely related to a number of orbital (altitude, trajectory etc.) and technical (spacecraft shielding) aspects of a mission in LEO.

The radiation exposure in LEO was described in detail by Reitz (2008), both inside and outside the spacecraft structure, *i.e.* in the presence or lack of, protective shielding. Radiation exposure inside the spacecraft is the result of the proton and heavy ion components of Galactic Cosmic Rays (GCR) and the protons inside the South Atlantic Anomaly (SAA), which is an area where a displacement of the axis of the geomagnetic field with respect to the axis of the geoid results to the radiation belt coming closer to the Earth's surface. This region is responsible for up to 90% of the total exposure in LEO (Horneck, 1992). Neutrons are also present, produced from the interaction of GCR

particles with atoms in the Earth's atmosphere. Outside the spacecraft, the dominant radiation elements are the electrons in the horns of the radiation belts located at a $\pm 60^{o}$ latitude at the Polar Regions (Reitz, 2008).

3.4 The ESA BIOPAN platform for exposure of biological experiments to Low Earth Orbit conditions

3.4.1.1 Current status of BIOPAN

The BIOPAN multi-user experimental platform for the exposure of scientific experiments to space conditions in LEO was designed in 1990-1991 (Demets *et al*., 2005). BIOPAN is a circular, pan-shaped aluminium structure (38 cm in diameter, 23 cm in height, 27 kg mass) with a hinged lid that can open to 180^{0} in orbit. A number of experimental modules can be mounted on two experiment support plates (top and bottom layer) (Figure 3-1).

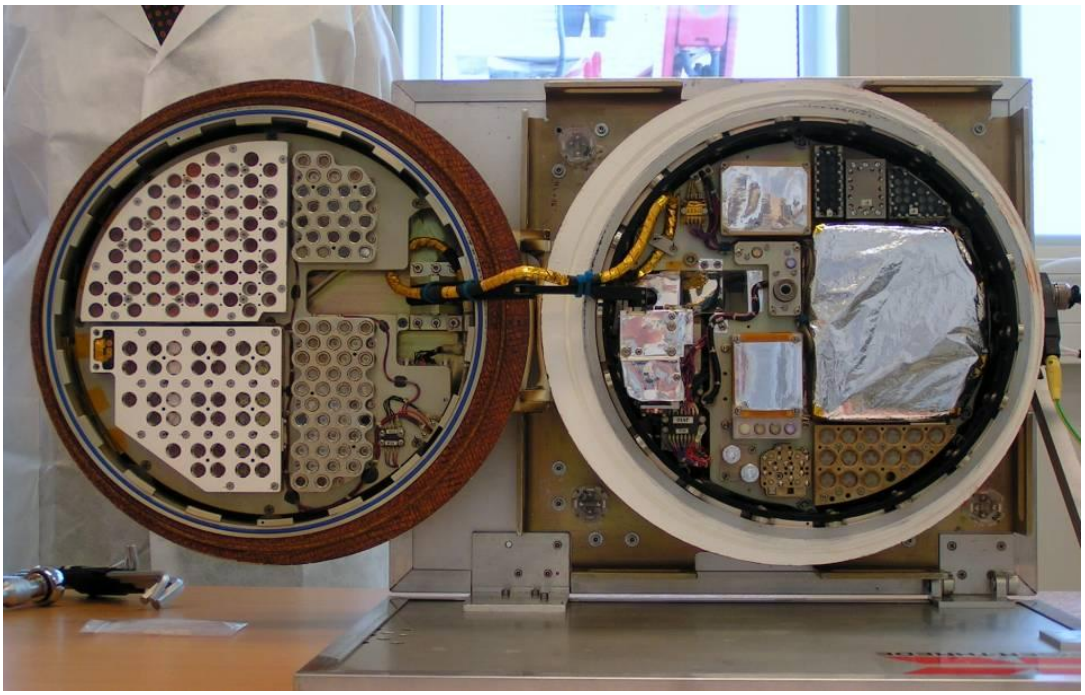


Figure 3-1: Opening of BIOPAN-6 loaded with experiments at ESTEC in the Netherlands after flight and recovery (October 2007)

Once in orbit, the structure opens by telecommand to expose the experiments to space radiation and vacuum, microgravity and extreme temperatures, as well as unfiltered solar light. BIOPAN can carry a variety of experiments (typically 10 different experiments) of up to 3.5 kg in combined weight. The platform is mounted on the exterior of a FOTON retrievable capsule (Figure 3-2) and is thermally insulated from the capsule. During launch and re-entry BIOPAN is hermetically sealed and secured with a locking ring, while the whole structure is covered by an ablative shielding material to protect the experiments from the heat generated during launch and re-entry (Schulte *et al*., 2007).

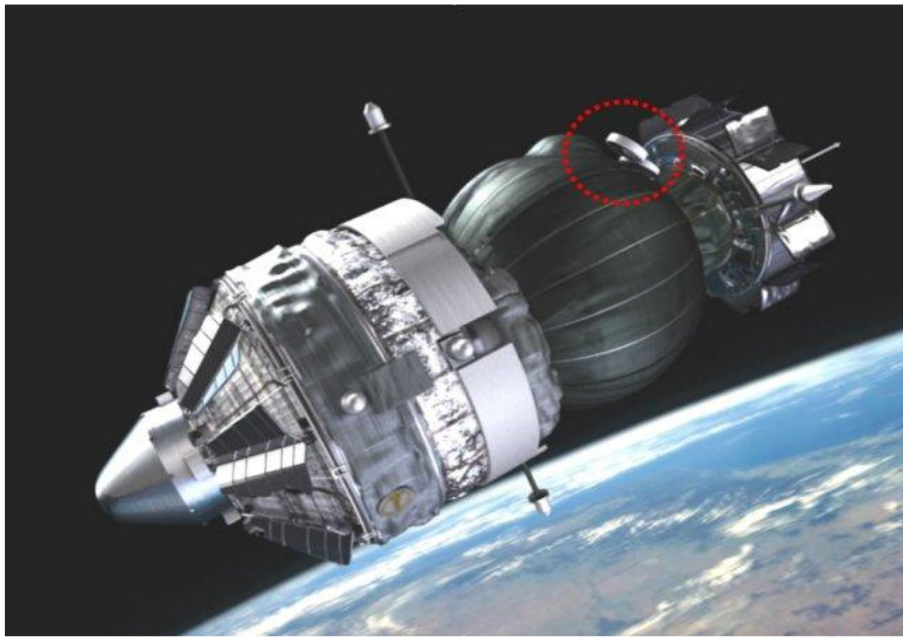


Figure 3-2: The retrievable FOTON capsule in LEO with BIOPAN exposing its contents to space conditions (red circle, top right)

In order to monitor the environmental conditions during the mission in detail, BIOPAN is equipped with a suite of built-in sensors (in addition to researcher-provided detectors of various types), including thermometers, a broadband radiometer, UV-B and UV-C sensors and pressure sensors. All data is recorded and stored onboard. BIOPAN provides thermal control between +15ºC and +25ºC, with temperatures normally never exceeding the biologically critical limit of +30 to +35ºC. In addition to temperature consistency, the orbital parameters selected for FOTON missions are fairly similar,

allowing experiments to be repeated under comparable conditions (Demets *et al*., 2005). After landing and recovery, all the experimental samples are returned to their owners for analysis at their respective laboratories.

3.4.1.2 History of the BIOPAN missions

Between 1992 and 2007, six BIOPAN missions had been completed successfully on FOTON missions on board Soyuz rockets, with an additional one (BIOPAN-4) on board FOTON M1 being lost due to launcher failure in 2002. BIOPAN 1-4 were launched from the Plesetsk Cosmodrome in Russia, while BIOPAN 5 and 6 were launched from the Baikonur Cosmodrome in Kazakhstan.

The BIOPAN platforms have each carried 4-9 experiments so far, covering four areas of research; astrobiology, chemical evolution, radiation biology and radiation dosimetry (Demets *et al.,* 2005). In addition to the biological experiments carried on the BIOPAN platforms, the mission setup provides an opportunity to perform experiments in other fields or test new technologies to determine their performance in space and their resistance to the environmental extremes in LEO. Examples include a variety of electronics and diagnostic systems, sensors for ionising or UV radiation and the various components (antibodies, microarrays, fluorescent dyes) of the LMC (biosensor technology) (Schulte *et al*., 2007).

3.4.1.3 Details and Description of BIOPAN-6

BIOPAN-6 on board the FOTON M3 mission went into LEO on the 14th of September of 2007 and was retrieved on the 26th of September 2007. The unit used in BIOPAN-6 was the refurbished unit from BIOPAN-5, after light modifications to accommodate the 9 experiments (7 single-module and 2 multi-module experiments) it would carry. Table 3-1 and Figure 3-3 provide details about the experiments on BIOPAN-6 and their location within the unit.

Table 3-1: List of experiments on BIOPAN-6

Experiment	Location	Description/Subject
UV-OLUTION I	Unit Lid	Chemical Evolution
UV-OLUTION II	Unit Lid	Chemical Evolution
LITHOPANSPERMIA	Unit Lid	Microbiology
TARDIS	Unit Bottom	Microbiology
ROTARAD	Unit Bottom	Microbiology
MARSTOX & HIGHRAD	Unit Lid	Microbiology
LIFE MARKER CHIP	Unit Bottom	Biosensor Technology
YEAST	Unit Bottom	Radiation Biology
RADO DEPTH DOSE	Unit Bottom	Radiation Dosimetry
RADO NEUTRON DOSE	Unit Bottom	Radiation Dosimetry
RADO PLASTIC	Unit Bottom	Radiation Dosimetry
R3D-B	Unit Bottom	Radiation Dosimetry

As with previous missions, the suite of instruments on board BIOPAN-6 corresponded to experiments of different nature and scientific context; some experiments had been included in previous BIOPAN missions (Marstox, RADO and R3D-B had also been flown in BIOPAN-5), while others, like the LMC experiment, had no previous LEO history.

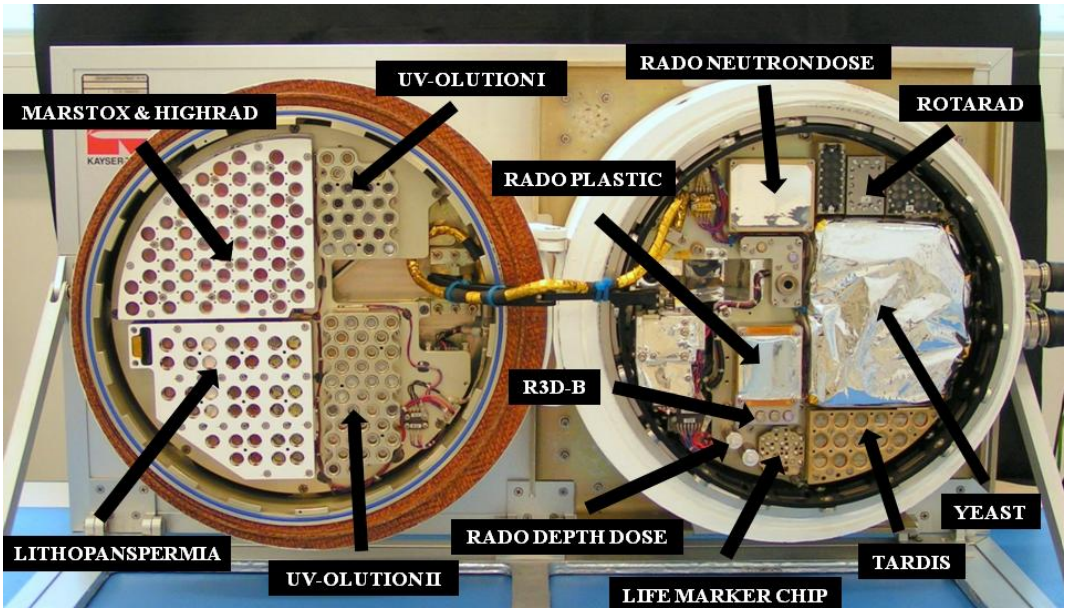


Figure 3-3: Location of the various BIOPAN-6 experiments in the flight unit

3.4.1.4 Space Radiation on BIOPAN

The experiments inside BIOPAN are exposed to the radiation environment in LEO, predominantly solar radiation trapped in the inner radiation belt and in the SAA (South Atlantic Anomaly). The composition of this radiation field is dominated by electrons in the range of 100 keV to 5 MeV and protons in the range of 1 MeV to 30 MeV which have been captured by the Earth's magnetic field and form a uniform radiation environment due to gyration movement in the magnetic field (Taken from document titled *Space Radiation in Biopan* (author not given) downloaded from the ESA Biopan FTP server on 5th June 2006).

The radiation in LEO, with the exception of the SAA where anisotropic fluxes are observed, is isotropic; for this reason the slow rotation of the FOTON capsule along its longitudinal axis does not influence the irradiation of BIOPAN. The irradiation of BIOPAN depends on how the spacecraft trajectory intersects the magnetosphere of the Earth and on the timing of the mission within the 12-year solar cycle. The way the FOTON spacecraft crosses the magnetosphere depends on the orbital parameters selected for that mission (inclination, altitude and flight duration). Those parameters for FOTON are highly consistent: the orbital inclination is always close to 63º, the spacecraft always cruises at an altitude around 300 km and the flight duration is always

close to two weeks. This consistency is particularly important whenever repetition of experiments is required; experiments can be performed under conditions that are quite similar, with the exception of solar cycle events.

As far as the absorbed dose of radiation is concerned for the experiments on BIOPAN, it was determined that shielding levels play a very important part in the protection of samples. It was observed that small changes in shielding density resulted to massive differences in the radiation dose received by samples. Over the first 0.05 g/cm^2 of shielding mass the radiation dose falls steeply down by one order of magnitude, over the first 0.25 g/cm^2 by two orders of magnitude, over the first 1g/cm^2 by three orders of magnitude, while at shielding densities beyond 2.25 g/cm^2 the absorbed radiation dose is more stable. For cosmic rays, it was observed that around 99.9 % was blocked by the first 1 g/ cm^2 of shielding mass. This 99.9 % consists of the softer components of the space radiation spectrum (belt electrons and low-energy protons). The residual 0.1 % consists of high-energy belt & solar protons, high-energy heavy ions (HZE) and secondary particles like neutrons.

Among the findings from BIOPAN-5 was the observation that the two parts of the BIOPAN unit (lid and bottom) did not receive the same dose of radiation, with the bottom part exposed to a lower dose (approx. 30% less), presumably due to partial "shading" by the cooling panels of the power supply unit (Fehér and Pálfalvi, 2008). The same authors reported that when the lid of BIOPAN is closed, more than 4.2 g per cm^2 of additional shielding mass is placed in front of the experiment packages pertaining to the heat shield and parts of the lid structure. The closed lid results to a dose reduction by more than 99.9 %, whereby the influx of electrons and low-energy protons is completely blocked. This means that when BIOPAN is closed, the exposure to cosmic rays is comparable to the dosage received by experiments inside the satellite.

3.5 Aims and Objectives

The objective of the present work was to de-risk the employment of LMC-related immunoassay reagents – especially antibodies – in a planetary exploration mission instrument. The LMC experiment on BIOPAN-6 was complementary to a detailed

series of ground-based irradiations (see Chapter 2) and offered additional information about the ability of antibodies to recognise and bind to their respective antigens after exposure to space radiation.

By exposing antibodies to the radiation environment in LEO, which is not uniform in composition and particle energy levels, we were aiming to achieve a more accurate representation of the radiation situation of an actual mission to Mars. In addition to the primary radiation (protons, electrons, heavy ions), LMC on BIOPAN allowed the study of the effects of secondary radiation (a result of the interaction of primary radiation particles with the spacecraft structure) and any spacecraft radiation sources.

The LMC on BIOPAN-6 experiment was also an opportunity to factor in the physical aspects of a space mission, as the LMC antibodies had no history of space flight. These included the conditions during launch and re-entry (shock loadings, acoustic energy during launch etc.) and the general ground handling of samples, from the point of sample preparation all the way to recovery and analysis.

Materials and Methodology

3.6 Experimental Design

3.6.1.1 Selection of antibodies and strategy for sample preparation

Two representative antibodies were used in the LMC experiment on BIOPAN; among the antibodies available at the time of studies (mid-2007), priority was given to those that existed within the lead authors' research group and had been confirmed to function in immunoassay format and recognise and bind to their targets with high sensitivity. The antibodies used were a polyclonal antibody against chaperonin 60 (GroEL) and a recombinant antibody fragment (scAb – single chain antibody) against atrazine. Chaperonin 60 and atrazine are both representative LMC targets and among the first assays to be developed from an extensive list of potential molecules that were of interest for the LMC instrument (Parnell *et al*., 2007)

In accordance with the sample preparation strategy throughout the development of the LMC, the antibodies were packaged and exposed in a lyophilised state, freeze-dried into

laser-cut glass fibre pads. This format, in addition to the stability and radioresistance advantages it was expected to offer, facilitated handling during various steps of the LMC assembly, and was used both in the BIOPAN experiment and the parallel ground-based radiation studies. Each antibody was freeze-dried into a separate pad and multiple replicates of each antibody pad were prepared, aiming to confirm the reproducibility of the results obtained.

The strategy concerning the preparation of control samples that was used in the ground-based experiments was also employed in the LMC on BIOPAN experiment; two sets of control sample pads were prepared, identical to the flight samples. These were a storage control set (kept at Cranfield University for the duration of the BIOPAN mission) and a shipping control set, transported to the Baikonur Cosmodrome in the same way as the flight samples but not flown on board the mission. The shipping control set was returned to Cranfield University along with the flight sample set at the end of the mission. All three sample sets were analysed in parallel in Cranfield University.

3.6.1.2 Exposure of antibodies to space conditions

The antibodies that were used in the LMC on BIOPAN-6 experiment had to be tested in a realistic simulation of the ExoMars mission. To this end, they were pre-treated and packed in an LMC-intended format and sealed in an inert gaseous atmosphere (no exposure to space vacuum). The gas chosen was argon, which would minimise the H_2O and O_2 content of the sample container and the gas pressure was constant at 1 atm.

Temperature during launch and re-entry was not expected to be extremely high, due to BIOPAN's heat shielding and temperature controls on board, while low temperatures (in orbit) were not expected to be an issue for the LMC antibodies.

Finally, as radiation exposure was the high-priority factor to investigate (except solar UV radiation, which is expected to be fully blocked by the structure of the ExoMars rover), it was decided that the antibodies would be exposed to space radiation under three different shielding thicknesses/materials. One of the shielding levels was deemed "zero shielding", blocking only UV radiation and helping to maintain the argon atmosphere around the samples, the second shielding level was labelled "ExoMars

shielding", as it corresponded to the level of shielding provided by the ExoMars rover and the final shielding level was "infinite shielding", which was expected to fully block all kinds of radiation, allowing the study of all other flight variables. The three shielding levels in detail can be seen in Table 3-2.

Table 3-2: Types of shielding of the antibody samples in the LMC on BIOPAN-6 experiment

Shielding Level	Description	Radiation Protection
"Zero" shielding	125 μm Kapton® foil	Minimal (blocks UV radiation, maintains atmosphere)
"ExoMars" shielding	4 mm of aluminium & 125 μm Kapton® foil	ExoMars rover equivalent
"Infinite" shielding	Stacked 4 mm of aluminium & 2 mm of stainless steel & 125 μm of Kapton® foil	Maximises shielding within the size and mass constraints of the experiment (blocks all types of radiation)

3.6.1.3 Dosimetry

In addition to the radiation measurements performed by other instruments on board BIOPAN-6 (RADO, R3D-B), the LMC sample antibody pads were each loaded in the sample holder unit with an individual Al_2O_3 Optically Stimulated Luminescence (OSL) dosimeter, which would offer supplementary data on the radiation levels the samples were exposed to. By contrast to the RADO experiment, the operation of which is based on thermoluminescent dosimetry (TLD) where readout is based on heating, in OSL dosimeters the readout is performed by controlled illumination of the dosimeter.

The operation principle of OSL dosimeters is based on electron/hole pairs created by exposure to ionising radiation and a fraction of these pairs elevated to the conduction band via ionisation becoming trapped in the OSL dosimeter structure. The subsequent recombination process leads to the emission of a light signal which can be detected. OSL dosimeters offer highly sensitive measurements, mainly due to the carbon doped aluminium oxide (Al_2O_3: C), which is their core component (Yukihara *et al*., 2006). The same authors report the use of OSL dosimeters (OSLDs) in space radiation

measurements for heavy charged particles (HCPs), where the OSL data combined with Linear Energy Transfer (LET) measurements are a practical way of characterising the radiation profile (total absorbed dose and dose equivalent) of a space mission.

The dosimeters used in the LMC on BIOPAN-6 experiment were hand-cut Luxel+® Al_2O_3: C dosimeters (2 x 4 x 0.3 mm^3) from Landauer Inc, provided by the Oklahoma State University (OSU).

3.7 Preparation of the LMC experiment for BIOPAN-6

3.7.1.1 Provision of LMC experimental hardware

A custom-made sample holder unit was prepared for the LMC on BIOPAN experiment by DLR (Germany). The machined aluminium (grade ISO 4762- M3x9-10.9) unit had two main parts; the base plate, with a number of cylindrical wells to accommodate the antibody-loaded glass fibre pads and a number of printed microarrays (not reported in this paper), and a top plate with areas of varying thickness (zero, 4mm Al and 4mm Al + 2mm stainless steel) to correspond to the different shielding levels of the various samples. The aluminium parts of the structure were passivated with an Alodine 1200 treatment, while the stainless steel inserts were bonded in place with a two-part epoxy adhesive (Araldite AV 100/HV100). Sandwiched between the two plates was a 125 μm thick Kapton® foil (grade HN polyimide film), aiming to seal the sample wells and offer a minimal level of radiation protection. Between the Kapton® foil and the bottom plate of the unit, a Viton® elastomer sheet gasket (grade GLT, 0.5 mm thickness) served as a gas-tight seal after mechanical clamping of the two parts with assembly bolts also provided by DLR. A schematic overview of the LMC on BIOPAN sample unit and a picture of the Development Model (DM) can be seen in Figure 3-4A and Figure 3-4B.

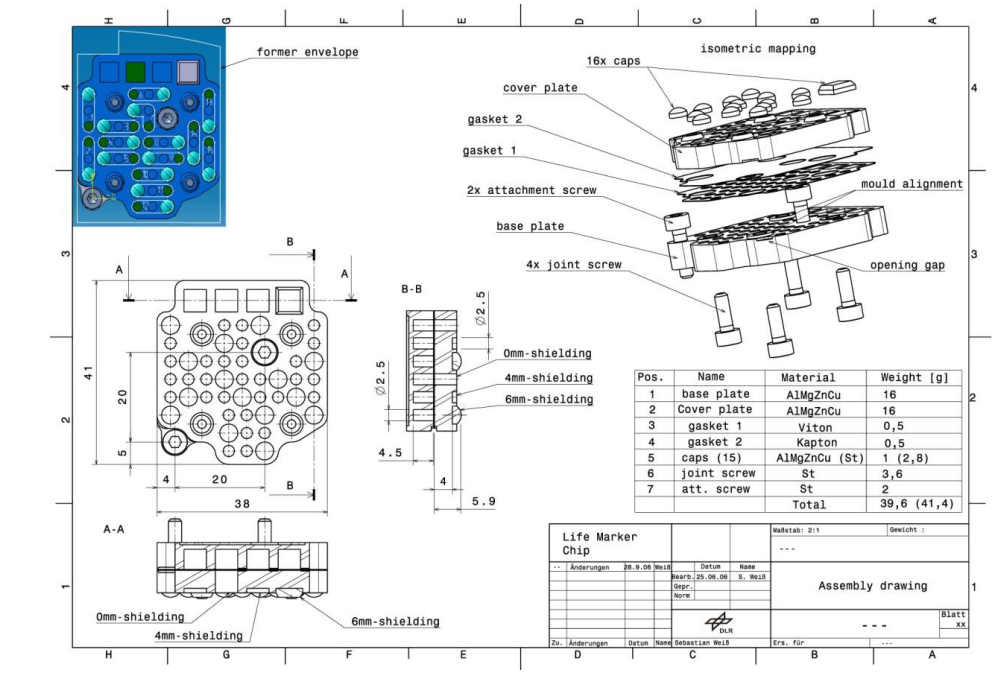


Figure 3-4: A. Design and B. Development Model of the sample holder unit for the LMC on BIOPAN-6 experiment (latter prior to Alodine treatment) (image credits: DLR Germany)

The final mass of the sample holder was 37.2 g prior to sample loading, at a volume of 41 x 38 x 12 mm^3.

In addition to the flight model, a second identical unit was constructed and used as a shipping control sample holder. A machining oversight on the shipping control sample

holder resulted in the shielding level of one of the microarray sample wells being much lower than the correct thickness of 4mm.

3.7.1.2 Preparation of glass fibre pads and sample integration

The antibodies used in the LMC on BIOPAN experiment were an anti-GroEL polyclonal antibody from Sigma Aldrich (cat. no. G6532) (stock concentration: 10 mg/ml) and a recombinant scAb against atrazine from Immunosolv Ltd. (Aberdeen, Scotland) (stock concentration: 5 mg/ml). The material for the glass-fibre pads was GF/D glass microfibre filter (47 mm ø) from Whatman. The 2 x 4 x 0.6 mm^3 rectangular pads were laser-cut using a Fenix Laser Marker from SYNRAD. Using a protocol identical to the one used in the preparation of samples for the ground-based studies, 6 µl of each antibody (in the buffer solution it was provided in by its manufacturer) were dispensed using pipettors and allowed to wick into the glass fibre pads. Assessed by visual inspection, this volume appeared to fully saturate the pad. Both antibodies were used at the stock concentrations they were provided in by their manufacturers. After the sample solution was added to each pad, the pads were immediately frozen by immersion into liquid nitrogen. Due to the pads' small size and practically no thermal inertia, it was observed that removing them from liquid nitrogen prior to the drying step of the process resulted in rapid thawing. The problem was overcome by keeping the frozen pads in liquid nitrogen, inside 20 ml plastic beakers, which were then placed in the vacuum chamber of a bench top freeze-drier (Coolsafe 55-4 bench-top freeze drier from ScanVac). Upon establishment of the vacuum inside the chamber, the liquid nitrogen quickly boiled away. The samples were freeze-dried overnight (approx. 12 hours).

The lyophilised samples were stored in Petri dishes secured with masking tape, in ambient conditions (ambient temperature and atmosphere) and kept in the dark until the assembly of the flight and storage control unit.

3.7.1.3 Assembly of flight model and shipping control unit

The assembly of the flight sample holder unit and shipping control unit were performed in parallel in a class 100,000 clean room at Cranfield University on the 28th of August 2007. The assembly protocol was provided by DLR. The Luxel+® dosimeters (Figure 3-5A) were loaded in the cells at the bottom part of the unit first (1 dosimeter/well), followed by all the lyophilised samples (Figure 3-5B) and the printed microarrays.

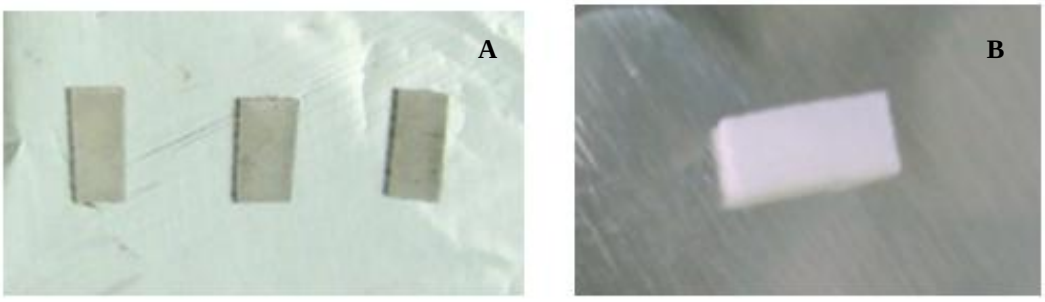


Figure 3-5: A. Hand-cut Luxel+ dosimeters (2 x 4 x 0.3 mm^3) and B. Laser-cut glass fibre pad (2 x 4 x 0.6 mm^3) for the LMC on BIOPAN-6 experiment

For the antibody samples, three replicates of each antibody type were loaded into wells (one glass fibre pad per well) of the same level of shielding (3x "zero" shielding, 3x "infinite" shielding and 3x "ExoMars" shielding), in order to confirm the reproducibility of the results obtained. Once the sample loading was complete (Figure 3-6A), the Viton® gasket (Figure 3-6B), followed by the Kapton® foil (Figure 3-6C) were put in their place over the sample area, before the cover plate was screwed in place (Figure 3-6D).

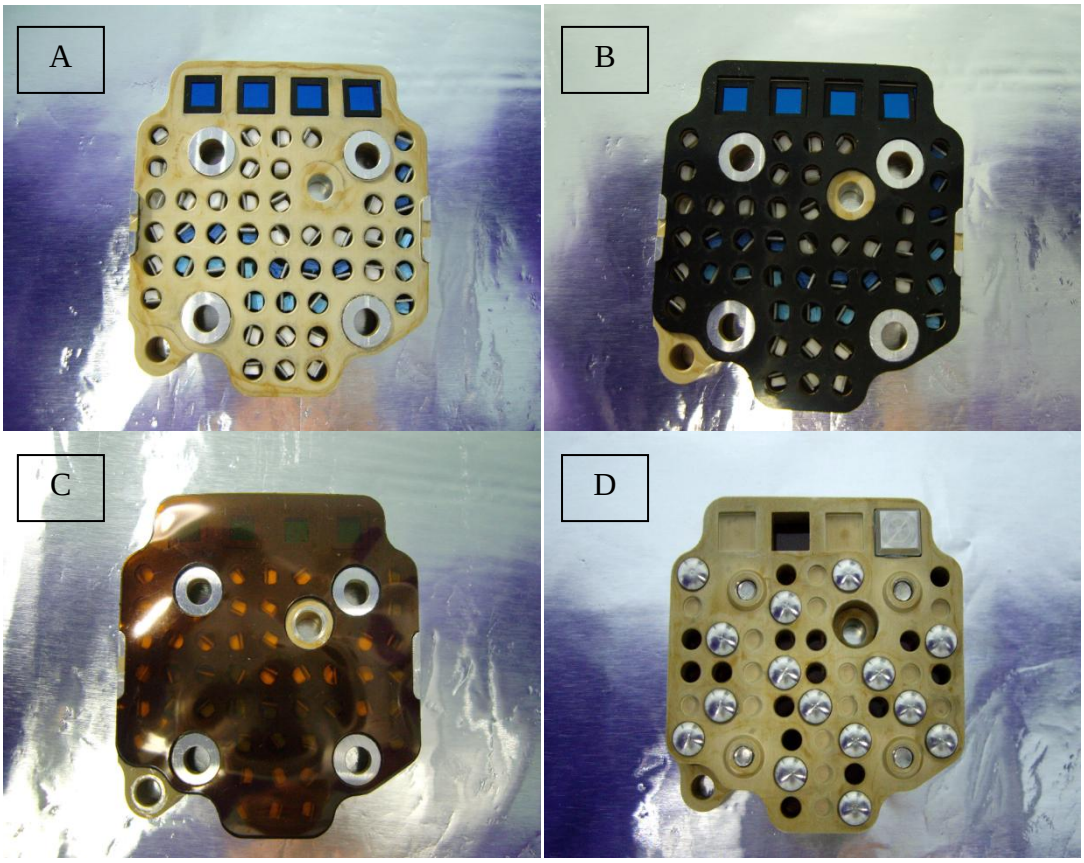


Figure 3-6: FM of the LMC on BIOPAN-6 experiment (A. unit loaded with samples, B. addition of Viton gasket, C. addition of Kapton foil, D. cover plate screwed in place). Note the "blue" squares at the top of A and B are microarrays printed on silicon nitride coated chips and the dark and light blue coloured glass fibre pads contain fluorescent dyes (Both fluorescent dyes and microarrays are not considered further in this paper).

An important step in the assembly was the establishment of an argon atmosphere in all the sample-loaded wells in the unit, which was handled by assembling the sealing covers for the unit inside an argon-filled box. The progress of the assembly process was recorded in detail – both photographically and via video capture. The Shipping Control (SC) was assembled identically and both units were kept at Cranfield University prior to being transported to ESTEC in the Netherlands and finally Baikonur in Kazakhstan.

3.8 The flight of the LMC experiment on BIOPAN-6

3.8.1.1 Transport from Cranfield to ESTEC

After assembly, the FM and SC of the sample holder unit were hand-carried via ground transport (although subjected to security X-ray scanning due to use of the Eurostar / Channel Tunnel) from Cranfield University to ESTEC in the Netherlands, where all the BIOPAN-6 experiments were integrated on the exposure platform (5^{th} to 8^{th} September 2007). The integration laboratory was a class 100,000 cleanroom and the room temperature was kept constant at +19ºC. Once the integration was completed, BIOPAN was stored in the TTC (Thermal Transport Container) at +13ºC between the 8^{th} and 10^{th} of September 2007, before being transported to Kazakhstan (Schulte and Faulstilch, 2008).

3.8.1.2 Transport from ESTEC to Kazakhstan

On the 11^{th} of September 2007, after 20 hours of transport, BIOPAN arrived at the Baikonur Cosmodrome in Kazakhstan, where it would be mounted on the descent module of the FOTON capsule. BIOPAN was integrated onto the FOTON capsule under ambient conditions (T inside BIOPAN after mounting and payload testing = +26 to +29°C) (Figure 3-7).

Figure 3-7: The FOTON-M3 capsule prior to mounting on the Soyuz rocket at Baikonur Cosmodrome (September 2007) (BIOPAN-6 in the red circle)

3.8.1.3 Launch of FOTON-M3

The FOTON-M3 mission was launched from the Baikonur Cosmodrome on the 14th of September 2007 at 13:00 (CEST) (Figure 3-8), with the lid opening in orbit approximately 3 hours later via a pre-programmed automatic mode (all mission timeline data from the BIOPAN-6 Mission Report, Kayser-Threde 2008).

Figure 3-8: Launch of the Soyuz rocket carrying the FOTON-M3 capsule with BIOPAN-6 (14th September 2007)

3.8.1.4 Orbit Phase

In total, BIOPAN was in LEO for 11 days, 20 hours and 25 minutes (14th to 26th September 2007). During orbital flight, the lid of BIOPAN remained open for 9 days, 23 hours and 44 minutes, before the lid was closed by telecommand on 24th September 2007. For the duration of the flight, FOTON completed a full Earth orbit every 90 minutes, at an altitude of 300 km (≈ 190 miles) from the Earth's surface.

3.8.1.5 Re-entry and landing

On 26th September 2007, the re-entry capsule with BIOPAN-6 landed 150 km south of Kostanay in Kazakhstan (Figure 3-9). Shortly before landing, the FOTON capsule was separated from the service module, resulting to a power cut-off of BIOPAN's external supply until FOTON landed and BIOPAN was retrieved and connected to the constant temperature TTC.

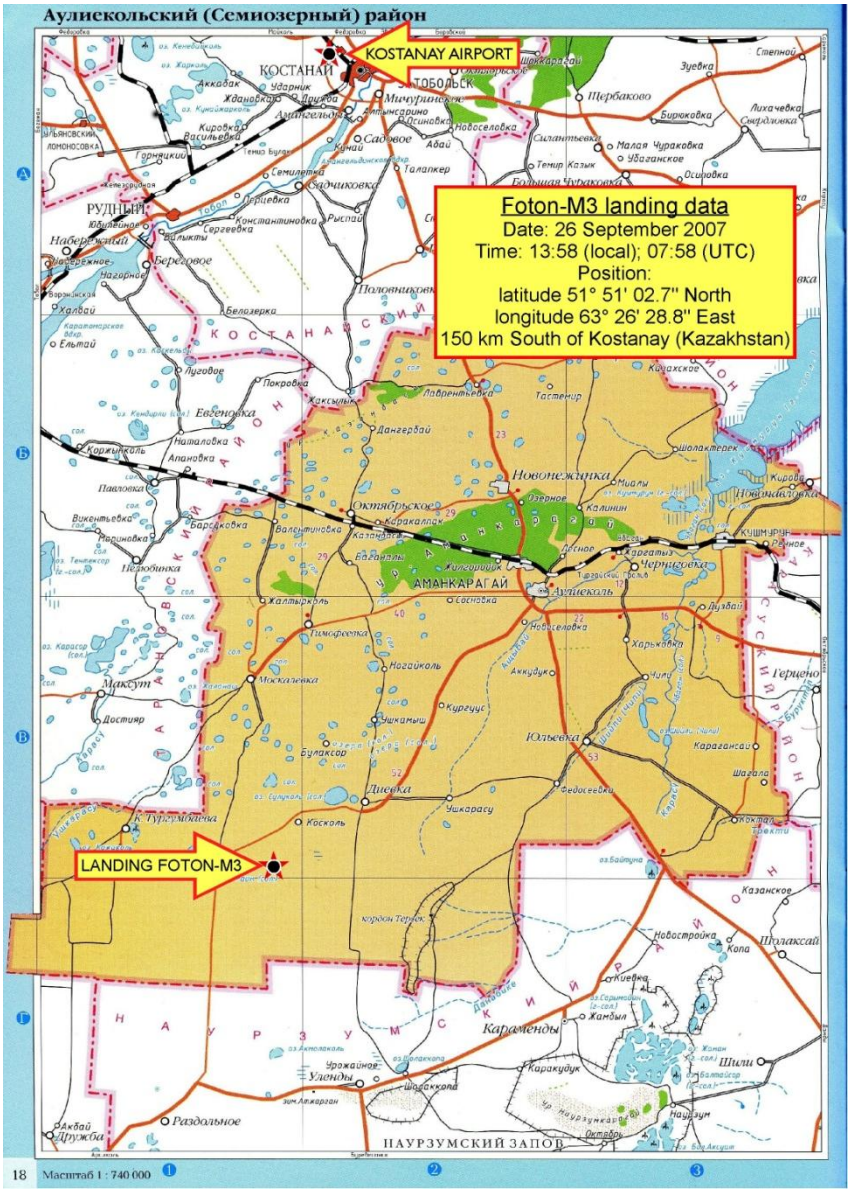


Figure 3-9: Landing site of the FOTON capsule in Kazakhstan

3.8.1.6 Platform recovery and transport to ESTEC

Shortly after landing, the re-entry capsule was retrieved and BIOPAN-6 was disconnected from it and connected to the TTC (T = +12 to +13°C). Figure 3-10 shows the re-entry capsule at the landing site with BIOPAN-6 (red circle).

Figure 3-10: FOTON re-entry capsule at the landing site (BIOPAN-6 can be seen in the red circle, bottom centre)

After retrieval, BIOPAN-6 was transported back to ESTEC in the Netherlands, where, between the 1st and 2nd of October 2007 the unit was disconnected from the TTC, the data from the various sensors on board were downloaded and the experiments were removed from BIOPAN-6.

3.9 Sample recovery from the LMC experiment on BIOPAN-6

3.9.1.1 Transport ESTEC-Cranfield

After disassembly from BIOPAN-6, the LMC experiment was collected from ESTEC and hand-carried back to Cranfield University in the same manner it had been transported to the Netherlands. The SC unit was also collected and transported to Cranfield University, after having travelled to the Baikonur Cosmodrome and remained there for the duration of BIOPAN's orbital flight.

3.9.1.2 Opening of the FM and SC units and sample recovery at Cranfield University

After arrival at Cranfield University, the FM and SC sample holder units were opened in a class 100,000 clean-room and the dosimeters and samples inside were recovered and stored until analysis. The dosimeters were removed first, in the dark, to prevent any illumination-induced alteration of the radiation data recorded (Figure 3-11A), followed by the antibody pads and microarrays (Figure 3-11B). Each pad and dosimeter was placed in a separate labelled container until their subsequent analysis.

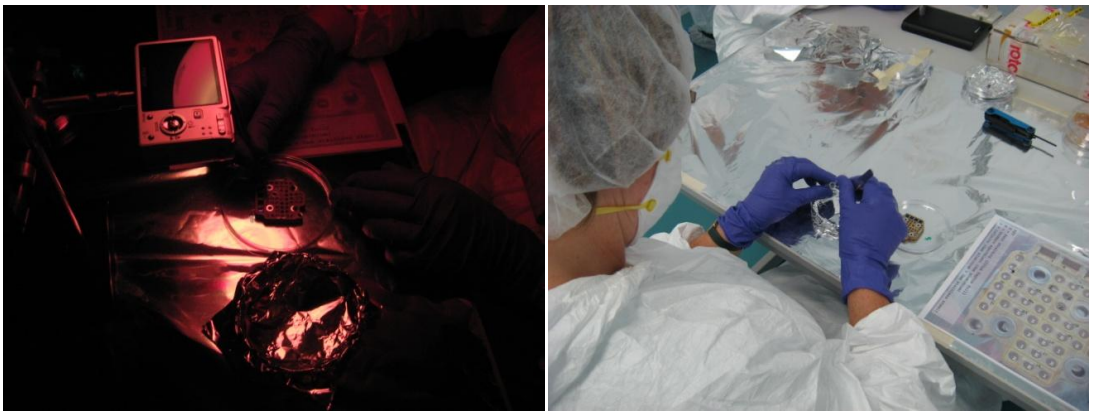


Figure 3-11: A. removal of dosimeters from FM and B. removal of glass-fibre pads from FM

The glass fibre pads, after being removed from the sample holder units, were stored in separate labelled Eppendorf tubes, in argon atmosphere, in an attempt to maintain a stable environment, similar to that of the FM and SC wells.

3.10 Analysis of samples from the LMC experiment on BIOPAN-6

3.10.1.1 Visual inspection

The first step in the data recording from the LMC experiment on BIOPAN-6 was the visual inspection of the sample holder units during their disassembly and sample recovery. The assembly process, with the loading of samples and the sealing of the units, had been recorded in detail, allowing a direct before/after comparison of the visual condition of the hardware and samples.

3.10.1.2 Sample Rehydration and ELISA

The antibodies in the glass fibre pads were rehydrated using a protocol which was developed during the ground-based radiation studies; initially, each was placed inside a 10 µl plastic pipette tip. This step prevented the pad coming in contact with any surface that could retain the newly-rehydrated antibodies and provided a stable position for the pad during the rehydration process. The 10 µl tips were then put inside 1.5 ml Eppendorf tubes. This facilitated the centrifugation step that followed and provided a means to collect the rehydrated material. The rehydration protocol involved placing 6 µl of phosphate buffered saline (PBS) (Sigma Aldrich – cat. no. P44I7) on the surface of each pad and allowing this to wick into each pad, followed by a centrifugation step using an MSE MicroCentaur centrifuge from DJB Labcare to flush out and recover the buffer. The procedure consisted of ten centrifuge runs of approximately one minute each, using 6 µl of PBS in each run, yielding a ~60 µl solution with an expected antibody dilution factor of 10, assuming 100% wash-out efficiency.

These solutions were used in Enzyme Labelled Immunosorbent Assays (ELISA). The ELISAs were all binding assays, aiming to test the antibodies' ability to recognise and bind to their respective targets.

Ninety six-well polystyrene microtitre plates from Nunc (Microplate Immuno Maxisorp, cat. no. DIS-971-030J) were coated with antigen with a working concentration of 10 µg/ml and incubated for one hour at room temperature. The antigen was Chaperonin 60 (GroEL) (Sigma Aldrich – cat. no. C7688) for the anti-GroEL antibody and an atrazine/BSA conjugate (purchased from GBC – Aberdeen, Scotland)

for the anti-atrazine antibody. 100 µl of antigen solution were added to each well. After the coating step the plates were emptied and tap-dried and unbound sites at the walls of the wells were blocked using a 1% (or 5%) blocking buffer of skimmed milk powder (Marvel brand) in phosphate buffer saline (PBS). 200 µl of blocking buffer were added to each well and the plates were incubated for one hour at room temperature. Once the blocking was complete, the plates were emptied and tap-dried. The sample antibody solutions were then split in triplicate wells, serially diluted in accordance to each assay's working range. 100 µl of sample antibody solution were added to each well and the plates were incubated for one hour at room temperature. Upon completion of this step, the plates were washed using a BW 50 plate washer from BioHit, and the washes consisted of three 200-µl 0.05% Tween 20 (Sigma Aldrich - cat. No P7949) in PBS (PBST) runs. Then the secondary antibody was added, in an appropriate dilution, at 100 µl per well and the plates were incubated for one hour at room temperature. The secondary antibody in the case of the GroEL assay was an anti-rabbit, HRP-labelled polyclonal antibody (Sigma Aldrich - cat. no. A6154) and the secondary antibody in the atrazine assay was an anti-human kappa light chain HRP labelled antibody (Sigma Aldrich - cat. no. A7164) (the recombinant single chain antibody was constructed with a human kappa light chain framework domain). Another washing step of three 200 µl PBST washes followed. The final stage of the ELISA consisted of the colour development reaction, using an HRP substrate of TMB (3,3′,5,5′-Tetramethylbenzidine dihydrochloride tablets – Sigma Aldrich - cat. no. T3405) in phosphate-citrate buffer (Sigma Aldrich – cat. no. P4922). 100 µl of this solution were added to all wells and colour was allowed to develop over 5-7 minutes (according to colour intensity). The reaction was stopped using 50 µl of 1M sulphuric acid (Fisher Scientific - cat. no. J/8420/17), and the optical density of the samples was measured at a wavelength of 450 nm, using a VarioSkan Flash reader from ThermoScientific.

3.10.1.3 Dosimeter Readout

All dosimeters from the LMC on BIOPAN experiment were removed from the sample wells in the sample holder units and stored in individual Eppendorf tubes wrapped in

aluminium foil to avoid accidental exposure to light and data alteration. They were then shipped to the Oklahoma State University for their controlled illumination readout.

Results

3.11 General mission sensor data

The various sensors on board BIOPAN stored data throughout the mission, which were collected after the unit's retrieval. A set of data collected by these sensors are shown in Table 3-3, reflecting the environmental conditions in which the LMC on BIOPAN-6 experiment with all the antibody samples was exposed to (Schulte and Faulstich, 2008). The temperature measurements for the experiments at the top lid and the bottom of the BIOPAN platform were considerably different; the temperature values presented in Table 3-3 correspond to the experiments at the bottom part of BIOPAN, where LMC was situated.

Table 3-3: General mission sensor data for BIOPAN-6 (data taken from the post-flight review by Schulte and Faulstich, 2008)

Accumulated dose of Solar Radiation (Radiometer)	**24 SCh**
Accumulated dose of Solar Radiation (UV-B Sensor)	36 SCh
Accumulated dose of Solar Radiation (UV-C Sensor)	29 SCh
Average Temperature during orbit (for bottom experiments where LMC was placed) – note that lid experiments saw av. temp of -2°C	+ 15°C
Temperature change between sunlight and darkness (for bottom experiments where LMC was placed)	± 2.5°C
Temperature change during the entire orbital flight (for bottom experiments where LMC was placed)	± 12.5°C
Range of Temperature change during orbital flight (for bottom experiments where LMC was placed)	+ 25°C
Minimum / maximum Temperatures (for bottom experiments where LMC was placed)	+ 10°C / +35 °C
Pressure during orbital flight and after landing/recovery	< 3 mbar

As far as the shipping controls for the LMC samples were concerned, temperature data from the 12th of September to the 2nd of October of 2007 show that the maximum temperature the samples were exposed to was approximately +30°C (for a very brief period of time, during transport to Samara), while the minimum temperature recorded was approximately +5°C (once again very briefly, during landing in Rotterdam).

3.12 Visual Inspection

The pictures taken during the assembly and disassembly of the LMC on BIOPAN hardware lead to a series of observations on the physical condition of the experiment before and after the BIOPAN mission. The first thing that was noted during disassembly was a slight colour alteration of the Kapton foil used in the FM compared to that used in

the SC (Figure 3-12) and where there is a difference in colour for the FM foil where unshielded and shielded by the metal cover.

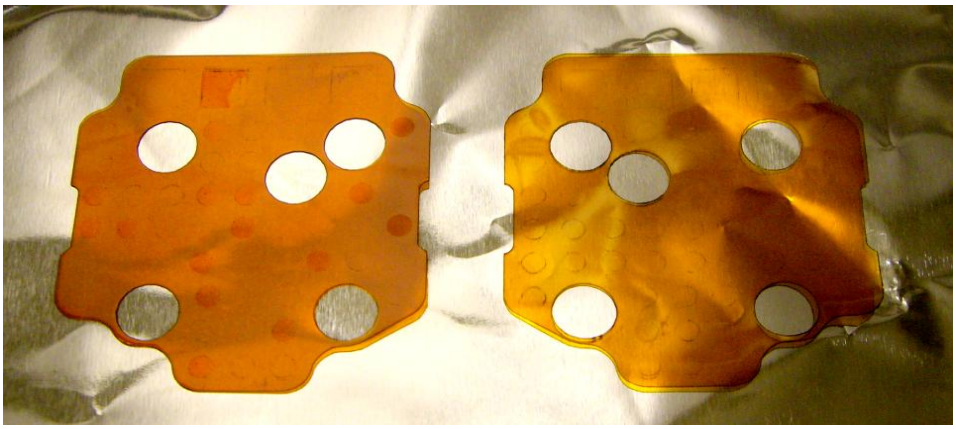


Figure 3-12: Comparison of Kapton foil used in the FM (left) and SC (right) unit. The FM component appeared darker than the SC one in areas unprotected by the metal cover (round spots)

Another physical observation was related to the position of the samples inside the unit wells; some pads and dosimeters had noticeably moved from their initial positions inside the wells (Figure 3-13).

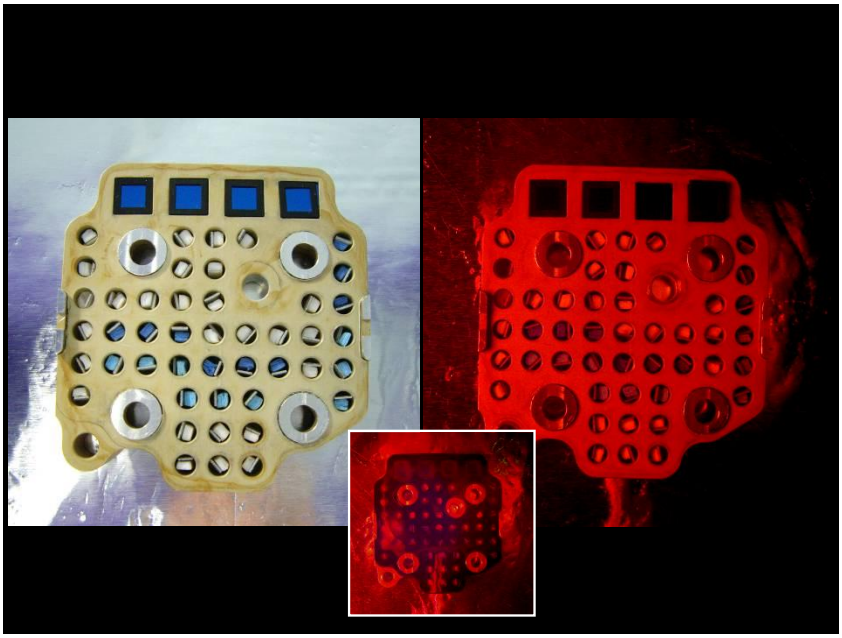


Figure 3-13: Movement of samples inside wells in the FM sample holder unit before (left) and after (right) the BIOPAN mission - insert picture shows the FM unit after the mission, prior to removal of the Viton gasket

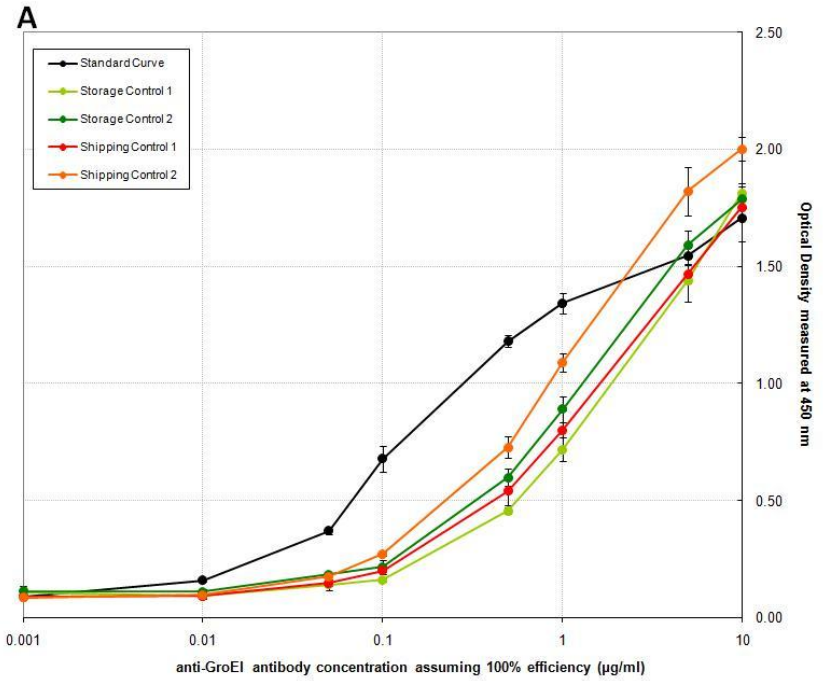
The visual inspection revealed no alterations or visible damage to the antibody sample pads/dosimeters/microarrays inside the units.

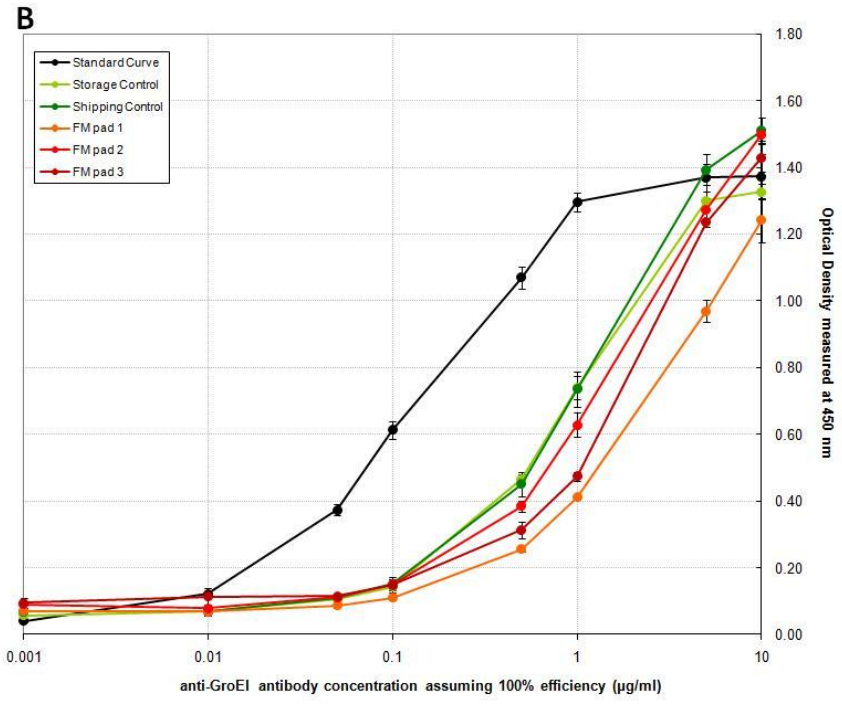
3.13 ELISA results for the anti-GroEL and anti-atrazine antibodies

ELISA provided the most definitive set of data concerning antibody performance after the BIOPAN mission. All the ELISA that were run on the LMC on BIOPAN samples were binding assays, aiming to test the antibodies' ability to recognise and bind to their respective targets. The assays were run in parallel for the samples from the FM unit, the SC unit and the set of storage controls which had remained in Cranfield University for the duration of the mission.

3.13.1.1 Anti-GroEL Antibody

The ELISA results for the anti-GroEL antibody can be seen in Figure 3-14. Figure 3-14A shows the comparison in the binding performance of anti-GroEL antibody storage and shipping control samples against a standard curve which was produced using an anti-GroEL antibody stock solution (not lyophilised). Figure 3-14B-D show the binding performance of the anti-GroEL antibody (compared to storage and shipping controls, the latter corresponding to the same shielding level as the FM samples) from the FM unit for the three levels of shielding.


A
Standard Curve
Storage Control 1
Storage Control 2
Shipping Control 1
Shipping Control 2
2.50
2.00
1.50
1.00
0.50
0.00
Optical Density measured at 450 nm
0.001
0.01
0.1
1
10
anti-GroEl antibody concentration assuming 100% efficiency (µg/ml)


B
Standard Curve
Storage Control
Shipping Control
FM pad 1
FM pad 2
FM pad 3
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Optical Density measured at 450 nm
0.001
0.01
0.1
1
10
anti-GroEl antibody concentration assuming 100% efficiency (µg/ml)

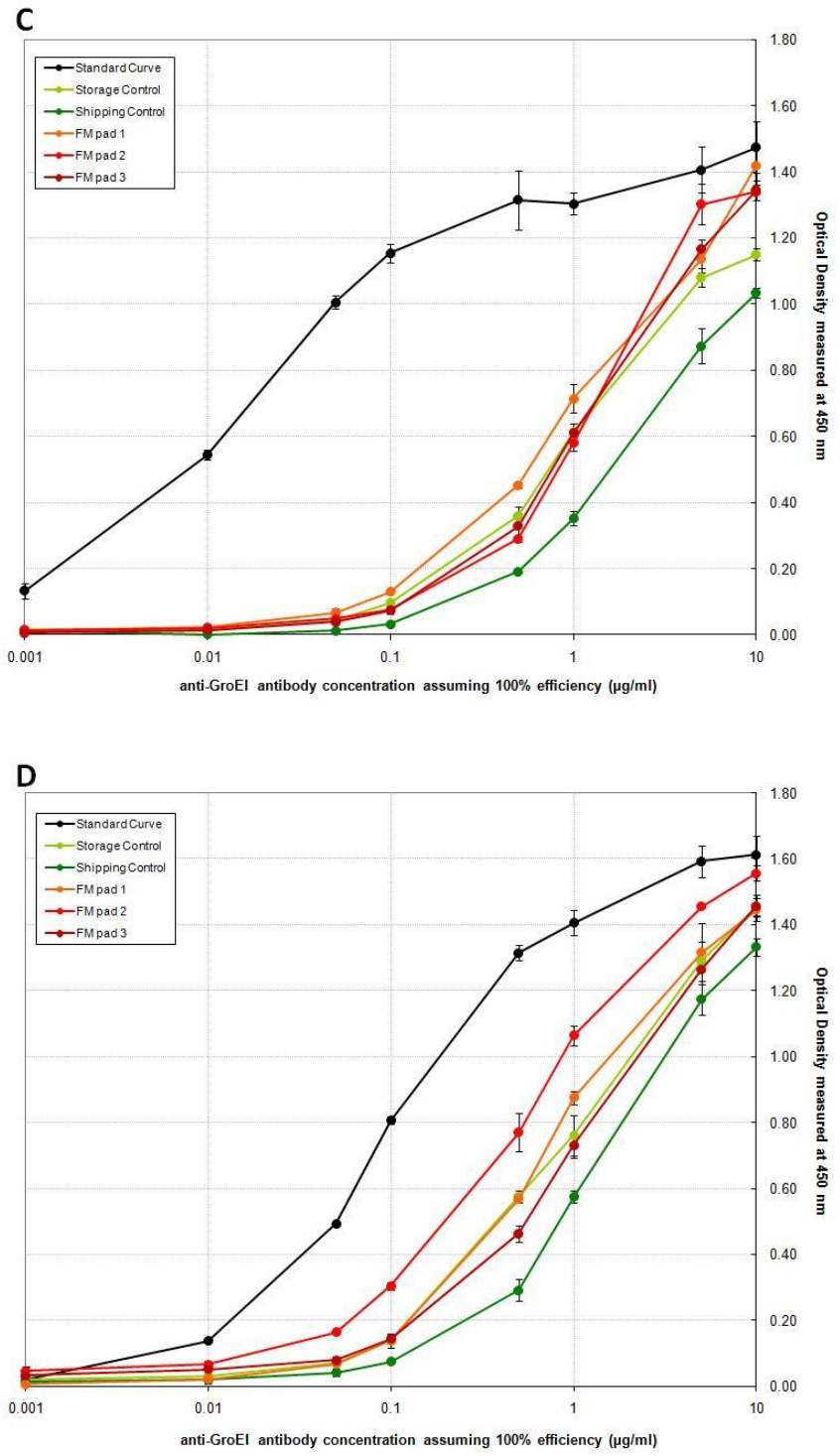


Figure 3-14: Binding performance of the anti-GroEL antibody samples in the LMC on BIOPAN-6 experiment – "efficiency" refers to pad wash-out efficiency (A. comparison of shipping and storage controls, B. "Infinite" shielding samples, C. "Zero" shielding samples, D. "ExoMars" shielding samples). Error bars reflect the standard deviation among the three replicates of each type of pad that were analysed.

From the figures above it is apparent that, despite a considerable loss of activity compared to the standard curves, the FM samples behave very similarly to the control samples.

In order to simplify the visualisation of the effect of the three different shielding levels, the ELISA signals of the samples from all three pads of each shielding level were averaged and drawn against each other in Figure 3-15.

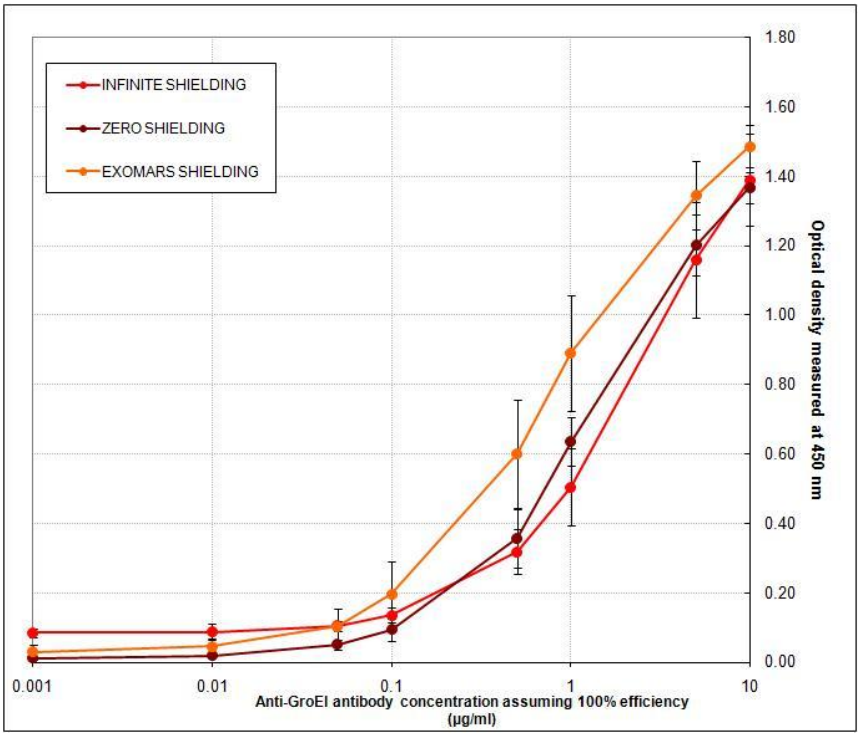
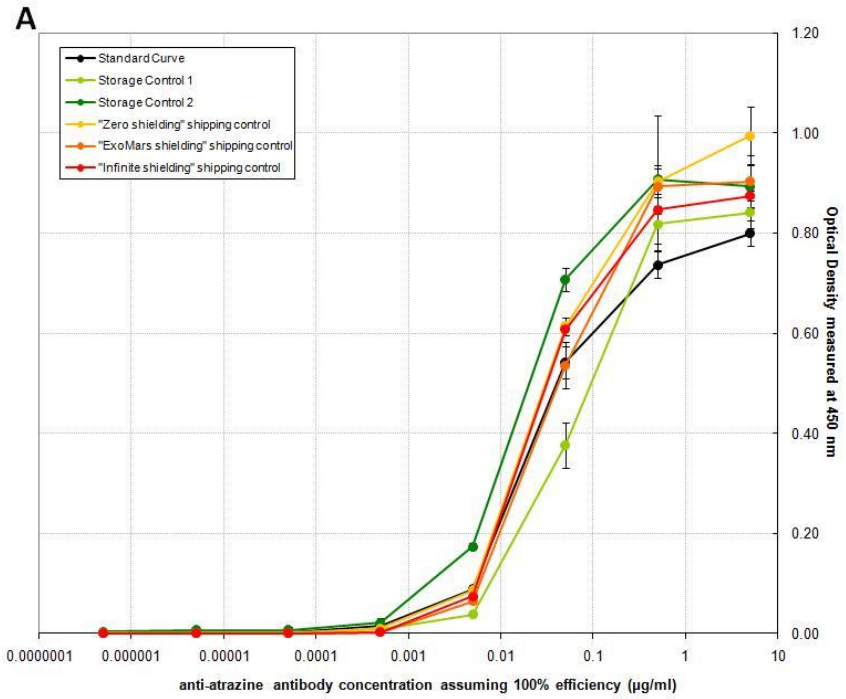


Figure 3-15: Binding performance comparison for the three levels of shielding in the LMC on BIOPAN-6 experiment. Error bars reflect the standard deviation among the three replicates of each type of pad that were analysed

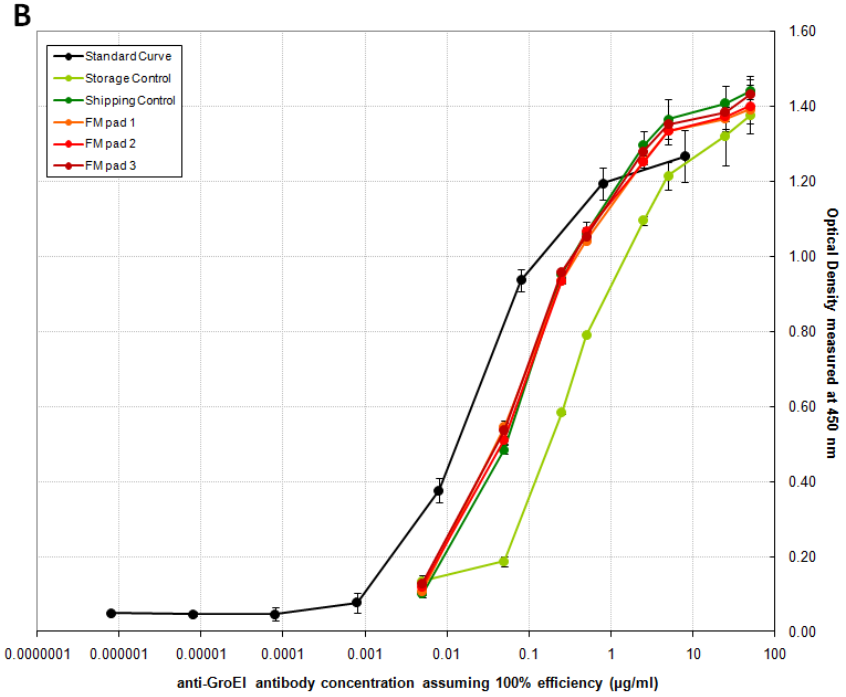
From Figure 3-15 it is apparent that the different levels of shielding of samples have no significant effect on their subsequent binding performance, and therefore the "ExoMars" level of shielding appears not to have a detrimental effect on the activity of the anti-GroEL antibody.

3.13.1.2 Anti-atrazine antibody

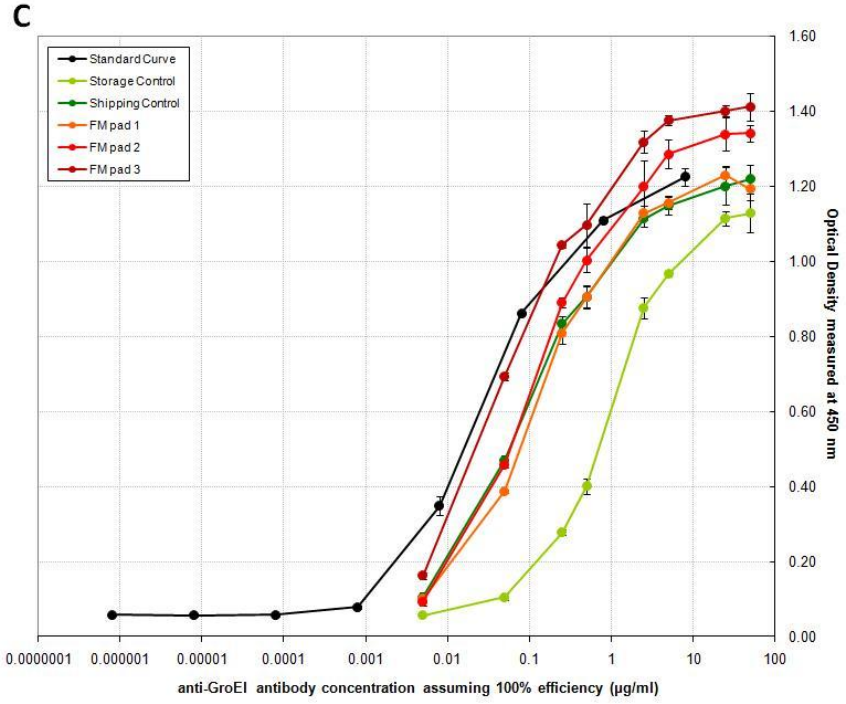
Similarly to the anti-GroEL antibody results, the ELISA signals for the anti-atrazine samples from the FM unit can be seen in Figure 3-16A-D. Figure 3-16A provides a comparison of control samples (storage and shipping), and once more, all curves are drawn against standard curves from stock antibody solutions. Figure 3-16B-D show the binding performance of the anti-atrazine antibody samples for the three different levels of shielding, compared to a standard curve and storage and shipping controls (the latter once again corresponding to the same level of shielding).



B


Standard Curve
Storage Control
Shipping Control
FM pad 1
FM pad 2
FM pad 3
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Optical Density measured at 450 nm
0.0000001
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
100
anti-GroEl antibody concentration assuming 100% efficiency (µg/ml)

C


Standard Curve
Storage Control
Shipping Control
FM pad 1
FM pad 2
FM pad 3
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Optical Density measured at 450 nm
0.0000001
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
100
anti-GroEl antibody concentration assuming 100% efficiency (µg/ml)

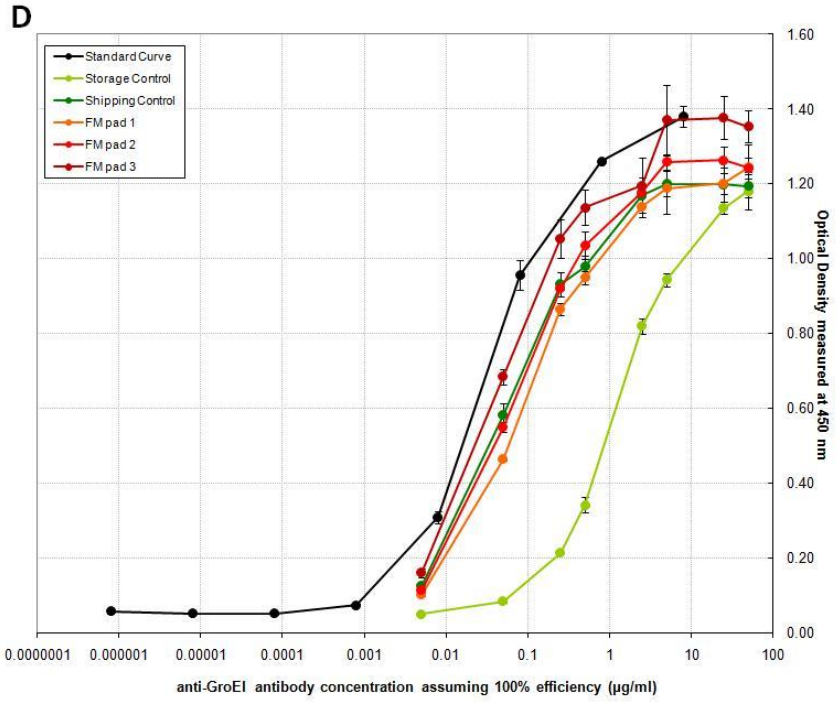


Figure 3-16: Binding performance of the anti-atrazine antibody samples in the LMC on BIOPAN-6 experiment – "efficiency" refers to pad wash-out efficiency (A. comparison of shipping and storage controls, B. "Infinite" shielding samples, C. "Zero" shielding samples, D. "ExoMars" shielding samples). Error bars reflect the standard deviation among the three replicates of each type of pad that were analysed.

From Figure 3-16B-D, it is obvious that the storage controls for the anti-atrazine antibody showed reduced activity compared to SC and FM pads.

Similarly to the anti-GroEL samples, the visualisation of the effect of the three different shielding levels was made possible by averaging the ELISA signals of the samples from all three pads of each shielding level and plotting them against each other in Figure 3-17.

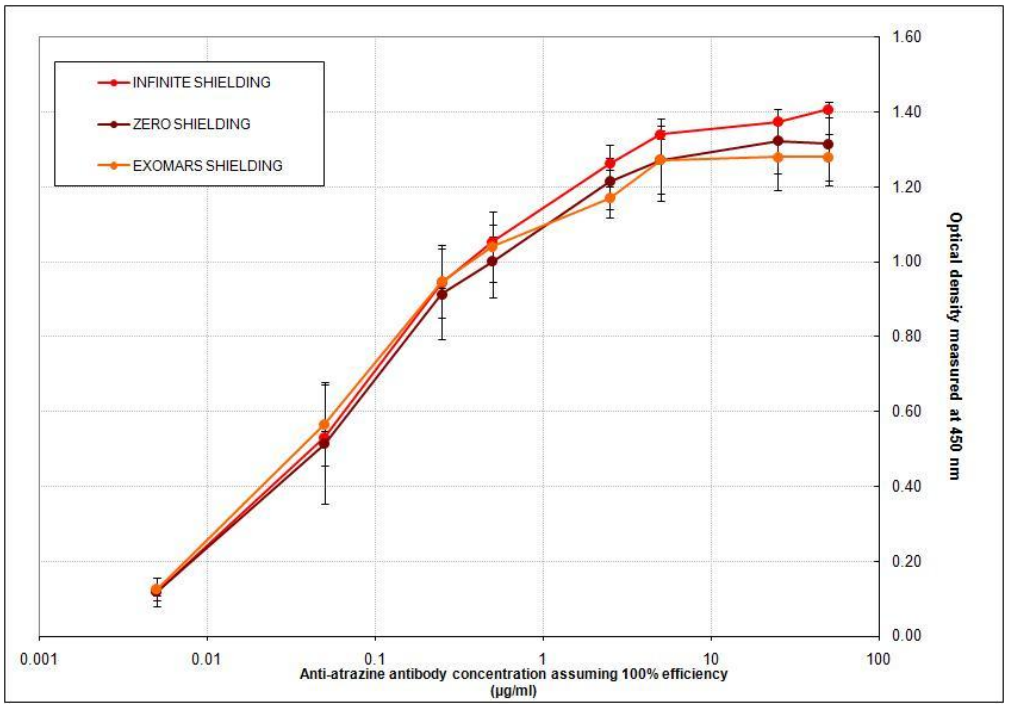


Figure 3-17: Binding performance comparison for the three levels of shielding in the LMC on BIOPAN-6 experiment. Error bars reflect the standard deviation among the three replicates of each type of pad that were analysed

From Figure 3-17 it became obvious that in the case of the anti-atrazine antibody, the level of shielding of the sample pads had practically no effect on the subsequent activity of the antibody in binding ELISA.

3.13.1.3 Data analysis

In order to quantify the changes in the antibodies' binding activity after recovery from the BIOPAN/FOTON mission,

Table 3-4 shows the percentage of activity retention in all samples for both antibodies. The remaining activity percentage was calculated by using the assay mid-points (subtracting an assay's mid-point value from the mid-point value of the standard curve).

Table 3-4: Binding activity retention (%) for the LMC on BIOPAN-6 antibodies

	Anti - Atrazine Antibody		Anti - GroEL Antibody	
	Assay mid-point	**Retained Antibody Activity (%)**	**Assay mid-point**	**Retained Antibody Activity (%)**
Standard curve	0.029	100%	0.079	100%
Storage control	0.835	4%	1.116	7%
Shipping control "Zero" shielding	0.118	25%	2.718	3%
Shipping control "Infinite" shielding	0.133	22%	1.538	5%
Shipping control "ExoMars" shielding	0.068	43%	1.496	5%
Flight model "Zero" shielding	0.117	25%	1.598	5%
Flight model "Infinite" shielding	0.123	24%	3.000	3%
Flight model "ExoMars" shielding	0.083	35%	0.843	9%

3.14 Dosimetry results

The OSL dosimeters from the LMC on BIOPAN experiment were shipped to the Physics Department of the Oklahoma State University in order to read out via controlled illumination the radiation data they had recorded. This type of dosimeters can be reset

by light, making it crucial to keep them in the dark at all times until analysis. Table 3-5 shows the radiation data collected by the dosimeters. The data from dosimeters of the same shielding level and sample unit have been averaged to facilitate data presentation.

Table 3-5: Accumulated radiation dose recorded by the dosimeters from the LMC on BIOPAN-6 experiment

Shielding Level/Sample Type	Accumulated radiation dose (mGy)[1]
"Zero" shielding / SC dosimeters	0.01 ± 0.1
"Infinite" shielding / SC dosimeters	0.05 ± 0.1
"ExoMars" shielding / SC dosimeters	0.13 ± 0.1
"Zero" shielding / FM dosimeters	2.2 ± 0.1
"Infinite" shielding / FM dosimeters	2.4 ± 0.1
"ExoMars" shielding / FM dosimeters	2.4 ± 0.1

[1]values are presented in each case accompanied by the standard deviation (n=2)

As the dosimeters were all hand-cut, slight variations in size and mass were inevitable. This variation was taken into account during the readout, and normalisation was necessary, in the form of weighing each dosimeter and normalizing results based upon dosimeter mass.

From Table 3-5, it is obvious that the radiation dose recorded for dosimeters coming from the same sample holder unit is very similar. All FM dosimeters appear to have recorded roughly twenty times the dose recorded by the SC dosimeters, and one interesting observation is that for the FM dosimeters, the dose from the "Zero" shielding dosimeters is almost identical to that from the "ExoMars" and "Infinite" shielding ones.

Discussion

3.15 Missions in LEO as planetary exploration mission analogues

Missions in LEO do not provide entirely accurate representations of an actual planetary exploration mission; some environmental conditions in LEO (temperature extremes, high vacuum) are very similar to what such a mission would encounter, while others, like the radiation environment are not direct analogues. LEO radiation is a mixture of trapped particles (electrons, heavy ions, protons etc.) of various fluxes and individual energies, that is mainly isotropic; this is not expected to be the case during the ExoMars mission.

The radiation environment for the ExoMars mission, which was modelled prior to the ground-based radiation studies (see Chapter 2), was simplified to be composed of Solar Particle Event (SPE)-related protons en route to Mars and back-scattered neutron radiation on the surface of the planet. This facilitated simulating it via a cyclotron and studying the individual and combined effect of these particles on components of the LMC. The radiation environment in LEO is more complex in the context of its effect on antibody performance. However, exposure of experiments to the environment in LEO is practically the most effective way to test response to a naturally-occurring radiation environment, free from the limitations posed by ground-based simulations.

Mission duration is also an important factor to consider, especially in terms of radiation dose deposition on the LMC components. Based on the current configuration of the ExoMars mission (circa 2010), the transit phase is expected to last nine months, followed by a minimum of six months of surface operations. The BIOPAN-6 mission lasted 12 days in its entirety, only 10 of which corresponded to full exposure to the radiation field in LEO (while the lid was fully open). Radiation data from BIOPAN-5 gave an overall radiation dose of 2.4 krad, which corresponds to roughly 1/3 of the total radiation dose expected for ExoMars (6.6 krad – calculation based on earlier estimations of a 2-year transit phase), while BIOPAN-6 “saw” much lower levels of radiation at 0.4 krad, primarily due to the low solar activity during the flight compared to previous BIOPAN flights. Therefore, despite being much shorter than an actual mission to Mars, the BIOPAN missions manage to provide antibody performance data based on

significant fractions of the radiation levels envisaged for ExoMars (1/3 ExoMars level on BIOPAN-5 and 1/16 ExoMars level on BIOPAN-6).

Where missions in LEO prove faithful to planetary exploration missions are the "physical" conditions of a space mission. The setting during payload transportation to the launch site and the exposure to all aspects of launch (and re-entry, for future sample/instrument recovery) are expected to be very similar, and offer valuable information on the potential -or lack of- of using certain materials in such missions.

Concerning the "physical" results – i.e. the visual observations of the condition of the LMC components upon sample recovery, it was observed that the movement of the pads inside the sample holder wells was minimal (Figure 3-13), as most of the pads did not appear to move whilst others did but without any obvious damage; this suggests that the disturbance of the pads during the mission was minimal. The harsh vibration and shock conditions during launch and re-entry therefore did not appear to cause any damage to the pads, rendering them an appropriate platform for antibody packaging.

As far as the protective Kapton foil was concerned, mild colour alterations were observed in areas which were not protected by the metal cover (Figure 3-12). Given the patterns seen on the flight foil, the colouration change is most likely due to the effects of the solar UV radiation that the Kapton foil saw through the zero shielding holes.

3.16 Choice of antibody integration method

The antibodies were used in the LMC on BIOPAN-6 experiment in a lyophilised format. This decision was based on literature data about the increase in shelf life and stability of antibodies when freeze-dried, compared to molecules in solution and was in accordance with the general strategy for antibody integration to the LMC instrument. Lyophilised samples were expected to resist the environmental conditions during transportation and storage before and after the BIOPAN-6 mission. Lyophilisation was also expected to increase resistance to inactivation due to radiation exposure in LEO, by eliminating, for instance the presence of radiolytic products due to the presence of water in a sample. The importance of an optimised procedure for lyophilisation, taking into

account the physical and chemical degradations that samples are exposed to during and after freeze-drying, became obvious from the ground-based experiments.

As far as the sample packaging format was concerned, the antibodies were lyophilised into glass fibre pads in accordance to the envisaged final design format in the LMC instrument and in order to facilitate handling and integration to the sample holder unit and subsequently the BIOPAN-6 platform.

It is noted that the flight of BIOPAN-6 took place prior to the majority of the results obtained from the ground-based irradiations (Chapter 2) and therefore no specific lyophilisation matrix was used, whereas in latter studies for the ground-based testing, sophisticated lyophilisation matrices were explored, as other work within the LMC team indicated that additional lyophilisation matrix features required study.

3.17 The effects of ground handling

After all the LMC on BIOPAN-6 samples had been prepared and loaded into the sample holder units, they left the controlled laboratory environment and were exposed to various environmental conditions and outside factors that could have an effect on the quality of their performance during the final ELISA analysis. Transportation from one place to another meant that the samples inside the FM and SC unit were not only physically handled and shaken (this could be the reason for the sample movement inside the wells that was observed during the visual inspection after recovery, an assumption which is further encouraged by inspection of the pads in the SC unit, which appear to have moved no less than the FM pads), but also subjected to X-ray scanning at airports. The latter is expected to have a negligible effect on samples as the doses are extremely low due to health and safety concerns [data from Aviation Online mention doses <1 mSv (Mitchell, 2010)].

After integration in the BIOPAN platform, the journey of the LMC samples was no longer directly monitored by our group, but the transportation conditions were documented in detail by the mission personnel and the values recorded for all the crucial factors (primarily the sample temperature) were not considered harmful to antibody

activity. The same principles applied to the transportation back to Cranfield University after the end of the BIOPAN-6 mission.

The consequent sample analysis via ELISA showed no significant difference in the binding activity of the FM and SC samples, compared to storage controls (this is clearly illustrated in Figure 3-14A and Figure 3-16A, with the comparison between storage controls and shipping controls from the SC unit), leading to the conclusion that the ground handling did not have a damaging effect on the LMC antibodies.

3.18 Sample rehydration

The rehydration of antibodies in order to assess the retention of binding activity after recovery from the BIOPAN mission was a process that was optimised during the ground-based radiation studies (the preparation of the BIOPAN samples took place before the majority of the ground-based studies while the analysis of the samples took place after the ground-based experiments, taking advantage of the optimised handling and analysis protocols that were developed during the latter). The importance of an efficient method for sample recovery lies in differentiating the effects of sample preparation/handling from the effects on performance due to exposure to the conditions of a space mission.

3.19 Effect of LEO on antibody performance

Overall, the two LMC-representative antibodies that were used in the LMC on BIOPAN-6 experiment were observed to maintain their binding functionality after exposure to space radiation and the physical conditions of the FOTON mission.

The antibody against atrazine appeared to retain higher levels of activity compared to the antibody against GroEL (Table 3-4).

Assuming that the binding activity of stock solutions of both antibodies corresponds to 100%, the anti-atrazine antibody showed almost 50% of activity retention after lyophilisation and ground handling (SC samples) and around 40% activity retention after exposure to the previous conditions plus space radiation (FM samples), compared

to the less than 10% activity retention for SC and FM samples of the anti-GroEL antibody.

The results obtained for the anti-atrazine antibody show a very close similarity between the SC and FM samples, as far as activity levels are concerned (Figure 3-16 and Table 3-4). This implies that the orbital flight phase of the mission, including shock loadings during launch and landing, did not have a damaging effect on the antibody. Activity loss compared to the standard assay (carried out using stock solutions) seems to be due to the lyophilisation and/or sample rehydration process. The effect of ground handling, which was not expected to have been harmful, could be assessed by comparing the shipping control (SC) samples to the storage controls. Unfortunately, the ELISA results for the storage controls revealed very low activity retention compared to the SC and FM samples (Figure 3-16B-D and Table 3-4), an issue that could be attributed to errors during the preparation of this particular batch of sample pads, handling errors (pad wash-out) or, in a less likely scenario, alterations to the storage control pads that occurred between their production and their analysis.

The effect of the three different shielding combinations can be observed in Figure 3-17. It is clear that, taking into account the error margins, the different shielding levels have no observable effect on antibody functionality, which leads to the conclusion that the degree of shielding provided by the ExoMars rover is sufficient from protection against inactivation, at least inactivation due to LEO levels of radiation. In conclusion, the anti-atrazine antibody was able to survive complete inactivation after participation in a space mission in LEO with exposure to 0.4krad of radiation and is considered a suitable candidate molecule for the LMC instrument. Note that an inhibition immunoassay for atrazine within the LMC context is considered a positive control assay, as atrazine, being a manmade molecule, is unlikely to be found on Mars.

As far as the anti-GroEL antibody is concerned (Figure 3-14A-D), the results from the storage controls seem to agree with the SC and FM samples, in terms of the effect of lyophilisation and rehydration (Table 3-4). The freeze-drying process alone appears to significantly affect the subsequent performance of the antibody, stripping it of around 90% of its binding activity. Lyophilisation aside, the ground handling seems to have no considerable effect on antibody activity, after comparison of the storage controls with

the SC samples (Figure 3-14A). Comparing the values obtained for the FM samples and the SC samples, the effect of the mission itself and the space radiation in LEO can be assessed (Table 3-4). The performance of both samples sets is similar, implying that this effect is not destructive to the anti-GroEL antibody.

Figure 3-15 allows the comparison of the effect of the three shielding combinations on the anti-GroEL antibody; the observations here are very similar to what was seen in the case of the anti-atrazine antibody, with the different shielding levels leaving antibody activity essentially unaffected. Therefore, the shielding level within the ExoMars rover has proven sufficient to protect the anti-GroEL antibody against inactivation due to LEO levels of radiation. The conclusion from the analysis of the anti-GroEL samples is that this antibody is another suitable tracer for the LMC instrument.

The LMC on BIOPAN-6 experiment was viewed as an important step in de-risking the use of antibodies in a space mission context, despite being exposed to much lower levels of ionising radiation (approximately 1/16) of those envisaged for the ExoMars mission. The different response of the two antibodies to treatment (lyophilisation) and exposure to the mission parameters highlighted the need to test all potential LMC antibodies in order to assess their lyo- and radio-resistance levels, prior to use in the final (flight) LMC module. This will naturally not be possible in a LEO environment, but ground-based experiments can provide comprehensive data sets. Ground-based testing, which involves the same sample preparation steps, ground handling and exposure to some elements of a space mission (i.e. components of space radiation).

3.20 Dosimetry Results

The radiation levels recorded by the dosimeters from the FM and SC sample holder units (Table 3-5) were the most accurate values that corresponded to the radiation levels encountered by samples from the LMC on BIOPAN-6 experiment. As it was expected, the radiation values that came from dosimeters from the SC unit were very low (≈0.5 mGy), in agreement with the normal radiation exposure on the Earth's surface. The data from the FM unit were approximately 20 times higher than the SC dosimeter data. The "zero" shielding dosimeter values from the FM unit were the only abnormal ones, *i.e.* it

was expected that these would be higher values, and the most likely interpretation is that the dosimeters were reset for the zero-shielding samples due to solar light being able to pass through the Kapton foil cover, albeit at a reduced level. It is also noted that the levels seen by the dosimeters were different from those seen by the dosimeters using an alternative and more established technology elsewhere in BIOPAN, *i.e.* 400 rad (equivalent to 4000mGy) versus 2.4 mGy. This discrepancy will require further study and may be due to inappropriate calibration of the OSL dosimeters or inappropriate handling pre- or post flight that allowed partial resetting of the dosimeters. The 400 rad data provided by other experiments which were previously flown on BIOPAN is therefore taken as the more reliable value.

3.21 Future Work

Presently, the European Space Agency has expressed no intention to go forward with another BIOPAN mission in the immediate future. Nevertheless, there is a need to study the performance of LMC-related immunoassay (and potentially hardware) components after exposure to space radiation. This issue will be resolved, at least to some extent, with more ground-based testing. Additional rounds of irradiations are currently being planned, following the development of more assays of interest to the LMC. Ground-based experiments, despite their limitations, allow studies with controlled levels of radiation and conclusions on the effects of combined or individual elements of space radiation.

Summary and Conclusions

The experiment that was designed in order to be part of the BIOPAN-6 platform onboard the FOTON-M3 mission lead to a number of conclusions concerning the ability of antibodies to form components of an instrument on a planetary exploration mission. Using this mission in LEO as an analogue of a mission to another planet, a variety of factors that could have a detrimental effect on antibody functionality were examined, including sample preparation, principally the lyophilisation of the antibodies into glass fibre pads, ground handling of the samples, orbital flight and sample recovery. The

analysis that took place at the end of the mission revealed that, aside from the effect that lyophilisation has on the ability of antibodies to recognise and bind to their respective targets, the effect of all physical conditions during the mission and exposure to LEO level of radiation are not destructive to these antibodies.

Within the context of the development of the LMC, it is important to note that the radiation levels measured for the Foton M3 mission were considerably lower than the radiation levels envisaged for an actual mission to Mars; the radiation environment was also different (more heterogeneous) than the radiation used in the ground-based studies (composed exclusively of proton and neutron radiation). Nevertheless, the results from the BIOPAN-6 experiment agreed with the results of the ground-based experiments in that they showed that antibodies did not undergo inactivation due to exposure to radiation, even after exposure to a broad spectrum of radiation like the one expected in LEO. Additionally, the BIOPAN-6 results showed that the antibodies used in this experiment remained functional after exposure to the combination of physical factors associated with a space mission (launch and re-entry conditions), which is another encouraging step in their use within a planetary exploration mission context.

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4 .

Development of recombinant antibodies against haptens with biosignature properties with the use of naïve and immunised phage antibody libraries

Introduction

4.1 Chapter Preamble: the LMC instrument

The Life Marker Chip (LMC) instrument is an antibody assay-based system which will attempt to detect molecular signatures of Life in the Martian subsurface as part of the payload on board the ExoMars mission rover (Sims *et al.*, 2005, Cullen and Sims, 2007). The ExoMars mission, a collaboration between the European Space Agency (ESA) and NASA, is ESA's flagship mission to Mars, currently scheduled for launch in 2018. The mission belongs to a greater framework for Mars planetary exploration that has recently been announced by ESA and NASA (Brown, 2009).

The LMC is frequently described in the general media as a "pregnancy test for Mars", a term which hails from the fact that both the LMC and commercial off-the-shelf pregnancy tests are based on lateral flow immunodiagnostic technology (Wilson and Howell, 2002). The operation of the LMC is based on the employment of dried-down fluorescently-labelled antibodies that dissolve into an aqueous-based liquid extract of a Martian regolith / crushed rock sample containing extracted organic molecules, initiating a multiplexed inhibition immunoassay before being flowed over a microarray composed of immobilised copies of the various multiplexed immunoassay targets. For a given antibody, if the corresponding target molecule is present in the liquid extract, the antibody binding site is occupied and thus inhibited from binding to the corresponding microarray spot which subsequently does not fluoresce. If the relevant target molecule is not present in the liquid extract, the antibody binding site is left vacant and therefore able to bind to the corresponding microarray spot which fluoresces.

One of the key stages in the development of the LMC is the selection of antibodies that will be used in the flight instrument. The challenge in the acquisition of these antibodies lies in the nature of the molecules or classes of molecules that are currently under examination as potential LMC targets.

4.2 LMC Target selection and challenges

The LMC will be part of the Pasteur payload on board the ExoMars rover and it will have the ability to detect up to 25 different molecular targets of different origins that are representative of ancient, preserved biomarkers of extinct life, biomarkers of extant life, those representing meteoritic input and other abiotic organic molecules as well as organic molecular markers of rover / instrument borne Earth contamination (Table 4-1, taken from Cullen and Sims, 2007).

Table 4-1: Categories of molecular biomarkers and other relevant targets for biosensor-based Life detection (taken from Cullen and Sims, 2007)

Category	Context	Typical Properties
Molecular biomarkers of ancient extinct life	Stable breakdown/preservation products of Life, typically membrane derived	Low molecular weight, apolar
Molecular biomarkers of extant/recent life	Full range of molecules from metabolites to macromolecules	Low to high molecular weight, polar
Molecular biomarkers of microbial spacecraft contamination	Species-specific targets, antigens or nucleic acid sequences	High molecular weight macromolecules
Abiotic organics	Meteoritic in-fall	Low molecular weight, polar and apolar
Other targets	Man-made synthetic molecules as positive controls Non-biological contamination	Various Various (cleaning materials, spacecraft exhaust products)

The need to select and prioritise the molecules that will represent the four major classes of LMC targets mentioned above, became clear at a very early stage of the LMC development, and a list of over sixty biomarker candidate compounds (both specific molecules and generic structures) was proposed, as a result of a 2006 international workshop focused on biomarkers for Life detection (Parnell et al., 2007). The search for, and even the understanding of what constitutes a biomarker is heavily influenced and directed by Earth biology (*i.e.* the assumption of "Earth-like" life on Mars), and this

has been the case with the list of potential LMC targets; nevertheless, an effort was made to keep it as wide-ranging as possible.

From Table 4-1 it is obvious that the LMC targets vary greatly in their physical properties and for some, if not most, of these molecules, acquiring high-affinity antibodies against them is not a simple procedure. "Off the shelf" antibodies can be obtained for molecules with commercial applications (medical, environmental, *etc*.), but the LMC-relevant molecules that also have such applications are mainly in the contamination/control categories of targets. On the other hand, in order to obtain antibodies via the traditional methods for producing polyclonal and monoclonal antibodies, the antigens that will be employed should ideally present a level of structural and compositional complexity that most LMC targets lack, especially the markers for extinct Life, that are mostly small apolar molecules (haptens).

Additionally, the antibodies that will be part of the LMC platform will be required to resist deactivation due to all factors related to a planetary exploration mission (primarily space radiation) and remain functional in the LMC solvent mixture that will be used – a combination of water, methanol and surfactant (Court *et al*., 2010) – in the extraction of organics from the regolith/crushed rock sample. Both the nature of the LMC target molecules and the harsh environment that are anticipated during the ExoMars mission suggest that the optimal option would be custom-made antibodies that would be engineered to recognise small haptens with high affinity, without being affected by the presence of unfavourable reaction conditions and phage display technology offers the potential to generate such molecules.

4.3 Phage display Technology

4.3.1.1 History and Principle

Since the first description of phage display by George Smith (Smith, 1985), the display of proteins or small peptides on filamentous phage has become one of the leading approaches in the identification of peptides with novel properties and the isolation of desired peptides from large "phage peptide libraries". The principle behind the method is that a foreign protein sequence (gene of interest) can be fused to the gene of one of

the coat proteins of filamentous phage, resulting to the creation of a fusion protein that is incorporated into the virion (the virus particle) (Parmley and Smith, 1989). The resulting virions (fusion phage) both display the foreign protein on their surface (phage display) and contain its encoding gene, providing a direct link between phenotype and genotype.

Among the many applications of phage display technology, the ability to generate high sensitivity antibodies against challenging targets is the focus of the present work. Display does not necessarily involve entire immunoglobulin molecules; antibody fragments have also proven extremely efficient (Figure 4-1). The most popular formats are the single-chain fragments composed of the antigen-binding variable domains of the IgG heavy (VH) and light (VL) chain regions connected by a flexible peptide linker (scFv), and fragments described as Fab, that consist of the variable domains plus the first part of the constant domains. Additionally, F(ab')2 fragments consist of two disulfide-connected Fab fragments. The entire scFv is encoded by a single gene, facilitating the construction of fusion proteins and subsequent expression. The term "recombinant antibodies" which is employed extensively in this chapter refers to these single-chain antibody fragments.

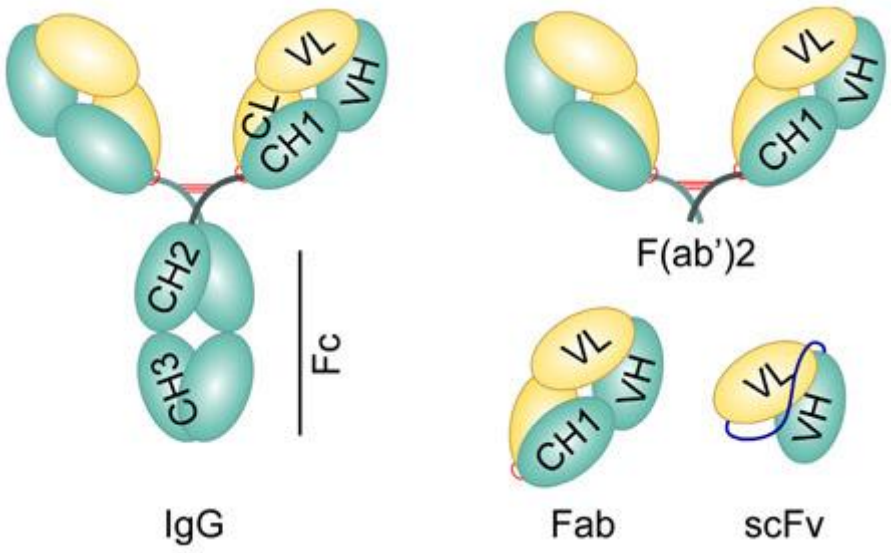

Figure 4-1: Structure of intact IgG molecule (left) and a variety of antibody fragments (right) (Image taken from Peterson et al., 2006)

4.3.1.2 Filamentous phage: characteristics and biology

Filamentous phage are a large family of bacterial viruses that infect gram-negative bacteria, but unlike most bacteriophage, their production and secretion does not involve cell lysis (destruction). The most well-studied filamentous phage are *E.coli*-infecting phage f1, fd and M13 (which was used in the present work) (Figure 4-2), often grouped as Ff phage. Phage particles have a cylindrical shape with a diameter of 6.5 nm containing the 6400-nucleotide, circular ssDNA genome. The tubular virions are composed of thousands of copies of a major coat protein (pVIII), with a few copies (3-5) of the minor coat proteins (pIII, pVI, pVII, pIX) at the two tips of the particle (Russel *et al*., 2004).

Figure 4-2: Structure of the filamentous bacteriophage M13 (Image taken from Arap, 2005)

All the Ff phage infect *E.coli* cells displaying F pili, which are the most visible feature of the F-plasmid mediated conjugation system in *E.coli* and is believed to allow stable DNA transfer between cells (Harrington and Rogerson, 1990). The amino-terminal domain of the minor protein pIII is exposed on the virion surface and binds to the F pilus (during the logarithmic phase of bacterial growth) in order to initiate the host cell infection.

Once inside the host cell, the viral DNA uses host resources (RNA and DNA polymerases, topoisomerase) in its replication and phage gene expression, which is

assisted by additional phage proteins (pII, pV and pX). All phage proteins are synthesised simultaneously, but their production rate is strictly moderated.

The distinctive feature of filamentous phage assembly is that it is a secretory process, with assembly occurring in the cytoplasmic membrane and new phage particles being secreted from the host cell as they are assembled.

Filamentous phage are considered ideal candidates for phage display for a number of reasons, including the stability of the phage particles to a wide range of selection conditions, the small size of their genome which allows insertions into non-essential regions and the facilitation of library construction by the ability to isolate both single- and double stranded DNA and the availability of phagemid vectors. Additionally, since filamentous phage are not lytic phage, high titres of phage can be accumulated from infected cells and the modification of the coat proteins does not induce functionality loss (Russel *et al*., 2004).

4.3.1.3 The minor coat protein pIII

The Ff phage genome contains nine closely packed genes (I to IX) plus one major non-coding region which contains the replication origins for the + and – DNA strand synthesis and the packaging sequence (PS). Gene III encodes the minor coat protein pIII which, as mentioned earlier, initiates host cell infection via its amino-terminal half (the carboxyl-terminal region remains buried in the virion and plays a part in morphogenesis). The foreign sequence can be inserted between the amino- and carboxyl-end of pIII without disrupting pIII's functionality (Smith, 1985). The protein pIII, which is present in five copies on one end of the phage particle, is the protein of choice for most phage display fusions; this is due to its tolerance for large insertions, its compatibility with monovalent display (see section 4.3.1.4) and the wide availability of suitable vectors (Russel *et al*., 2004).

4.3.1.4 Phage and phagemid vectors for peptide display

The vectors that can be employed in the display of peptides on the phage surface can either be based on the natural Ff phage sequence (phage vectors) or be plasmid-based vectors (phagemids) that only contain the fusion protein gene without other phage genes

(Lowman, 1997). When using phage vectors, the foreign sequence is inserted directly into the pIII sequence (or another coat protein); upon introduction to *E.coli*, phage particles will have all copies of the coat protein displaying the foreign peptide (polyvalent display).

Phagemids were used in the present work; when phagemid vectors are used, the fusion gene is cloned into a plasmid under the control of a weak promoter. The phagemid vector has a plasmid origin of replication and an Ff origin, to allow phage genome replication and subsequent assembly. An overview of phage display via phagemid vectors can be seen in Figure 4-3 (image taken from Sidhu, 2001).

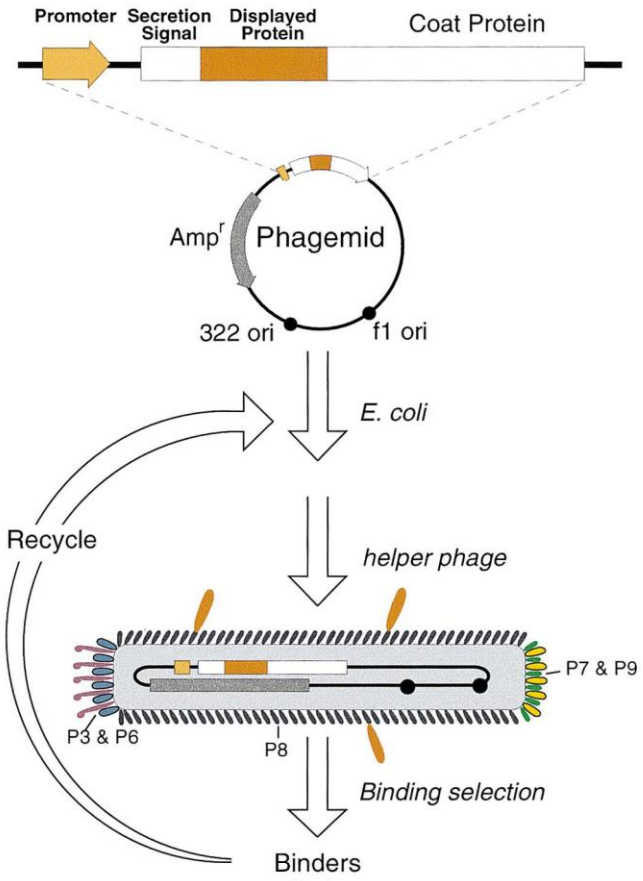

Figure 4-3: Phage display using a phagemid vector. The vector contains a double-stranded DNA origin of replication (322 ori) and a selectable marker (ampicillin resistance, Ampr) to replicate and maintain the vector as a double-stranded plasmid in *E. coli.* (Image taken from Sidhu, 2001)

When using phagemid vectors, the host cells are superinfected with a helper phage (M13KO7 was used in the present work), which belongs to the same family (Ff) but has

undergone mutations to reduce its packing efficiency and ensure that phagemid genomes are preferentially packaged during phage assembly and secretion. The helper phage also carries a gene that offers kanamycin resistance, which allows antibiotic selection of superinfected cells.

The resulting phage population in phagemid systems display between zero and five copies of the desired peptide, with the majority of particles displaying no copy followed by particles displaying one copy (monovalent display) (O' Connell *et al.*, 2002).

Phagemid vectors present a number of advantages in the display of antibody fragments on filamentous phage, including their small size and ease of cloning (compared to phage vectors, where the structure of genes, promoters and terminators must not be disrupted) (Russel *et al.*, 2004). This results to the construction of antibody libraries of much bigger sizes when using a phagemid system rather than a phage vector (this problem was noted by Parmley and Smith when describing using phage vectors – Parmley and Smith, 1989). Additionally, the monovalent display of antibody fragments on the phage surface facilitates the isolation of high-affinity binders, as opposed to multivalent display, which is based on avidity rather than pure affinity of binder to target on a one-to-one basis.

4.4 Phage antibody libraries

4.4.1.1 Mimicking the immune system

The success of phage display technology in the generation of sensitive antibodies against targets of interest is greatly dependent on the phage antibody library that will be used in the selection process. A library of poor quality (small size, low diversity of clones) could prove detrimental to the isolation of high-quality antibodies.

The antigen-binding site of an antibody is composed of six complementarity-determining regions (CDRs) or hypervariable regions; three within the light-chain variable domain (VL) and three within the heavy-chain domain (VH). In the immune system of living organisms, a great variety of antibody binding sites can be generated, via the combinatorial assembly of germline-encoded fragments.

In phage display technology, diverse libraries of immunoglobulin heavy (VH) and light (Vκ and Vλ) chain variable genes can be prepared from peripheral blood lymphocytes via polymerase chain reaction (PCR) amplification. It is believed that with a library size of $\geq 10^8$ clones all possible VH/VL pairings can be included (even the pairs originating from B cells selected by the immune system) (Winter and Milstein, 1991).

The genes encoding the scFv fragments are generated by random recombination of heavy and light chains and the resulting library of recombinant clones is displayed on the surface of a population of phage. The construction and subsequent screening of a combinatorial phage display library mimics the immune system of living organisms (Figure 4-4); in the immune system, diverse combinatorial libraries of antibodies are displayed on the surface of B-cells. Exposure to a certain antigen triggers cell proliferation and differentiation (antibody generation or memory pathways). A phage display library is displayed on the surface of the virions (equivalent of the B cells) and selection is performed via exposure of the entire library to the target of interest, followed by amplification of the relevant clones.

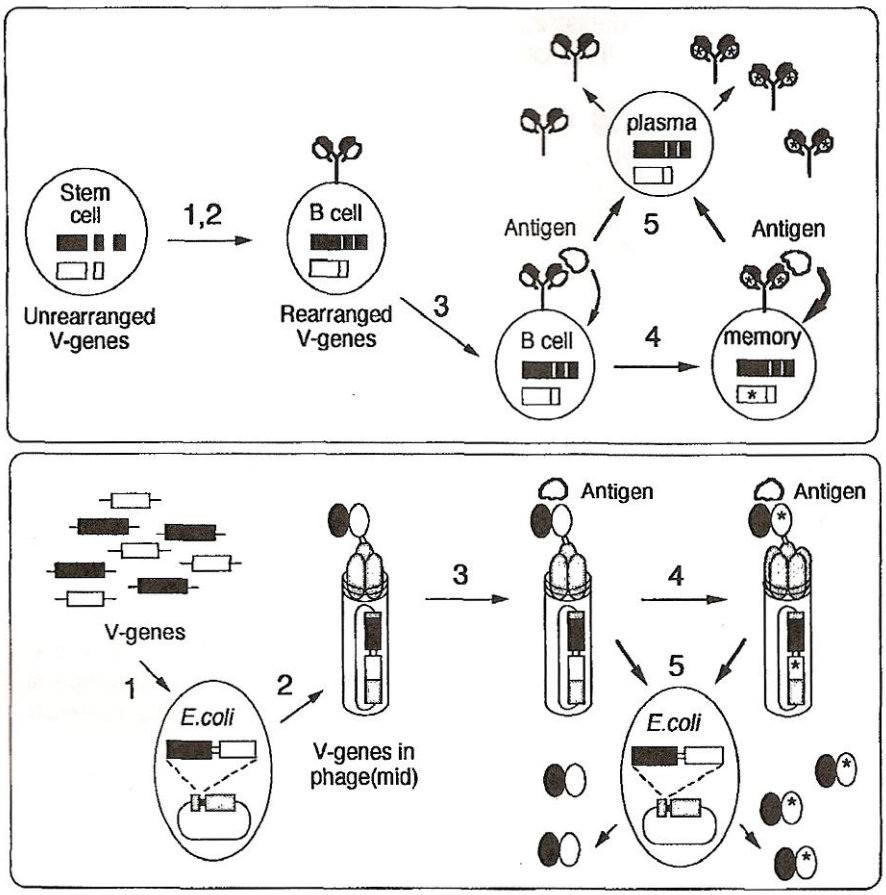

Figure 4-4: Generation of antibodies in the immune system (top) and via phage display technology (bottom). The steps include rearrangement or assembly of germline V genes (1), surface display of antibody (or antibody fragment) (2) antigen-driven or affinity selection (3) affinity maturation (4) and production of soluble antibody (5) (Image taken from Winter et al., 1994)

4.4.1.2 Naïve and immunised phage antibody libraries

Phage antibody libraries are heterogeneous mixtures of recombinant DNA fragments, the expression of which is a population of fusion phage particles, each carrying a different recombinant DNA insert and displaying the corresponding antibody on its surface.

Naïve phage antibody libraries are composed of antibody fragments from a pool of genes that are not explicitly biased to contain clones that bind to a certain antigen. The lack of bias makes naïve libraries appropriate for selection for a wide variety of antigens (including self, non-immunogenic or even slightly toxic molecules). In the construction of naïve libraries, V genes are amplified from B-cell cDNA, and heavy and light chains are randomly recombined and cloned in appropriate vectors, in order to encode a

combinatorial scFv or Fab library of fragments (Hoogenboom *et al*., 1998). The use of a naïve library in the search for binders against particular antigens offers access to some of the germline gene antibodies (non-recombinant molecules), but it is understood that their occurrence in the library is extremely rare. The affinity of antibodies isolated from a naïve library is proportional to its size (Hoogenboom *et al*., 1998), but a library of efficient size and diversity can yield antibodies against a great number of targets.

Immunised phage antibody libraries are collections of immune antibody fragments, showing a bias for certain specificities as a result of coming from an immunised donor organism (Hoogenboom, 2005). Immunisation leads to an increase in the number of cells producing an immune response, especially in the levels of mRNA produced. Spleen, lymph nodes, tonsils, and bone marrow provide a rich source of plasma cells and Ig mRNA. Repertoires of VH or VL genes amplified from the mRNA of spleen cells of an immunised organism are therefore greatly enriched in V genes encoding part of an antigen binding site (Winter *et al*., 1994). Similarly to the naïve libraries, the random recombination of VH and VL gene repertoires leads to a loss of the original pairings, but this recombination, combined with the bias created by the immunisation, leads to the isolation of antibodies with extremely high affinities for the targets of interest.

As far as the donor organisms for the construction of immunised libraries are concerned, immunised libraries of mouse (Clarckson *et al*., 1991), rabbit (Lang *et al*., 1996), chicken (Davies *et al*., 1995), camel (Ghahroudi *et al*., 1997) and human (Barbas *et al*., 1993) origin have been reported. Immunised libraries from sheep are also promising, as studies have revealed high diversity in the antibody repertoire, despite a smaller overall gene pool size than humans or mice (Charlton *et al*., 2000). A large number of immunoassays and biosensors make use of polyclonal antisera (Wust and Hock, 1992), and it has been shown that the affinity of sheep antibodies for their targets is high, with slow dissociation rates from antigen, explaining their success as diagnostic molecules (Li et al., 2000). Both the naïve and the immunised library used in the present work were derived from sheep.

4.4.1.3 Selection of antibodies from a phage display library - Biopanning

The step that follows the decision of the library type to use (naïve/immunised) is the screening process, which will isolate the antibodies with desired properties from the pool of displayed fragments. The selection procedure focuses on obtaining a subpopulation of phage-borne peptides with desired properties, from a heterogeneous starting population. In most cases, the first round of selection begins with a very large initial library and the selected subpopulation is a small fraction of the original population, within which the fitter clones are being overrepresented. This population can be "amplified" by infecting fresh bacterial host cells, so that each individual phage in the subpopulation is represented by millions of copies in the amplified stock. The amplified population can then be subjected to further rounds of selection to obtain an ever-fitter subset of the starting peptides (Figure 4-5).

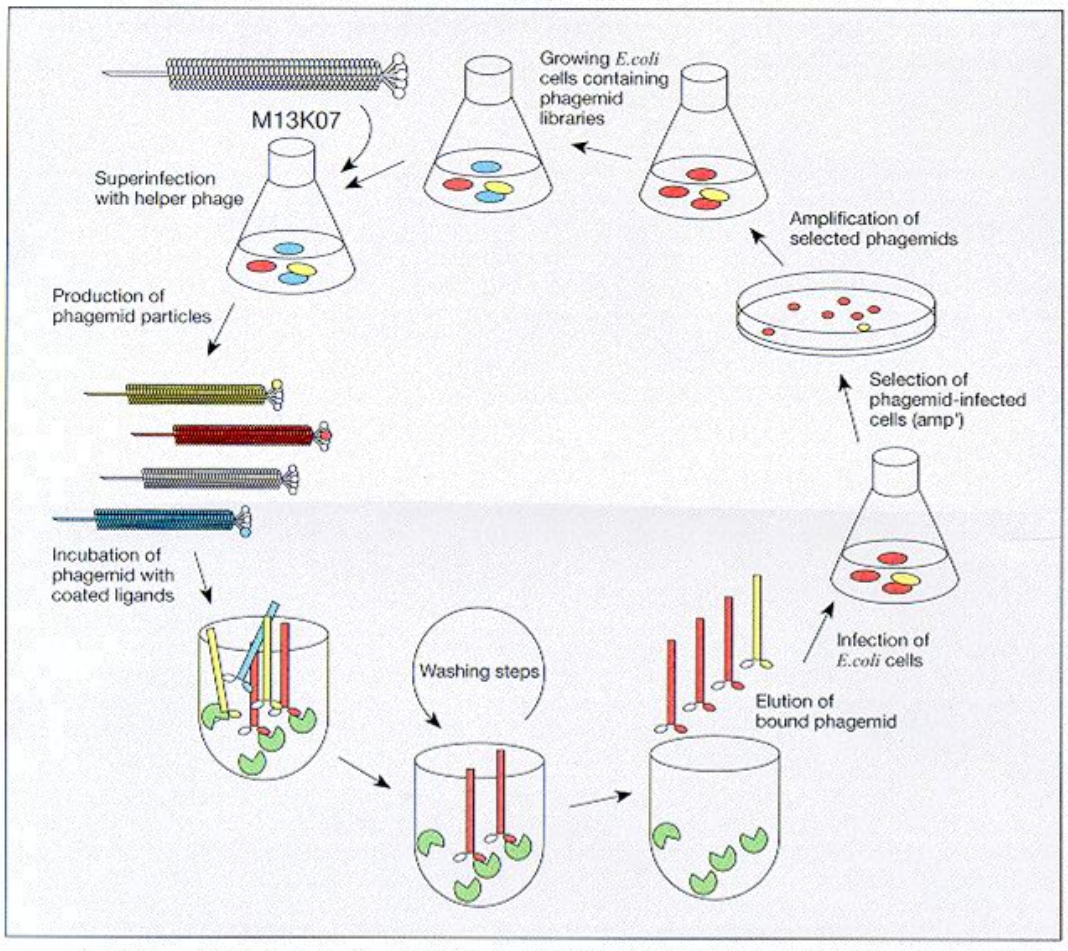

Figure 4-5: Screening of a phage display library for binders of interest (biopanning) (Image taken from http://ymbc.ym.edu.tw/proteome/interact/phage_display.htm)

In a review of phage display technology, Smith and Petrenko highlight two parameters of selection which can be manipulated to some extent in order to enhance the efficiency of selection process. These parameters are stringency, which is defined as the degree to which peptides with higher "fitness" are favoured over peptides with lower fitness; yield is the fraction of particles with a given fitness that survive selection. The ultimate goal of selection is to isolate peptides with high fitness, but this does not mean that stringency should be increased without bound, as increases in stringency result in reduced yield (Smith and Petrenko, 1997).

The most common selection pressure imposed on phage-displayed peptide populations is affinity for a target receptor, a procedure known as "biopanning" (Figure 4-5). In this method, the antigenic target is immobilised on a solid support, and the phage library is passed over the immobilised receptor. The phage particles from the library whose displayed peptides bind the receptor are captured on the surface; unbound phage particles are then washed away. Finally, the bound phage are eluted in a solution that loosens receptor-peptide bonds, yielding an "eluate" population of phage that is greatly enriched (often a million fold or more) for receptor-binding clones. The eluted phage particles are still infective and are propagated by infecting fresh bacterial host cells, yielding an "amplified" eluate that can serve as input to another round of affinity selection. Phage clones from the final eluate (typically after 2-3 rounds of selection) are propagated and characterised individually. The amino acid sequences of the peptides responsible for binding the target receptor are determined simply by determining the corresponding coding sequence in the phage DNA. In general, high stringency is favoured by low densities of the target receptor and by monovalent display of the foreign peptide; high stringency is almost invariably accompanied by relatively low yield (Smith and Petrenko, 1997).

4.5 Application of phage display technology in the generation of anti-hapten antibodies

The production of sensitive monoclonal antibodies via hybridoma technology against compounds that are poor immunogens (viral coat proteins, carbohydrates, small organic

molecules) is a very difficult procedure. However, the use of antibody libraries (naïve, immunised or synthetic) in combination with phage display technology offers an efficient alternative.

Haptens are defined as low molecular weight (<1000Da) compounds that are not immunogenic by themselves, but are able to elicit an immune response when they are conjugated to much larger carrier proteins (Kuby, 1997). Haptens include a number of molecules with everyday applications, like dyes, pesticides and antibiotics; therefore, the acquisition of antibodies that would allow the rapid detection of potentially harmful haptens in the environment has always had commercial interest. Most anti-hapten immunoassays have been developed for environmental contaminants and utilise polyclonal antibodies, rather than monoclonal or recombinant molecules (Charlton *et al*., 2001). Conversely, the vast majority of recombinant antibodies used in biosensor and diagnostics technology have been raised against large molecules, like proteins and peptides (Charlton *et al*., 2001).

In recent years, the generation of high-affinity antibodies against hapten targets has seen an impressive increase, as noted in a 2007 review by Sheedy and colleagues (Sheedy et al., 2007), greatly due to the parallel advances in phage display technology. Naïve, immunised and synthetic (built artificially via *in vitro* assembly of antibody gene fragments) antibody libraries have been employed in the production of anti-hapten antibodies, with various degrees of success, depending on the target and library used. The employment of phage display in the isolation of anti-hapten antibodies offers flexibility through the potential to modify the screening strategy (biopanning) to fit the requirements of different hapten targets.

In addition to using certain libraries to screen for binders against certain haptens, the ability to construct a focused "anti-hapten" library which is biased for the recognition of a variety of antigens of pre-defined size has been reported (Persson *et al*., 2006, Persson *et al*., 2008). A library of this kind is constructed using a hapten template, but has the ability to produce antibodies against a range of other haptens, closely related to the template in size.

4.6 Phytane and β-Carotane

The two proposed LMC targets that were used for the generation of recombinant antibodies in the present work are phytane (2, 6, 10, 14 - tetramethylhexadecane) and β-carotane (3, 7 – dimethyl-9-2, 2, 6 trimethylcyclohexylnonan) both of them belong to the category of fossil biomarkers (markers of extinct Life) and are stable breakdown products of more complex precursor molecules.

Phytane ($C_{20}H_{42}$) (Figure 4-6) is a diterpenoid alkane which is a component of archeal membranes and is believed to be characterised by high stability over long periods of time; phytane and the closely related pristane ($C_{19}H_{40}$) have been found in ancient sedimentary deposits containing fossil organic matter (Brocks *et al*., 1997).

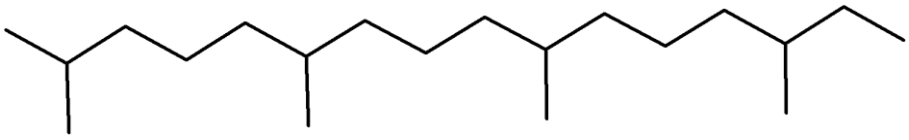

Figure 4-6: The structure of phytane

A number of precursor molecules can become modified to form phytane, with a possible diterpenoid intermediate, like phytol. Chlorophyll and the tetraether lipids that are cell membrane components in archea may both yield phytol during the breakdown process (Parnell *et al*., 2007); phytane can derive from phytol after structural modifications. The high stability and the wide range of biomolecules that can have phytane as their final breakdown product make the latter a high priority LMC target molecule.

The second LMC target which was used in the generation of recombinant antibodies described in the present chapter was β-carotane (Figure 4-7). β-Carotane is a shorter chain, fossil derivative of the carotenoid class of large isoprenoid molecules (> C_{40}). While certain fossil carotenoids are specific to particular kingdoms, β-β carotane (the dimer structure of the hapten used in this work) is produced by a number of different microorganisms, making it another promising LMC target.

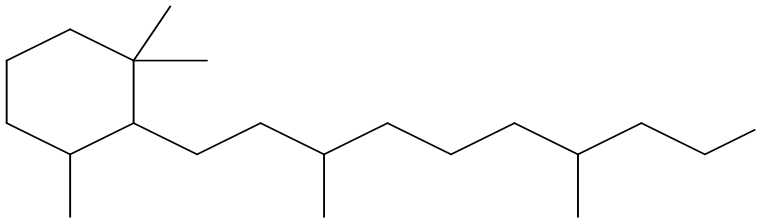

Figure 4-7: Structure of β-carotane

4.7 Aims and Objectives

The objective of the present work has been to generate recombinant antibodies against the two potential LMC targets (phytane and β-carotane) with the employment of phage display technology. The outcome of this work, which was done in parallel for six LMC targets in total (the two targets reported here and four other targets), was viewed as a means to determine whether phage display can be the main method of acquiring antibodies against high priority LMC target haptens.

Constrictions in time and resources are a major issue in the development of the LMC; given that one of the main advantages of phage display technology is its efficiency in terms of both cost and time, this was viewed as an opportunity to test this efficiency and determine whether phage display would be the optimal method of LMC antibody generation.

By using both a naïve and an immunised phage antibody library, their potential could be directly compared; the use of naïve libraries does not involve the construction cost and time delay of obtaining an immunised library, and could therefore prove more resource-efficient if successful. Additionally, the naïve library which was used had been constructed as an immunised library against another mixture of small molecules (lactone-type haptens), and this was seen as a chance to examine whether there would be any kind of positive bias in acquiring binders against our own haptens.

Given that this was the first time that phage display technology was to be used in the context of the LMC development, the present work was also an opportunity to adjust

and optimise the protocols related to the screening of the libraries to fit the requirements of specific targets, in order to isolate high-quality binders against them.

Materials and Methodology

4.8 Culture media – Buffers – Other reagents

The main culture media that were used throughout the screening of the phage antibody libraries were TYE, 2xTY and LB broth. All media are standard *E.coli* cultivation mixtures of similar ingredients that promote bacterial growth via the provision of elements that are necessary for crucial metabolic processes. Detailed descriptions of all three media can be seen in Table 4-. In order to prepare the media, the amounts of all their respective components were weighed out, dH_2O was added to a final volume of 500 ml and the medium was autoclaved prior to use. In the case of TYE where agar is present, the medium will quickly solidify in room temperature; in order to avoid this, the bottles of TYE medium were kept in a 40^o C waterbath after being autoclaved and prior to being used.

Table 4-2: Details of the culture media used during phage antibody library rescue and panning (all component weights correspond to 500 ml final volume of medium in dH_2O)

Component Details	***Component Function***	**TYE (500 ml)**	**2xTY (500 ml)**	**LB broth (500 ml)**
Bacto-tryptone (Oxoid – cat. no. LP0042)	Provision of peptides, amino acids and peptones	4g	8g	5g
Bacto-yeast extract (Oxoid – cat. no. LP0021)	Provision of vitamins, trace elements, various organics	2.5g	5g	2.5g
Sodium Chloride (NaCl) (Fisher – cat. no. BP358-1)	Provision of sodium ions for transport and osmotic balance	2.5g	2.5g	10g
Agar Bacteriological (Oxoid – cat. no. LP0011)	Solid culture substrate	7.5g	-	

The 2xTY medium was generally used wherever liquid culture growth was the objective, while TYE was used in the preparation of solid media for colony analysis (i.e.

for counting and selecting colonies for further work). Three main categories of solid colonies were prepared and used in the form of solid medium in Petri dishes, with the addition of antibiotics to prevent culture contamination; TYE_{AG} (500 ml TYE, 10 ml glucose 50%, 500 μl ampicillin, the latter from Sigma Aldrich – cat. no. A5354), TYE_{AKG} (500 ml TYE, 10 ml glucose 50%, 500 μl ampicillin, 500 μl kanamycin, the latter from Sigma Aldrich – cat. no. K0254) and TYE_{KG} (500 ml TYE, 10 ml glucose 50% and 500 μl kanamycin). The 50% glucose mixture that was used was prepared with the addition of dH_2O to 100 g glucose (Sigma Aldrich – cat. no. G5767) for a final solution volume of 200 ml and autoclavation prior to use. Ampicillin is an antibiotic that functions as a competitive inhibitor of the enzyme transpeptidase, an essential component of bacterial cell walls. The gene for ampicillin resistance is inserted to all the phage library antibody sequences during the construction of the libraries. Kanamycin is an antibiotic that indirectly inhibits protein synthesis in bacteria via interactions with the 30S subunit in prokaryotic ribosomes. Kanamycin resistance comes from the helper phage, helping library cells superinfected with helper phage survive over cells without the helper. Finally, the LB (Lysogeny Broth) medium is one of the best media for the growth and preservation of recombinant *E.coli* strains, and it is used during library rescue and plasmid preparations for sequencing or long-term storage.

The soft agar that was required for plaque purification of the helper phage M13KO7 was prepared by mixing 6.4 g of bacto-tryptone, 4 g of bacto-yeast extract, 4 g of NaCl and 6 g of agar bacteriological (for suppliers, see Table 4-) and adding dH_2O to a final volume of 500 ml. Soft agar was autoclaved prior to use and after autoclavation it was kept at a 42^oC waterbath before using it in the production of helper phage.

As far as the common buffer solutions were concerned, phosphate buffer saline (PBS) was prepared from PBS tablets (Sigma Aldrich – cat. no. P4417) and a 0.1% solution of PBS-Tween 20 (PBST) was prepared via the addition of 500 ml Tween 20 (Sigma Aldrich – cat. no. P1379) to 500 ml PBS. Finally, TRIS buffer was used during the small scale expression (Severn Biotech – cat. no. 20-7901-10). All the buffer solutions were autoclaved prior to use.

Phage precipitation was facilitated by the use of a mixture of 20% PEG 6000/2.5M NaCl. 80g of PEG (polyethylene glycol) 6000 (Fisher Scientific – cat. no. P/3681/53) were mixed with 58.44g NaCl (Fisher Scientific – cat. no. BP358-1) and dH_2O was added, to a final volume of 400 ml. The solution was autoclaved prior to use.

4.9 Naïve phage antibody library

The naïve phage antibody library which was used in the first attempt to acquire recombinant antibodies against β-carotane and phytane was provided by Grampian Bioconsultants and had been constructed a few months earlier as an immunised library against lactone-type molecules. The library comprised κ and λ light chains (approximately 2 x 10^7 clones each). The κ and λ libraries were pooled together and used in the panning procedure.

4.10 Immunised phage antibody library construction

The animal immunisation and the subsequent construction of the immunised library were carried out by Immunosolv Ltd. At the first stages, the phytane-KLH conjugate that had been synthesised earlier was used by Immunosolv Ltd. to immunise two Norfolk sheep (CF68 and CF69) on the 3rd February 2009. The sheep were immunised initially with 100 µg /ml of a mixture of the phytane-KLH conjugate with four additional LMC target molecules conjugated to KLH. The carotane-KLH conjugate was not available at the time, so immunisation with carotane took place during the final one of four additional boosts that took place every 4 weeks with 50 µg /ml of the same mixed antigen. Binding ELISA were performed on each test bleed (taken 2 weeks after the sheep were boosted {week 6, 10 and 14}) to determine whether an antibody response was occurring to the target antigens. The ELISA plates were coated with 2 µg/ml antigen conjugated to BSA (bovine serum albumin) or BSA alone to rule out for any effect seen for binding to the carrier protein alone. Four days after the final of the four additional boosts, a production bleed was carried out, and B lymphocytes from this

bleed were used for the isolation of RNA, the library starting material. Following RNA collection, the immunised library was constructed by Immunosolv Ltd.

4.11 Phage antibody library rescue

4.11.1.1 Production of helper phage M13KO7

The production of a high-titre helper phage M13KO7 stock is an essential first step to the screening of phage antibody libraries, as it is M13KO7 that provides the "building blocks" for the production of phage particles displaying the library antibodies. In order to produce large amounts of a high-titre M13KO7 stock, a commercially available stock of M13KO7 (New England Biolabs – cat. no. NO315S) at a titre of 10^{11} phage/ml was used, and dilutions of this stock in PBS buffer (10^{-2} to 10^{-12}) were prepared. 50 µl of each dilution were used to infect 500 µl E.coli cells, TG1 strain (Stratagene – cat.no. 200123) at logarithmic phase (log phase) of growth (OD_{600} = 0.4). In order to grow the cells to log phase, a small inoculum of the stock material was added to an autoclaved 250 ml baffled flask containing 2xTY medium and incubated in a 37^{o}C shaking incubator (IKA KS4000i) at 280 rpm until OD_{600} reached a value of 0.4 (approximately 2 hours). The OD_{600} values were measured using a portable cell density meter (WPA – cat. no. CO8000). Infection of the TG1 cells with the helper phage was allowed to develop over a 30-minute period in a 37^{o}C Clifton waterbath (Fisher Scientific). After this period, the infected TG1 cells of each helper phage dilution were added to 3 ml of soft agar (see section 4.8) and poured over TYE plates, without the addition of glucose or antibiotics. The plates were incubated overnight in a 37^{o}C static incubator.

The following day, plaque purification of the helper phage took place; one plaque of M13KO7 from the dilution plates from the day before was inoculated into 10 ml of TG1 cells (OD_{600} = 0.4) in a sterile 50 ml plastic tube (Fisherbrand – cat. no FB55957). The contents of the tube were incubated for 2 hours in a 37^{o}C shaking incubator at 280 rpm. After this incubation period, the contents of the tube were transferred to an autoclaved 2l baffled flask with 500 ml of 2xTY medium and incubated for 1 hour in a 37^{o}C shaking incubator at 280 rpm. At the end of the hour, kanamycin was added, to a final

concentration of 50 μg/ml, and overnight incubation followed, in a 30^{o}C shaking incubator, at 280 rpm.

On the third day of the M13KO7 production, the newly-formed helper phage particles were concentrated and collected in order to be used in the subsequent screening process, via a "PEG precipitation" method involving polyethylene glycol 6000 (PEG 6000). PEG precipitation is a well-established method of phage concentration from crude bacterial lysates (Yamamoto *et al.*, 1970). For the PEG precipitation, the culture that was incubated overnight was centrifuged using a Heraeus Megafuge 11R centrifuge (Thermo Fisher Scientific) at 8000 rpm for 15 minutes and 400 ml of the resulting supernatant were transferred to a new 2l baffled flask. 100 ml of a 20%PEG/2.5M NaCl solution were added to the supernatant and the flask was placed in a plastic container filled with ice for 1 hour. After this period, the contents of the flask were split between two autoclaved 330 ml plastic centrifuge tubes (Fisher Scientific) and centrifuged at 8000 rpm for 30 minutes using a Sorvall centrifuge (model no. RC5C). After centrifugation, the supernatants were discarded and the pellets were resuspended in 8 ml of PBS buffer. The two suspensions were pooled together into a sterile 50 ml plastic tube, where 2 ml of the same PEG/NaCl solution were added, before placing the tube in ice for 20 minutes.

Another centrifugation step followed, at 8000 rpm for 30 minutes and the supernatant was discarded. The pellet was resuspended in 5 ml PBS buffer and split into 1 ml volumes in sterile 1.5 ml Eppendorf tubes. The tubes were placed in a bench top minifuge and centrifuged at 12000 rpm for 10 minutes to remove any traces of bacterial debris. After centrifugation, the supernatants were moved to sterile cryo-vials (Fisherbrand – cat. no. 10-500-26) and 10 μl of material was removed to be used in the titration of the newly-made stock. The remaining volume of M13KO7 stock was stored at -80^{o}C in the form of 15% glycerol (Sigma Aldrich – cat. no. G5516) stocks.

In order to titrate the M13KO7 stock, the 10 μl volume that was taken before storing the stock was used to prepare a set of serial hundred-fold dilutions in PBS (10^{-6} to 10^{-18}). 50 μl of each of these dilutions were transferred to sterile 10 ml plastic tubes and 450 μl of TG1 cells in log phase of growth (OD_{600} = 0.4) were added. Infection of the TG1 cells was allowed to take place over a 30-minute period in a 37^{o}C waterbath, followed by a

second 30-minute period in a 37^{o}C shaking incubator, at 280 rpm. After infection, two volumes of 50 µl of each dilution were spread onto TYE_{KG} plates (TYE medium with glucose and kanamycin) (duplicate plates) and the plates were incubated overnight in a 30^{o}C static incubator. The helper phage M13KO7 has a gene that offers kanamycin resistance, so it is expected that the cells that grow on the TYE_{KG} plates are cells infected with M13KO7. The following day, colonies were counted and titres were obtained. Titres of $10^{12\text{-}13}$ or higher are considered satisfactory. In our case, application of this protocol resulted in a helper phage stock with a titre of 2.8 x 10^{13} phage/ml.

4.11.1.2 Naïve phage antibody library

The process that is used in order to "rescue" the phage antibody libraries prior to screening them for binders against the desired targets presented some variations between the naïve and immunised libraries. In the case of the naïve phage antibody library, 500 µl of library stock (see section 4.9) were spread onto TYE_{AG} bioassay dishes (140 mm diameter – Sterilin, cat. no. 501V) and incubated overnight in a static 30^{o}C incubator (Thermo Fisher Scientific Heraeus Incubator). The following day, 3 ml of 2xTY medium were used to scrape the cell colonies from the dishes and the resulting cell suspensions were transferred to autoclaved 250 ml baffled flasks where 100 ml of LB broth, 1 ml of 50% glucose in dH_2O and 50 µl of ampicillin (100 µg/ml) were added. The contents of the flasks were incubated for 1 hour in a shaking 37^{o}C incubator at 280 rpm.

After this incubation period, the optical density of the cell suspensions at 600 nm (OD_{600}) was measured using a Varioskan Flash reader from Thermoscientific. A high OD_{600} (>2) means there is sufficient number of cells to use for the subsequent screening process, the preparation of "back-up" cultures for future use and the DNA preparations (clean-up) for long-term library storage.

4.11.1.3 Rescuing library material for immediate use

Once the OD_{600} of the library culture had reached the desired value (>2), an appropriate volume of the culture was transferred to a sterile 50ml plastic tube containing 40 ml

2xTY medium for a starting OD_{600} value of 0.1 (approx. 1ml of library culture). 2xTY was added for a final volume of 50 ml and 1 ml of 50% glucose in dH_2O and 50 µl (100 µg/ml) ampicillin were added to the tube. The contents of the tube were transferred to an autoclaved 250 ml baffled flask and incubated for in a 37°C shaking incubator at 280 rpm until OD_{600} reached 0.4 (corresponding to the log phase of cell growth).

Once the OD600 reached 0.4, the culture was transferred to a sterile 50 ml plastic tube and infected with the helper phage M13KO7 at a cell to helper phage ratio of 1:20. The culture was incubated for 30 minutes in a 37°C waterbath to allow infection. At the end of this period, 100 µl of the infected library were removed in order to be serially diluted in 2xTY medium (ten-fold 10^{-1} to 10^{-7} dilutions of the starting material in 2xTY) and used for cell titrations. Two volumes of 50 µl of each dilution were spread onto TYE_{AKG} plates (duplicates) and all plates were incubated overnight in a 30°C static incubator. Control TYE_{AKG} plates without cells and with M13KO7-uninfected library cells were also prepared to allow possible contamination detection. These titrations provide a means of measuring the number of library cells successfully infected with M13KO7, as kanamycin resistance comes from the helper phage. Titration results of 10^8 to 10^{10} cells/ml of culture are generally considered satisfactory, while lower titres suggest that repetition of library rescue and infection with the helper phage is necessary.

After having removed the material for titration, the remaining culture volume was centrifuged at 6000 rpm for 20 minutes. The resulting supernatant was discarded and the pellet was reconstituted in 50 ml 2xTY with the addition of 50 µl of ampicillin (100 µg/ml). This solution was transferred to an autoclaved 250 ml baffled flask and incubated for 1 hour in a 37°C shaking incubator. This incubation period allowed the M13KO7-infected cells to fully develop their resistance to kanamycin, and at the end of the hour 50 µl (50 µg/ml) of kanamycin were added to the flask. The contents of the flask were incubated overnight in a 30°C shaking incubator at 250 rpm.

The following day, the library phage particles were concentrated using the PEG-precipitation method which was also used in the production of helper phage M13KO7 (see section 4.11.1.1). After the overnight shaking incubation at 30°C, the library culture was transferred to a 50 ml sterile plastic tube and centrifuged at 6000 rpm for 20 minutes. After centrifugation, the supernatant was split between new 50 ml tubes in

order to facilitate further handling of the material, and 1/5 volume of 20%PEG/2.5M NaCl were added to each tube (each tube had 25 ml of culture, so 5 ml of 20%PEG/2.5M NaCl were added). The tubes were left in polystyrene box filled with ice for 1 hour to allow phage precipitation. At the end of the hour, "clouds" of phage should be visible in the tubes upon manual shaking. Another centrifugation step followed, at 8000 rpm for 30 minutes.

The tubes were removed from the centrifuge under careful handling to avoid resuspending the "soft" pellet that was created. The supernatants were discarded and the pellets containing the phage particles were each resuspended in 10 ml autoclaved dH_2O and pooled together in a new sterile 50 ml tube. The contents of the tube were centrifuged for 10 minutes at 6000 rpm as a further clean-up step. After the centrifugation, the supernatants were moved to a new tube and once more, 1/5 volume of 20%PEG/2.5M NaCl (approximately 5 ml) was added. The tube was placed in ice for 20 minutes and then centrifuged at 8000 rpm for 30 minutes. The supernatant was discarded and the pellet was reconstituted in 2 ml PBS buffer.

10 μl of this phage particle suspension, which had now been cleaned of cellular debris, were used for titrations in order to quantify the amount of phage in the resulting stock. The 10 μl volume was serially diluted (hundred-fold dilutions in 2xTY medium, 10^{-2} to 10^{-12} of the starting material) and used to infect TG1 strain *E.coli* host cells. 100 μl of each dilution were transferred to sterile 1.5 ml Eppendorf tubes and were used to infect 900 μl TG1 cell culture in 2xTY medium (OD_{600} = 0.4). The infection was allowed to take place over a 30-minute period in a 37^{o} C waterbath. At the end of this incubation period, two volumes of 100 μl of each dilution were spread onto TYE_{AG} plates (duplicates) and all plates were incubated overnight in a 30^{o} C static incubator. Titres of 10^{12} cells/ml or higher are considered satisfactory at this stage.

After removing material for the titrations, the remaining volume was split between two 1.5 ml sterile Eppendorf tubes (Eppendorf – cat. no. 05-402-25) and centrifuged using a bench top minifuge (Heraeus Fresco 17 from ThermoScientific) at maximum speed (13000 rpm for the specific model) for 5 minutes. The pure phage supernatant was transferred to sterile cryo-vials and stored at -80^{o}C in the form of 20% glycerol stocks until the time came to use it in the panning process.

4.11.1.3.1 Preparation of "back-up" cultures (B cultures)

After removing 1 ml of library material from the growing culture in order to rescue it for immediate use, another 1 ml of library culture was inoculated in an autoclaved 250 ml baffled flask containing 50 ml 2xTY medium, 1 ml 50% glucose and 100 μg/ml ampicillin. The contents of the flask were incubated overnight in a 37^{o}C shaking incubator at 280 rpm.

The following day, the culture was transferred to a sterile 50 ml plastic tube and centrifuged at 6000 rpm for approximately 30 minutes until a solid pellet was formed. The supernatant was discarded and the pellet was resuspended in 10 ml 2xTY medium with 1 ml 50% glucose and 1.5 ml glycerol (for a 5% glucose, 15% glycerol stock solution). These B culture stocks were moved to sterile cryo-vials and stored at -80^{o}C as bacterial backup material in case any problems or contaminations appeared during the library screening.

4.11.1.3.2 DNA preparations for long term library storage

In order to store the phage antibody library for long periods of time, the corresponding DNA sequences are isolated from the *E.coli* cells and stored separately, as it has been observed that stored library cells have a tendency to reject the DNA inserts after a certain period of time. In order to do this, the remaining library volume (after the removal of 2 ml for rescue of material for immediate use and the preparation of B cultures) was further incubated in a 37^{o}C shaking incubator for approximately 40 minutes. The library culture was then moved to sterile 50 ml plastic tubes and centrifuged at 6000 rpm for 20 minutes.

The isolation of DNA was performed using a plasmid preparation kit from Qiagen (HiSpeed Plasmid Maxi Kit – cat. no. 12663), following the kit instructions. The method is a modified alkaline lysis procedure followed by DNA binding to an anion-exchanging resin for the isolation of supercoiled plasmid DNA. After clean-up, plasmid DNA was stored at -20^{o}C.

4.11.1.4 Immunised phage antibody library

The protocol used in the rescue of the immunised phage antibody library followed the same overall principle as the one used in the naïve library rescue; the library cells were grown and infected with the helper phage M13KO7 in order to induce library phage production. The procedure used library stock material (see section 0), 1 ml of which was inoculated into 300 ml of 2xTY medium (1% glucose and 100 μg/ml ampicillin) inside a 2l autoclaved baffled flask. The contents of the flask were incubated in a 37^{o} C shaking incubator at 280 rpm until OD_{600} reached 0.4 (logarithmic phase of cell growth). When OD_{600} reached 0.4, 50 ml of culture were transferred to a sterile 50 ml plastic tube and infected with the helper phage M13KO7 at a cell to helper phage ratio of 1:20. The tube was placed in a 37^{o}C waterbath for 30 minutes to facilitate helper phage infection of the library cells, and the infection was completed via an additional 30-minute incubation in a 37^{o}C shaking incubator at 280 rpm.

At the end of the infection time, 100 μl of culture were removed to be used in titrations that would determine the number of successfully infected library cells. Serial ten-fold dilutions of culture in 2xTYmedium (10^{-2} to 10^{-10}) were prepared and two volumes of 50 μl of each dilution were spread on TYE_{AKG} plates (duplicates), which were incubated overnight in a 30^{o}C static incubator. Control TYE_{AKG} plates without cells and with uninfected library cells were prepared in parallel in order to monitor contamination. Similarly to the naïve library rescue titrations, titres of 10^{10} to 10^{11} cells/ml of culture are generally considered satisfactory for further work, while lower cell counts suggest that repetition of the rescue process is needed.

After removing material for the titrations, the remaining library volume was centrifuged at 4000 rpm for 10 minutes at 20^{o}C and the supernatant was discarded. The pellet was resuspended in 100 ml 2xTY medium (100 μg/ml ampicillin), moved to an autoclaved 250 ml baffled flask and incubated for 1 hour at a 30^{o}C shaking incubator at 280 rpm. At the end of the hour, 100 μl kanamycin (50 μg/ml) were added to the culture and it was incubated overnight in the 30^{o}C shaking incubator.

The following day, PEG-precipitation of the library phage took place, in a very similar protocol to the one used for the naïve library. After the overnight incubation of the infected library cells, the culture was transferred to sterile 50 ml plastic tubes and

centrifuged at 6000 rpm for 30 minutes. The supernatant was moved to a new 50 ml tube and 1/5 volume of 20%PEG/2.5M NaCl (approximately 10 ml) was added to the tube. The tube was put in ice for 2 hours, at the end of which "clouds" of phage were visible in the tube. Another centrifugation step followed, at 8000 rpm for 30 minutes. The supernatant was discarded and the pellet containing the library phage particles was resuspended in 40 ml of autoclaved dH_2O. This suspension was centrifuged at 6000 rpm for 10 minutes. The supernatant after this centrifugation step was transferred to a new 50 ml plastic tube and once more, 1/5 volume of 20%PEG/2.5M NaCl (8 ml) was added. The tube was placed in ice for 20 minutes before another round of centrifugation at 8000 rpm for 30 minutes. The supernatant was discarded and the pellet containing the phage was resuspended in 2 ml PBS.

10 μl of this phage particle suspension, free of cellular debris, were used for titrations in order to quantify the amount of phage in the resulting stock. The 10 μl volume was serially diluted (hundred-fold dilutions in 2xTY medium, 10^{-2} to 10^{-12} of the starting material) and used to infect TG1 strain *E.coli* host cells. 100 μl of each dilution were transferred to sterile 1.5 ml Eppendorf tubes and were used to infect 900 μl TG1 cell culture in 2xTY medium (OD_{600} = 0.4). The infection was allowed to take place over a 30-minute period in a 37^{o} C waterbath. At the end of this incubation period, two volumes of 100 μl of each dilution were spread onto TYE_{AG} plates (duplicates) and all plates were incubated overnight in a 30^{o} C static incubator. Titres of 10^{12} cells/ml or higher are considered satisfactory at this stage.

The remaining volume of phage in PBS buffer was split between sterile 1.5 ml Eppendorf tubes and centrifuged in a bench top minifuge at maximum speed (in this case 13000 rpm) for 5 minutes. The supernatant was transferred to sterile cryo-vials and stored at a -80^{o} C freezer in the form of 20% glycerol stocks until the time came to be used in the panning process. The eight different libraries were rescued and stored separately, and were only pooled together immediately before being used in the screening. The pooling of the κ and λ libraries took into consideration the representation of κ and λ chains in the active immune system of sheep (approx. 90% λ and 10% presence of κ).

4.11.1.4.1 Preparation of "back-up" cultures (B cultures)

The preparation of backup cultures (B cultures) from the immunised libraries was very similar to the procedure used for the naïve library. After removing 50 ml of library material from the growing culture in order to infect with the helper phage M13KO7, the remaining 250 ml of material were incubated for an additional 2-hour period in a 37^{o}C shaking incubator at 280 rpm. The culture was transferred to sterile 50 ml plastic tubes and centrifuged at 6000 rpm for 30 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml 2xTY medium (total volume) (5% glucose and 15% glycerol). This library stock was split between sterile cryo-vials and stored at -80^{o}C.

4.12 Library Panning

The principle for panning the libraries for binders against targets of interest (in this case phytane and carotane) is the same regardless of whether the naïve or the immunised library is being used.

A plastic immunotube (Fisher Scientific – cat. no. 444202) was coated with the target of interest (phytane, in the form of phytanic acid, or carotane) in its conjugated form to a carrier protein (BSA or KLH, although generally the BSA conjugate was used in the first round of panning). In order to coat the immunotube, 4 ml of a 10 μg/ml solution of the target conjugate in PBS buffer were added to the immunotube, which was placed at 4^{o}C overnight to allow binding of the conjugate to the inner surface of the tube. The following day, the coating solution was discarded and the immunotube was washed 3 times using 10 ml PBS buffer, in a sequential filling and emptying procedure. Then the immunotube was filled with 4 ml of a "blocking" buffer aiming to coat any parts of the inner surface that had not been covered by the target conjugate. The blocking buffer consisted of Marvel skimmed milk powder in PBS buffer (MPBS - 2% solution). The coated immunotube was incubated at 37^{o}C in a static incubator for 2 hours. At the end of this period, another washing step followed, identical to the previous one, with 3 washes with PBS buffer. The tube was tap-dried carefully before the addition of the library material.

After the immunotube was dried off, the naïve or immunised library was added, in a mixture of MPBS buffer (4%) and BSA in PBS buffer (2 mg/ml). 1 ml of library material was mixed with 2 ml MPBS 4% and 1 ml BSA solution. The immunotube was sealed with parafilm and its contents were incubated rotating, by placing it on a rotating plate base (Stuart SB3) for 30 minutes. A static incubation step followed, at ambient temperature for 90 minutes (standing on the laboratory bench). At the end of this step, the contents of the tube were discarded along with unbound phage particles and the immunotube was washed with a washing buffer consisting of 0.1% Tween 20 in PBS buffer (PBST), in 10 washes of 10 ml volumes. Another 10 washes of 10 ml volumes of PBS buffer followed, and the immunotube was tap-dried carefully.

In order to elute the phage particles bound to the surface of the immunotube, triethylamine (TEA) was used. This elution procedure is based on altering the pH value of the interior of the immunotube (alkaline elution), causing the detachment of the phage particles. 1 ml of 100 mM TEA (Sigma Aldrich – cat. no. T0886) solution in dH_2O was added to the tube and the latter was mounted on the rotating plate base and rotated for 10 minutes exactly. In the meantime, two volumes of 0.5 ml Tris buffer (see section 4.8) were added to two sterile 1.5 ml Eppendorf tubes and at the end of the 10-minute rotation the contents of the immunotube were immediately transferred to the Eppendorf tubes, in order to neutralise the TEA solution. An additional volume of 200 μl of Tris buffer was added to the immunotube in order to neutralise any remaining TEA/phage mixture.

0.75 ml of the eluted phage solution was used in order to infect 9.25 ml of TG1 strain *E.coli* cells (OD_{600} of 0.4) in a sterile 50 ml plastic tube. An additional volume of 4 ml TG1 cells was added to the immunotube and both tubes were placed in a 37^{o}C waterbath for 30 minutes to allow infection of the host TG1 cells with the eluted phage particles. The remaining volume of eluted phage suspension was stored at -80^{o}C in the form of a 15% glycerol stock. After 30 minutes in the waterbath, the contents of the immunotube and the 50 ml plastic tube were pooled together and 10 μl of material was removed in order to be used in titrations.

The titration procedure consisted of the dilution of this starting material in 2xTY medium (serial ten-fold dilutions from 10^{-2} to 10^{-7}) and spreading of two 100 μl

volumes of each dilution onto TYE_{AG} plates. The plates were incubated at 30°C (static incubation) overnight. This titration is a means of measuring the number of eluted phage particles and even low titres of 10^3-10^4 cells/ml are considered acceptable.

After removing the 10 µl of material of the titrations, the remaining volume of infected cells was centrifuged at 4000 rpm for 10 minutes, the supernatant was discarded and the pellet was resuspended in 0.5 ml 2xTY medium. This suspension was spread on TYE_{AG} large bioassay dishes and incubated overnight at 30°C (static incubation).

The following day, 4 ml of 2xTY medium were used in order to scrape the grown cell colonies off the surface of the bioassay dishes and the cell suspension was collected in sterile 1.5 ml Eppendorf tubes. 100 µl of this suspension were used to inoculate 100 ml 2xTY medium (1% glucose, 100 µg/ml ampicillin) in an autoclaved 250 ml baffled flask, while the remaining material was stored at -80°C as a 5% glucose, 15% glycerol stock. The contents of the baffled flask were incubated in a 37°C shaking incubator at 280 rpm until OD_{600} reached 0.4.

Once OD_{600} reached 0.4 (after approximately 90 minutes), 50 ml of the cell culture were transferred to a sterile 50 ml plastic tube and infected with helper phage M13KO7 at a cell to helper phage ratio of 1:20. The tube was placed in a 37°C waterbath to allow infection, followed by an additional infection period of 30 minutes in a 37°C shaking incubator at 280 rpm. At the end of the infection time, 10 µl of culture were removed to be used in titrations that would determine the number of successfully infected library cells. Serial ten-fold dilutions of culture in 2xTYmedium (10^{-2} to 10^{-10}) were prepared and two volumes of 50 µl of each dilution were spread on TYE_{AKG} plates (duplicates), which were incubated overnight in a 30°C static incubator. Control TYE_{AKG} plates without cells and with uninfected library cells were prepared in parallel in order to monitor contamination. At this stage of eluted phage rescue, titres of 10^8 to 10^{10} cells/ml of culture are generally considered satisfactory for further work, while lower cell counts suggest that repetition of the rescue procedure is needed.

After removing material for the titrations, the remaining library volume was centrifuged at 4000 rpm for 10 minutes at 20°C and the supernatant was discarded. The pellet was resuspended in 50 ml 2xTY medium (100 µg/ml ampicillin), moved to an autoclaved

250 ml baffled flask and incubated for 1 hour at a 30^{o}C shaking incubator at 280 rpm. At the end of the hour, 50 µl kanamycin (50 µg/ml) were added to the culture and it was incubated overnight in the 30^{o}C shaking incubator.

The following day, PEG-precipitation of the rescued phage took place. After the overnight incubation of the infected cells, the culture was transferred to sterile 50 ml plastic tubes and centrifuged at 6000 rpm for 30 minutes. The supernatant was moved to a new 50 ml tube and 1/5 volume of 20%PEG/2.5M NaCl (approximately 5 ml) was added to the tube. The tube was put in ice for 1 hour, at the end of which "clouds" of phage were visible in the tube. Another centrifugation step followed, at 8000 rpm for 30 minutes. The supernatant was discarded and the pellet containing the library phage particles was resuspended in 10 ml of autoclaved dH_2O. This suspension was centrifuged at 6000 rpm for 10 minutes. The supernatant after this centrifugation step was transferred to a new 50 ml plastic tube and once more, 1/5 volume of 20%PEG/2.5M NaCl (5 ml) was added. The tube was placed in ice for 20 minutes before another round of centrifugation at 8000 rpm for 30 minutes. The supernatant was discarded and the pellet containing the phage was resuspended in 2 ml PBS.

10 µl of this phage particle suspension were used for titrations in order to quantify the amount of phage in the resulting stock material. The 10 µl volume was serially diluted (hundred-fold dilutions in 2xTY medium, 10^{-2} to 10^{-12} of the starting material) and used to infect TG1 strain *E.coli* host cells. 100 µl of each dilution were transferred to sterile 1.5 ml Eppendorf tubes and were used to infect 900 µl TG1 cell culture in 2xTY medium (OD_{600} = 0.4). The infection was allowed to take place over a 30-minute period in a 37^{o} C waterbath. At the end of this incubation period, two volumes of 100 µl of each dilution were spread onto TYE_{AG} plates (duplicates) and all plates were incubated overnight in a 30^{o} C static incubator. Titres of 10^{12} cells/ml or higher are considered satisfactory at this stage.

The remaining volume of phage in PBS buffer was split between sterile 1.5 ml Eppendorf tubes and centrifuged in a bench top minifuge at maximum speed (in this case 13000 rpm) for 5 minutes. The supernatant was transferred to sterile cryo-vials and stored at a -80^{o} C freezer in the form of 20% glycerol stocks before being used in the second round of panning.

The PEG precipitation concluded the first round of panning of the phage antibody library; an additional 2 rounds of panning were performed in order to obtain specific and sensitive binders. Between panning rounds, the conjugate used to coat the immunotube was altered between the BSA- and KLH-bound format and the concentration of coating conjugate was lowered (from 10 μg/ml in the first panning round to 1 μg/ml for the subsequent rounds) in order to ensure the capture of sensitive binders. Representative plates from the phage titrations after the PEG precipitation step in each round of panning were stored at 4^{o}C and were used to select colonies for the monoclonal ELISA (see section 4.13.1.2).

After 3 rounds of panning using TEA in order to elute the phage bound to the target conjugate on the surface of the immunotube, free antigen elution was the next phase in the library screening. In our case, free antigen elution only took place during the screening of the immunised library, using phytane and carotane (10 μM solutions in PBS buffer, using phytane from Sigma Aldrich –cat. no. 80165 and an appropriate β-carotane derivative supplied by Dr. Manisha Sathe, see Figure 4-).

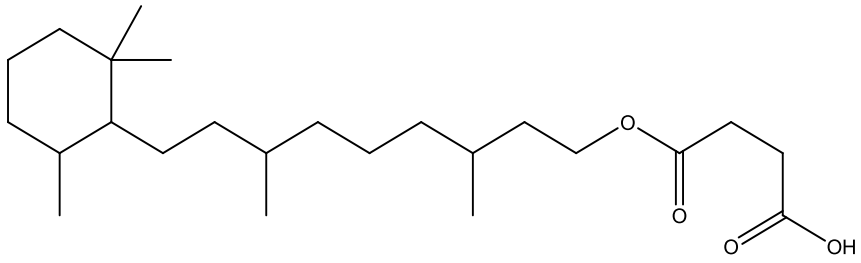

Figure 4-8: Structure of the carotane derivative used in the free antigen elution round of panning of the immunised phage antibody library

The derivatives that are used in the free antigen elution should ideally be identical to the molecules that antibodies will be raised against; in this case this was true for phytane, but the carotane derivative available at the time of studies (Figure 4-) had a carboxyl group on one end of the molecule, making it different to the LMC target carotane molecule (Figure 4-).

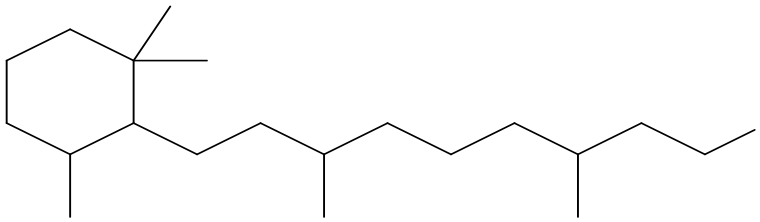

Figure 4-9: Structure of carotane (LMC target)

Panning using free antigen was identical to the previous rounds with the exception of the elution step where, instead of 1 ml of 100 mM TEA solution, 4 ml of the 10 μM antigen solution in PBS buffer were added to the immunotube. After addition of the free antigen solution, the immunotube was rotated for 30 minutes and 2 ml of the contents were removed and split between two sterile 1.5 ml Eppendorf tubes. The immunotube was kept static on the laboratory bench for another 30 minutes, and the remaining 2 ml volume of its contents was transferred to two new Eppendorf tubes. The 30-minute and 60-minute eluted material was used separately to infect two batches of TG1 *E.coli* cells, and the rest of the protocol was the same as for a normal round of panning. The two-step elution separates binders with a high disassociation constant (quick but potentially loose binding to their target) from binders with a lower disassociation constant (slower and potentially stronger binding to their target), allowing the choice of the most appropriate antibodies at later stages of the selection process.

4.13 Enzyme-linked immunosorbent assays (ELISA)

After all panning rounds were complete, enzyme-linked immunosorbent assays (ELISA) were performed, in order to determine whether an enrichment in relevant binders has occurred through the sequential rounds of panning. These diagnostic ELISA are divided into “polyclonal” and “monoclonal” phage assays, based on whether the material used in the assay comes from a mixture of different phage clones or a unique clone.

4.13.1.1 Polyclonal phage ELISA

Polyclonal ELISA were the first assays to employ, and came right after the completion of the three rounds of the library panning process. The material used in these assays was the suspension of phage that resulted from the PEG precipitation. This suspension was a mixture of different phage clones displaying different antibody fragments, so this assay is by no means conclusive about the quality of individual antibodies, but more of a method to acquire an idea of the results from the library panning.

For the polyclonal ELISA, separate rows of wells in a 96-well microtitre plate (Fisher Scientific – cat. no.442404) were coated with 1 μg/ml solutions of the target molecules (carotane and phytane) in their BSA and KLH conjugate format and the carrier proteins (BSA and KLH) alone and incubated overnight at 4^{o}C. All the solutions were in PBS buffer and 100 μl of solution were added to each well. Two rows of wells were coated with each solution, with columns corresponding to the number of panning rounds that had been performed (3 rounds for the naïve library panning and 4 rounds for the immunised library panning). The layout of the coated wells in the microtitre plate for the polyclonal ELISA can be seen in Figure 4-.

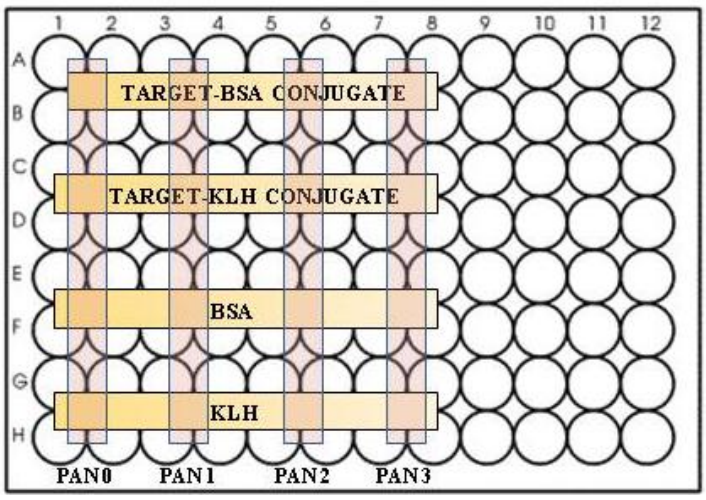

Figure 4-10: Polyclonal ELISA well layout

The next day, the plates were washed two times with PBS buffer (200 μl/well) and tap-dried, before a blocking buffer was added to all wells, consisting of 2% Marvel skimmed milk powder in PBS buffer (2% MPBS). 200 μl of the blocking buffer were

added to each well and the plates were incubated at 37^{o}C for 2 hours. At the end of this incubation period, the plates were washed two times with 0.1% PBST buffer and two times with PBS buffer (200 µl/well) and dried.

The phage suspension was added next, in a mixture of 50 µl 4% MPBS blocking buffer and 50 µl of phage suspension from panning rounds 0-3 ("panning round 0" was the PEG precipitated phage suspension that resulted from the library rescue, before any actual screening for binders). The phage were allowed to bind to the coated wells over 1 hour at room temperature. After this period, the plates were washed as before (2x PBST buffer and 2x PBS buffer) and dried, before the addition of the secondary antibody.

100 µl of an HRP-labelled, anti- bacteriophage M13 antibody (GE Healthcare – cat. no 27-9421-01), at a dilution of 1:1000 of the stock material were added to all wells and incubated for 1 hour at room temperature. After this incubation step, the plates were washed 4x PBST buffer and 2x PBS buffer and dried off.

The addition of the HRP substrate solution followed, where 100 µl of substrate solution of 1 TMB tablet (Sigma Aldrich – cat. no. T3405) in 100 ml of a phosphate-citrate buffer with sodium perborate (Sigma Aldrich – cat. no. P4922) were added to all wells and incubated at room temperature for 1 hour. Blue colour from the reaction of the enzyme and its substrate was allowed to develop over 5-10 minutes, before the reaction was stopped with the addition of 50 µl of a 1M sulphuric acid solution (Fisher Scientific – cat. no. J/8420/17) to all wells. The optical density of the samples at 450 nm was measured using a VarioSkan Flash reader from Thermoscientific.

4.13.1.2 Monoclonal phage ELISA

The TYE_{AG} plates that corresponded to the highest dilutions of phage material from the post-PEG precipitation titrations were stored at 4^{o}C after colonies were counted, and were used to select colonies for the next stage in the antibody development, which is based on individual "phage clones". In order to select clones displaying antibodies that bind to their target with high sensitivity and specificity, unique clones are randomly selected from the last rounds of panning and used in monoclonal ELISA, which is the decisive factor in the choice of clones that are taken forward to antibody production.

In our case, the selection process using the naïve library stopped after the polyclonal ELISA results and monoclonal ELISA data are only available for the immunised library panning.

4.13.1.2.1 Growing individual clones for monoclonal phage ELISA

The first step in the monoclonal ELISA was the picking of 95 different clones (leaving one of the 96 wells in a standard microplate empty to use as a control) from the titration plates using sterile pipette tips to scrape the cells from the solid medium and inoculate them into individual microtitre plate wells (Nunclon 96-well sterile plates from ThermoScientific – cat. no. TKT – 180 – 070U) with 100 µl 2xTY medium (1% glucose and 100 µg/ml ampicillin). The colonies were incubated overnight in a 37^{o}C shaking incubator, at 280 rpm. The following day, a transfer device (VWR – cat. no. 738-0252) was used to transfer a small inoculum from the overnight plate to a new 96-well plate containing 200 µl 2XTY medium (1% glucose and 100 µg/ml ampicillin) in each well. The new plate was incubated in a 37^{o}C shaking incubator, at 280 rpm for 2 hours, while the overnight plate was stored at -80^{o}C after addition of glycerol to a final concentration of 15% in each well.

After the incubation step, 25 µl of 2xTY medium (1% glucose and 100 µg/ml ampicillin) containing 10^{9} *pfu* helper phage M13KO7 were added to all wells and helper phage infection was allowed to take place during a 30-minute incubation in a 37^{o}C static incubator, followed by 1 hour in a 37^{o}C shaking incubator, at 280 rpm. After infection, the plates were centrifuged at 2500 rpm for 10 minutes, the supernatants from each well were discarded and the pellets were resuspended in 150 µl of 2xTY medium (100 µg/ml ampicillin and 50 µg/ml kanamycin). The plates were incubated overnight in a 30^{o}C shaking incubator at 280 rpm.

The following day, the contents of the microtitre plates were centrifuged at 2500 rpm for 10 minutes and the resulting supernatants were transferred to new microtitre plates and stored at -20^{o}C before being used in the monoclonal ELISA.

4.13.1.2.2 Monoclonal phage ELISA protocol

96-well microtitre plates (Fisher Scientific – cat. no. DIS 971 030J) were coated with 1 µg/ml solution of target/BSA conjugate and BSA alone (one full plate of each coating solution), both in PBS buffer. 100 µl of solution were added to each well and the plates were incubated at 4^{o}C overnight. The following day, the plates were washed three times with 0.1% PBST washing buffer (200 µl/well) followed by three washes with PBS buffer (200 µl/well) and tap-dried. The wells were blocked using 200 µl/well of 2% MPBS blocking buffer and incubated at 37^{o}C for 2 hours (static incubation).

The plates were washed as before (3x PBST and x PBS buffer) and dried, before a mixture of 50 µl 4% MPBS and 50 µl of phage supernatant was added to all wells. The plates were incubated for 1 hour at room temperature, then washed as before and dried. 100 µl of the HRP-labelled, anti- bacteriophage M13 antibody, at a dilution of 1:1000 of the stock material were added to all wells and incubated for 1 hour at room temperature. After this incubation step, the plates were washed as before and dried off.

The addition of the HRP substrate solution followed and incubation took place at room temperature for 1 hour. Blue colour from the reaction of the enzyme and its substrate was allowed to develop over 5-10 minutes, before the reaction was stopped with the addition of 50 µl of a 1M sulphuric acid solution to all wells. The optical density of the samples at 450 nm and 650 nm was measured using a VarioSkan Flash reader from Thermoscientific.

The monoclonal phage ELISA was repeated using the KLH conjugates of both targets, before conclusions could be drawn concerning the quality of the selected clones.

Results

4.14 Immunisation ELISA (polyclonal sera)

During the immunisation period, serum samples from blood collected from the two sheep (CF68 and CF69) at regular intervals (test bleeds) were used in binding ELISA, in order to determine the progress of the immunisation (all ELISA performed by Immunosolv Ltd.). During these "test bleeds", both sheep showed an increase in antibody response with increase in number of boosts but sheep CF68 showed a higher antibody response than sheep CF69, therefore sera from sheep CF68 were the ones that were taken forward and were used in the library construction. The ELISA results can be seen in Figure 4- for phytane (which had been present in the immunisation mixture from the beginning) (Figure taken from Immunosolv Ltd.). As β-carotane was only added at the final immunisation boost, its immunogenic potential only became apparent in later stages, during the screening of the immunised library.

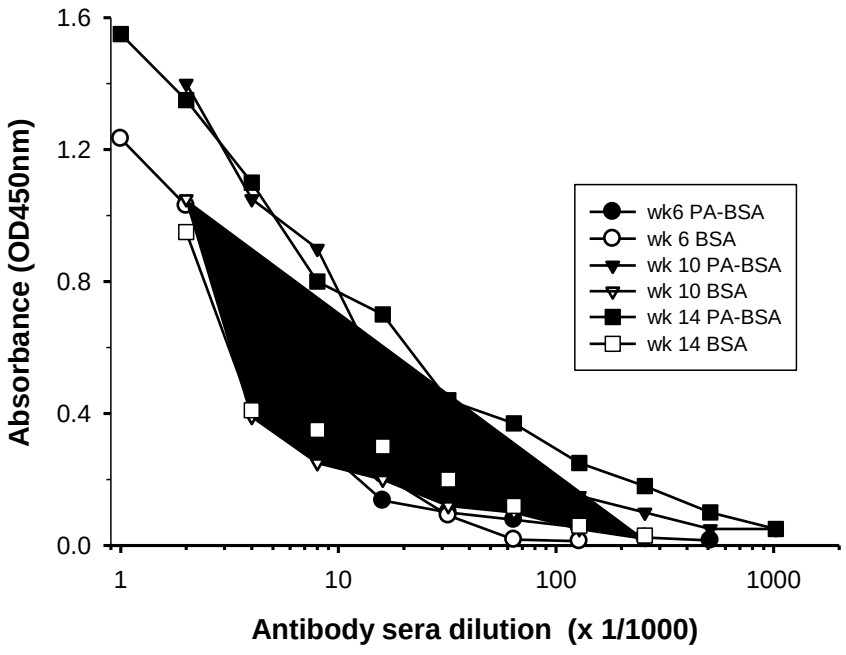

Figure 4-11: Progress of the antibody binding response of sheep CF68 blood sera to phytanic acid conjugated to BSA from week 6 to week 14 of the immunisation process (Figure provided by Immunosolv Ltd.)

As far as the immunogenic potential of phytane is concerned, it is apparent from Figure 4- that the antibody response to it was increasing between boosts.

4.15 Naïve phage antibody library

In the rescue and screening of the naïve phage antibody library, it was deemed necessary to grow the κ and λ light chains separately. This decision aimed to increase the chances of selecting relevant binders from the library later in the panning process, although it is generally accepted that λ light chains present more utility potential than the κ light chains (Charlton personal communication).

4.15.1.1 Titration results: library rescue and pan rounds 1-3

The titrations that were carried out during the rescue and panning process allowed the quality monitoring of the procedure and the control of contaminations. The titration results from the library rescue can be seen in Table 4- and the titrations from the panning for binders against carotane and phytane can be seen in Table 4-.

Table 4-3: Naïve phage antibody library rescue titration results

	Library rescue	
	Pre- PEG titration	**Post-PEG titration**
κ	5.8×10^7	4.29×10^{12}
λ	3×10^7	5×10^{12}

Table 4-4: Titrations from the naïve library panning for binders against carotane and phytane

	Pan 1			Pan 2			Pan 3		
	Pre-PEG titration	**M13KO7 infection**	**Post-PEG titration**	**Pre-PEG titration**	**M13KO7 infection**	**Post-PEG titration**	**Pre-PEG titration**	**M13KO7 infection**	**Post-PEG titration**
CAROTANE	3×10^3	3.5×10^6	3.2×10^{11}	5×10^3	2×10^7	Confluent growth	1.27×10^8	1.68×10^8	Confluent growth
PHYTANE	6×10^3	7.1×10^6	3.3×10^{11}	5×10^4	1.7×10^7	Confluent growth	1.5×10^8	1.9×10^8	Confluent growth

The titration results for the library rescue were reasonable, considering the library's initial size (2×10^7 clones for both the κ and λ component of the library). It is generally acceptable that in the beginning of each round of panning – especially for the first rounds – the titration results from the eluted phage are low, followed by an increase in the number of colonies in the subsequent steps of a panning round, i.e. amplification and concentration. As far as the panning process was concerned, titrations from pan 1 are within the values normally expected for a first round of screening, with a low number of binders binding to the immunotube and being amplified in the next steps of the panning round, and the same could be said for pan 2 and pan 3 results, with the exception of the post-PEG precipitation titration plates which showed confluent bacterial colony growth. Confluent growth is not the result of a successful PEG-precipitation, but usually the result of contamination. It may also be an indication of a failed round of panning, as excessive stringency in the selection process can result to the suppression of the clones containing the inserts of interest and the domination of more resistant clones with different inserts.

4.15.1.2 Polyclonal phage ELISA results

At the end of the three rounds of panning, polyclonal ELISA was performed, in accordance with the procedure described in section 4.13.1.1. The ELISA results from panning for binders against phytane can be seen in Figure 4-A, while Figure 4-B shows the results obtained from panning for binders against carotane.

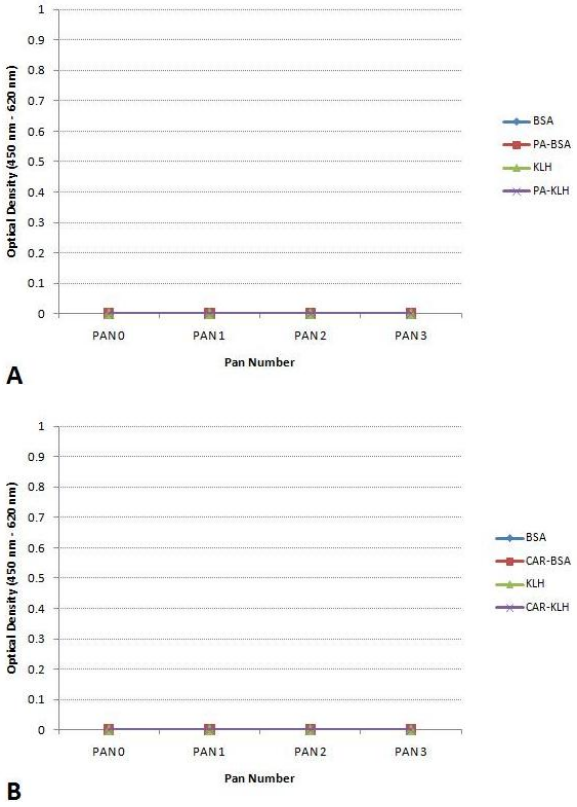

Figure 4-12: Polyclonal phage ELISA results from the panning of the naïve phage library for binders against phytane (A) and β-carotane (B)

It is obvious from the polyclonal ELISA results that the panning of the naïve library did not result in the isolation of binders against either one of the two targets.

4.16 Immunised phage antibody library

4.16.1.1 Library rescue

Similarly to the rescue of the naïve phage antibody library, the immunised library was rescued not as a whole, but the 8 sub-libraries were grown and stored separately, only to be pooled together immediately before the first round of panning. This practice is designed around preserving the diversity of the sub-libraries, despite the higher utility potential of the λ families. The titration results from the rescue of the 8 sub-libraries can be seen in Table 4-.

Table 4-5: Immunised library rescue titration results

	Library rescue	
	Pre- PEG titration	**Post-PEG titration**
MuVH1-Vλ	4×10^8	1.7×10^{13}
MuVH1-Vκ	2.7×10^8	2.5×10^{13}
Hu4a-Vλ	3×10^8	2.6×10^{13}
Hu4a-Vκ	6×10^8	8×10^{12}
VH1-Vλ	3.3×10^8	5×10^{13}
VH1-Vκ	1.03×10^8	1.9×10^{13}
VH2-Vλ	4×10^8	8.4×10^{13}
VH2-Vκ	1.7×10^8	6.2×10^{13}

The library rescue titres were considered reasonable and the 8 sub-libraries were pooled together in order to be screened for binders against carotane and phytane. The representation ratio of λ to κ libraries in the mixture that was used in the panning was 9:1, corresponding to the κ/λ ratios in the active immune system of sheep. The titration results from the immunised library panning can be seen in Table 4-6.

Table 4-6: Titrations from the immunised library panning for binders against carotane and phytane (results in Pan 4 are presented for both free antigen elution periods; the upper line corresponds to the 30-minute elution and the lower line to the 60-minute elution period)

	Pan 1			**Pan 2**		
	Pre-PEG titration	**M13KO7 infection**	**Post-PEG titration**	**Pre-PEG titration**	**M13KO7 infection**	**Post-PEG titration**
CAROTANE	7×10^3	5.3×10^8	1×10^{12}	2×10^5	7×10^7	4.3×10^{13}
PHYTANE	4.5×10^3	6×10^7	8.2×10^{13}	5×10^3	1.1×10^8	4.8×10^{13}
	Pan 3			**Pan 4**		
	Pre-PEG titration	**M13KO7 infection**	**Post-PEG titration**	**Pre-PEG titration**	**M13KO7 infection**	**Post-PEG titration**
CAROTANE	3×10^7	1×10^7	Confluent growth	2×10^7 1.5×10^8	5×10^8 1×10^{10}	4.8×10^9 3.1×10^9
PHYTANE	2.5×10^3	4×10^7	Confluent growth	2.5×10^6 1.3×10^7	3.5×10^9 5×10^9	1×10^{12} 8×10^8

The overall conclusion from Table 4-6 is that the titration results generally followed a reasonable trend according to what would be expected from panning an immunised library against similar targets (gradual enrichment in clones of interest between Pan 1 and Pan 4) with the exception of the post-PEG precipitation titrations in Pan 3, where confluent bacterial growth was observed (for possible explanations of confluent bacterial colony growth, see section 4.15.1.1).

4.16.1.2 Library Panning

4.16.1.2.1 Polyclonal phage ELISA

Similarly to the steps that followed the panning of the naïve phage antibody library, at the end of the three rounds of the immunised library panning, polyclonal ELISA were

performed, in accordance with the procedure described in section 4.13.1.1. The ELISA results from panning for binders against phytane can be seen in Figure 4-8A, while Figure 4-8B shows the results obtained from panning for binders against carotane.

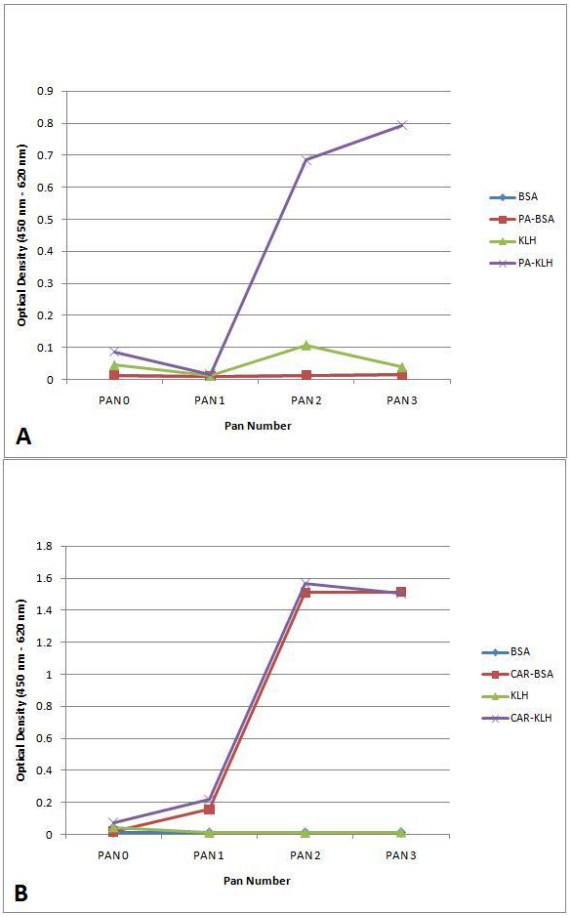

Figure 4-8: Polyclonal phage ELISA results from the immunised library panning for binders against phytane (A) and carotane (B)

From the polyclonal ELISA results it is obvious that enrichment for relative binders has occurred for both targets between Pan 1 and Pan 3, with carotane being recognised in both its BSA- and KLH-conjugate form, while phytane is only recognised in its KLH-conjugate form. The recognition of free carrier proteins is very low for both targets and both carriers used (BSA and KLH). The polyclonal ELISA was performed before Pan 4 (free antigen elution), and after Pan 4 had been completed, to confirm the recognition of the target conjugates, and the results from Pan 4 confirmed the results from Pan1-3. The

phage suspension from Pan 4 recognised carotane in its conjugated form (both the BSA- and KLH-conjugate) but not free BSA or KLH. As far as phytane was concerned, Pan 4 phage recognised phytane-KLH, didn't appear to recognise phytane-BSA and did not recognise free BSA or KLH (results not shown).

4.16.1.2.2 Monoclonal phage ELISA

After the completion of the polyclonal ELISA, individual "phage clones" were selected to be taken forward in the selection process, and the quality of the binders they were displaying was examined via monoclonal phage ELISA (see section 4.13.1.2.2). The detailed results from the monoclonal ELISA can be seen in Figure 4-9 to Figure 4-11. These results are presented in table format as this is a screening assay and not a complete curve for each clone. For carotane, where polyclonal phage ELISA had shown recognition of both conjugates (BSA- and KLH-based), monoclonal ELISA were performed using both conjugates and both carrier proteins (Figure 4-10 for carotane-KLH and Figure 4-11 for carotane-BSA). In the case of phytane, where only phytane-KLH appeared to be recognised by the library phage, monoclonal phage ELISA was performed using only the KLH conjugate (Figure 4-9).

Binding of 95 Phytane clones to KLH (4i)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.009632	0.011943	0.016552	0.012351	0.012033	0.011718	0.014021	0.011215	0.015466	0.01512	0.014827	0.01522
B	0.009783	0.012392	0.01029	0.01224	0.010714	0.008495	0.009982	0.024307	0.015865	0.010178	0.022703	0.019148
C	0.009766	0.009226	0.00951	0.011007	0.009836	0.020014	0.015327	0.01447	0.014	0.011277	0.009991	0.015755
D	0.012653	0.010735	0.012071	0.01016	0.009746	0.011009	0.010435	0.011361	0.009801	0.010825	0.011248	0.015551
E	0.012712	0.013735	0.010758	0.011388	0.009079	0.063678	0.014614	0.014398	0.011606	0.021343	0.011231	0.013676
F	0.025623	0.009201	0.014787	0.024807	0.024926	0.011413	0.010984	0.026465	0.015845	0.010837	0.012182	0.027267
G	0.013591	0.011901	0.009921	0.010342	0.015032	0.01117	0.012748	0.014927	0.019504	0.011747	0.01035	0.018519
H	0.02586	0.025972	0.014645	0.012358	0.035131	0.011508	0.053303	0.014637	0.010691	0.01695	0.029908	0.008937

Binding of 95 Phytane clones to Phytane-KLH (4i)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.010474	0.309631	0.445951	0.009196	0.010013	0.009332	0.513346	0.01135	0.009683	0.012818	0.881162	0.02376
B	0.725668	0.736618	0.286643	0.009344	0.008726	0.00867	0.009603	0.332307	0.009861	0.010146	0.014938	0.017454
C	0.643936	0.350358	0.008828	0.008737	0.0088	0.008531	0.132895	0.010221	0.414316	0.010212	0.010176	0.012408
D	0.675394	0.218898	0.009403	0.008283	0.008823	0.009058	0.009083	0.19798	0.01013	0.010229	0.018062	0.451815
E	0.011916	0.288318	0.009267	0.360463	0.009359	0.076766	0.00986	0.009326	0.011797	0.607728	0.016951	0.088391
F	0.460598	0.00994	0.009751	0.274527	0.013387	0.010999	0.010158	0.009561	0.010454	0.009952	0.788701	1.272859
G	0.088912	0.009801	0.010203	0.008966	0.009536	0.009094	0.010673	0.009488	0.01114	0.010805	0.011318	0.310401
H	0.066552	0.010791	0.009743	0.009922	0.428112	0.011558	0.767319	0.010967	0.010609	0.009947	0.010211	0.009511

Binding of 95 Phytane clones to KLH (4ii)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.013466	0.013067	0.020403	0.0135	0.017739	0.011735	0.018461	0.017154	0.01113	0.015879	0.013617	0.014813
B	0.020842	0.01225	0.020945	0.014191	0.010087	0.008659	0.009005	0.009226	0.015079	0.01239	0.015961	0.016471
C	0.022283	0.010083	0.009481	0.011263	0.010268	0.012887	0.009782	0.009767	0.013404	0.011572	0.012405	0.010603
D	0.018857	0.009813	0.022508	0.009273	0.009475	0.010159	0.010259	0.012343	0.010719	0.01249	0.011615	0.019157
E	0.009857	0.009601	0.01009	0.010385	0.010593	0.009905	0.009083	0.014989	0.010888	0.014938	0.019196	0.016616
F	0.010223	0.013032	0.009608	0.010804	0.00856	0.010333	0.008808	0.011972	0.009791	0.013465	0.010938	0.01102
G	0.010294	0.009857	0.007883	0.00912	0.016171	0.010566	0.01079	0.011362	0.011814	0.010619	0.012859	0.010962
H	0.012391	0.010407	0.009643	0.01123	0.013323	0.011543	0.017584	0.018357	0.045246	0.018736	0.010477	0.008858

Binding of 95 Phytane clones to Phytane-KLH (4ii)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.014058	0.009201	0.349961	0.05958	0.009117	0.008274	0.143576	0.012268	0.015935	0.010957	0.014641	0.014098
B	0.00884	0.077127	0.405891	0.012759	0.014304	0.00828	0.280702	0.01113	0.62199	0.080002	0.367907	0.020241
C	0.009968	0.010569	0.341628	0.68418	0.014308	0.009348	0.751011	0.02215	0.009955	0.009437	0.010595	0.013437
D	0.009443	0.009105	0.170025	0.283958	0.009547	0.011018	0.008625	0.015071	0.008737	0.009009	0.00934	0.013672
E	0.020154	0.008834	0.00857	0.381889	0.01195	0.00815	0.404601	0.324597	0.009696	0.009662	0.009263	0.01084
F	0.11902	0.855951	0.009621	0.449815	0.009085	0.009737	0.008433	0.010046	0.013259	0.009506	0.009761	0.010342
G	0.012326	0.410647	0.022549	0.00968	0.010074	0.008853	0.009499	0.008893	0.009436	0.010161	0.010118	0.009548
H	1.055243	0.405315	0.546457	0.634924	0.008984	0.008828	0.609608	0.748247	0.009978	0.009457	0.010007	0.009023

Figure 4-9: Monoclonal phage ELISA data for phytane (phytane-KLH). The data in green colour correspond to OD measurements between 0.05 and 0.3, the data in orange colour correspond to OD measurements between 0.3 and 0.5 and the data in red colour correspond to OD measurements >0.5 (4i/4ii correspond to the two free antigen incubation periods of 30 and 60 min respectively)

Binding of 95 Carotane clones to KLH (4i)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.016736	0.029969	0.015013	0.015105	0.017102	0.012834	0.01322	0.021096	0.011599	0.032869	0.013689	0.023747
B	0.012762	0.010953	0.022091	0.029785	0.013345	0.013711	0.01069	0.011288	0.00928	0.022602	0.011455	0.01452
C	0.015709	0.013524	0.014644	0.012815	0.011129	0.011474	0.010762	0.010542	0.011128	0.012312	0.013227	0.012761
D	0.015404	0.012023	0.012526	0.01087	0.01032	0.011186	0.012497	0.013589	0.009754	0.010944	0.013189	0.021537
E	0.011008	0.01177	0.009482	0.012999	0.011038	0.015606	0.011873	0.012476	0.010176	0.014253	0.011014	0.024101
F	0.015174	0.010091	0.011358	0.011027	0.021908	0.011502	0.012458	0.012773	0.009816	0.011374	0.012382	0.013531
G	0.010284	0.012417	0.01034	0.013199	0.010091	0.008404	0.011305	0.011358	0.010965	0.011658	0.01609	0.011881
H	0.011099	0.014325	0.010309	0.010927	0.011915	0.012001	0.01139	0.014754	0.01162	0.01555	0.013354	0.010086

Binding of 95 Carotane clones to Carotane-KLH (4i)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.12343	0.409459	0.011338	0.011532	0.012541	0.013225	0.012829	0.012157	0.012065	0.355279	0.26948	0.364928
B	0.010792	0.010573	0.169471	0.291849	0.087288	0.01205	0.012374	0.013021	0.011007	0.749297	0.010358	0.016175
C	0.010447	0.078276	0.095181	0.010787	0.010312	0.010425	0.011077	0.010421	0.011049	0.301556	0.010466	0.860987
D	0.466562	0.010251	0.229116	0.009827	0.01046	0.010759	0.010703	0.010958	0.010436	0.011041	0.012139	0.989566
E	0.027722	0.101817	0.010186	0.011041	0.010626	0.250859	0.011313	0.017601	0.012148	0.203814	0.011522	0.793357
F	0.261296	0.010216	0.18188	0.01	0.011586	0.010584	0.342547	0.465384	0.01045	0.010552	0.331226	0.854058
G	0.01434	0.043236	0.305321	0.01101	0.05573	0.01056	0.010705	0.368303	0.011533	0.012291	0.747358	0.031922
H	0.240857	0.110043	0.011505	0.240723	0.016846	0.011327	0.011929	0.392644	0.011387	0.208544	0.011717	0.011

Binding of 95 Carotane clones to KLH (4ii)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.010227	0.010114	0.010222	0.010932	0.01171	0.012102	0.011332	0.010553	0.010508	0.060683	0.012172	0.015119
B	0.009874	0.0141	0.011284	0.009922	0.011367	0.010057	0.009654	0.010022	0.012346	0.010987	0.014453	0.014384
C	0.013044	0.009924	0.008818	0.011826	0.016509	0.010784	0.009894	0.009941	0.018967	0.013943	0.012846	0.01411
D	0.01296	0.010728	0.0094	0.010786	0.009388	0.010819	0.010292	0.014654	0.009836	0.011759	0.012503	0.019051
E	0.012037	0.010132	0.009976	0.011287	0.009768	0.010676	0.010691	0.011432	0.010772	0.012571	0.026699	0.019693
F	0.01941	0.013757	0.015037	0.010721	0.009281	0.012884	0.010311	0.010414	0.010867	0.01253	0.014886	0.015194
G	0.010385	0.010629	0.008963	0.010483	0.010704	0.011356	0.011585	0.011671	0.010956	0.011426	0.010641	0.019909
H	0.019475	0.017287	0.01063	0.012256	0.042684	0.016554	0.014022	0.013884	0.013066	0.023593	0.01795	0.012875

Binding of 95 Carotane clones to Carotane-KLH (4ii)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.011808	0.011053	0.011214	0.010491	0.011853	0.217922	0.011412	0.458921	0.011067	0.153416	0.155793	0.337206
B	0.010006	0.228221	0.010879	0.010687	0.18851	0.203353	0.20567	0.011423	0.010369	0.011088	0.402052	0.011211
C	0.135447	0.009743	0.010099	0.009918	0.096136	0.184014	0.535283	0.010212	0.364252	0.249109	0.30338	0.263863
D	0.010197	0.010484	0.00977	0.214694	0.009466	0.158891	0.122654	0.268693	0.009978	0.954132	0.013739	0.664707
E	0.010309	0.011039	0.010443	0.028971	0.010048	0.256015	0.22934	0.440114	0.012245	0.252119	0.250344	0.828575
F	0.010129	0.065195	0.506966	0.010198	0.010782	0.188112	0.262728	0.012324	0.009944	0.154888	0.011541	0.41917
G	0.009538	0.009864	0.010534	0.010141	0.156113	0.106252	0.314038	0.147386	0.016119	0.011564	0.06527	0.269642
H	0.448534	0.010819	0.010548	0.010694	0.178484	0.269218	0.011425	0.94436	0.09649	0.279379	1.015357	0.0112

Figure 4-10: Monoclonal phage ELISA data for carotane (carotane-KLH). The data in green colour correspond to OD measurements between 0.05 and 0.3, the data in orange colour correspond to OD measurements between 0.3 and 0.5 and the data in red colour correspond to OD measurements >0.5 (4i/4ii correspond to the two free antigen incubation periods of 30 and 60 min respectively)

Binding of 95 Carotane clones to BSA (4i)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.010831	0.00924	0.010335	0.010091	0.009659	0.009566	0.010243	0.010515	0.009946	0.009925	0.010563	0.009994
B	0.009171	0.009933	0.009593	0.009648	0.009908	0.009251	0.009481	0.009603	0.009678	0.009779	0.010099	0.009589
C	0.01032	0.009433	0.008916	0.009478	0.008914	0.010675	0.009367	0.009357	0.010057	0.009841	0.009365	0.00956
D	0.010003	0.009801	0.009658	0.009661	0.009326	0.009324	0.009125	0.009285	0.009366	0.009788	0.009063	0.010279
E	0.00985	0.009531	0.009977	0.009495	0.008518	0.009193	0.009758	0.009872	0.009297	0.010043	0.009105	0.010871
F	0.010244	0.010541	0.009329	0.009571	0.009552	0.009971	0.008632	0.010001	0.009874	0.010423	0.009879	0.010152
G	0.009889	0.009889	0.009416	0.009814	0.009551	0.009667	0.009718	0.010073	0.010555	0.01184	0.009823	0.010466
H	0.00848	0.011241	0.00957	0.010295	0.00987	0.009881	0.010144	0.01018	0.010069	0.010336	0.010455	0.010125

Binding of 95 Carotane clones to Carotane-BSA (4i)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.155093	0.125815	0.010684	0.00828	0.009376	0.008351	0.009923	0.008675	0.010445	0.06904	0.125672	0.128423
B	0.010631	0.01061	0.133202	0.215102	0.020784	0.008573	0.009369	0.010563	0.010031	0.21798	0.010477	0.009799
C	0.021588	0.221427	0.158629	0.009715	0.009755	0.010304	0.00905	0.008666	0.009743	0.193884	0.010497	0.550845
D	0.345859	0.009201	0.070267	0.009287	0.01564	0.008993	0.008522	0.01018	0.009297	0.010624	0.009787	0.491809
E	0.104695	0.12163	0.058969	0.009221	0.009081	0.09539	0.010914	0.01054	0.00797	0.117851	0.009772	0.597033
F	0.227478	0.009763	0.081233	0.008993	0.011217	0.010024	0.114473	0.158403	0.010257	0.010715	0.185112	0.662614
G	0.128673	0.13968	0.124145	0.010064	0.052892	0.009994	0.009016	0.111797	0.011152	0.010735	0.188003	0.019915
H	0.567722	0.131512	0.009841	0.363796	0.010415	0.010604	0.010814	0.327467	0.011957	0.237929	0.010945	0.011227

Binding of 95 Carotane clones to BSA (4ii)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.010037	0.011308	0.011666	0.011618	0.012646	0.01117	0.01221	0.016435	0.012238	0.010529	0.010345	0.012067
B	0.010173	0.010425	0.009862	0.010579	0.010126	0.015778	0.014797	0.016076	0.012555	0.009762	0.011821	0.012493
C	0.010294	0.012311	0.009593	0.020367	0.016445	0.012259	0.012	0.009865	0.011705	0.009423	0.010087	0.009963
D	0.00929	0.009409	0.013055	0.010866	0.022162	0.016686	0.012586	0.009941	0.009954	0.012135	0.010823	0.011153
E	0.010626	0.012631	0.009749	0.009227	0.00797	0.009496	0.00996	0.009559	0.009938	0.012509	0.010183	0.012829
F	0.013317	0.010038	0.009234	0.009369	0.009285	0.010053	0.009828	0.010696	0.010463	0.012984	0.010335	0.010735
G	0.011105	0.013379	0.010049	0.009881	0.00882	0.009314	0.010458	0.010694	0.010671	0.0138	0.011311	0.011884
H	0.010717	0.014901	0.013085	0.011257	0.009476	0.010045	0.011613	0.013174	0.011519	0.01101	0.01153	0.011488

Binding of 95 Carotane clones to Carotane-BSA (4ii)

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.008618	0.009577	0.009181	0.00782	0.01074	0.377297	0.010253	0.171724	0.010598	0.14197	0.121503	0.168369
B	0.007533	0.134124	0.008185	0.008891	0.144831	0.127263	0.153288	0.010506	0.011372	0.008612	0.212748	0.014456
C	0.315036	0.007961	0.011227	0.017549	0.129149	0.094043	0.149567	0.008681	0.168278	0.118926	0.241236	0.168672
D	0.008655	0.008437	0.008558	0.117907	0.015149	0.101716	0.038599	0.103787	0.009591	0.226962	0.013749	0.665582
E	0.00833	0.14727	0.012177	0.046077	0.01186	0.23069	0.213675	0.077186	0.013236	0.102662	0.065422	0.340716
F	0.009366	0.103836	0.155635	0.009513	0.011332	0.144762	0.166382	0.194271	0.194618	0.094579	0.010769	0.386959
G	0.016845	0.00992	0.009783	0.009749	0.206096	0.127596	0.239385	0.150225	0.019916	0.011165	0.026667	0.221462
H	0.231666	0.011114	0.01006	0.010426	0.148041	0.233855	0.011159	0.273259	0.137563	0.259236	0.290112	0.030267

Figure 4-11: Monoclonal phage ELISA data for carotane (carotane-BSA). The data in green colour correspond to OD measurements between 0.05 and 0.3, the data in orange colour correspond to OD measurements between 0.3 and 0.5 and the data in red colour correspond to OD measurements >0.5 (4i/4ii correspond to the two free antigen incubation periods of 30 and 60 min respectively)

It is evident from Figure 4-9, Figure 4-10 and Figure 4-11 that for both phytane and carotane, there appear to be individual clones that bind to the hapten-carrier protein conjugate, but not the carrier protein alone, suggesting recognition of the hapten (target) end of the conjugate. More specifically, in the ELISA screening for clones that could recognise phytane-KLH approximately 10% of clones recognised it with low affinity (signal between 0.05 and 0.3), another 7% recognised it with medium affinity (signal between 0.3 and 0.5) and a 9% of clones recognised it with high affinity (signal over 0.5). In the case of the recognition of carotane-KLH, approximately 9% of clones recognised it with low affinity, 24% with medium affinity and approximately 6% recognised it with high affinity. For carotane-BSA the results were similar, with 4% of clones recognising it with low affinity, 39% of clones with medium affinity and approximately 3% with high affinity. In all ELISAs, it was observed that the clones that were isolated after the 60-minute incubation with free antigen presented an overall increased recognition of their respective conjugates.

Due to time restrictions, none of the clones that were shown to recognise their respective conjugate were taken forward to competition ELISA to determine whether they were able to recognise free antigen. Nevertheless, these studies showed that clones that were isolated from the use of an immunised library were able to recognise the conjugated forms of their antigens, while the use of a naïve library failed to give such clones.

Discussion

4.17 Limitations of recombinant antibody technology for hapten targets

4.17.1.1 The design and quality of libraries

Library quality is paramount to the isolation of high affinity antibodies. A library of insufficient size or diversity is likely to lack the potential to produce the best quality binders against a certain target. This is especially true for naïve libraries, particularly when they are used for hapten target antigens. In naïve libraries, the affinity of the

antibodies that can come from it is proportional to the size of the library, and this fact combined with the structural and compositional nature of haptens, suggests that only a library of significant size/diversity of clones could yield acceptable results.

In the case of immunised libraries, the design of the immunogens that will be used in the immunisation of the donor organism will greatly influence the quality of the resulting library of clones. Conjugates using the wrong carrier protein, conjugates bearing spacers of insufficient or excessive length or unfavourable structure, and conjugates where hapten exposure is not optimal can all result to a collection of antibody fragments that lack the ability to recognise the hapten end of the conjugate.

Where anti-hapten antibodies are concerned, it is quite usual that the antibodies developed are able to recognise a combination of the hapten and carrier protein molecule ('interface binders"), or other parts of the conjugate (spacer etc.), but not the free hapten in the absence of the carrier protein. In many cases, a low molecular weight target will not occupy the entire binding site on the surface of an antibody. The hapten will insert itself into a cavity formed by the antigen-binding site, leaving room for more contacts to be made between the carrier and the residues of the binding site. As a consequence, the affinity for the free hapten is lower than that of the hapten-carrier conjugate (Persson *et al.*, 2006). These issues can be overcome by designing -wherever possible- conjugates to be used as immunogens where everything is taken into consideration, from the optimal carrier protein to be used, to the choice of the spacer and the orientation/position of the hapten. Some problems arising from the use of non-ideal immunogens can be overcome during the selection process, especially if alternative versions of the hapten conjugate (different carrier protein, different spacer, different connection to the hapten) are available for the screening.

4.17.1.2 pIII as an insertion frame

The insertion frame that was used in the present studies was the minor coat protein pIII. In library design and construction, the use of pIII as the insertion frame for the foreign peptides may cause certain problems. Although pIII is more tolerant to insertions than other phage proteins (pVIII for instance), this may lead to infectivity reduction of the

resulting phage (Russel *et al*., 2004). The use of phagemid vectors, which results to the production of phage particles which also have wild-type protein pIII, helps to overcome this issue. These hybrid virions do not rely on the infectivity of the fusion pIII protein, and this allows the fusion to be designed around the specifications of the foreign peptide.

4.17.1.3 Issues with the use of phagemid vectors/monovalent display

The display system that was used in the present work was based on monovalent display of the antibody fragments on the surface of the virions. Despite the advantages of monovalent display, especially for LMC-type targets where the production of high affinity antibodies is crucial, it has been reported that it may lead to the selection of clones with unexpected structures (e.g. internal duplications resulting to bivalency, weak affinity clones present in high numbers) (Russel *et al*., 2004). This is the result of over-selection within the library, via many rounds of selection for instance, past the point where the clones of desired properties were dominant in the library population. Issues like these can be overcome by careful design (number of rounds, stringency of each round) and strict monitoring of the selection process, as well as collection of samples from each round of selection that can be analysed as a means of tracing the beginning of the problem.

4.17.1.4 Expression editing

Another issue to consider when using a library for phage display is that all library clones will not be displayed with equal efficiency. The fitness of the library clones is by no means identical, and some sequences will not be represented at all. This could mean that for a given target, the optimal (highest affinity) antibody could not be displayed at all and subsequently never isolated. Other sequences could be toxic to the host cells or obstruct phage assembly or be sensitive to digestion from bacterial proteases. These issues with clone (and subsequent antibody) representation in a library are collectively referred to as "expression editing" by Russel and colleagues (Russel *et al*., 2004), and it must be taken into consideration in the evaluation of the results from a given library,

even more so when the library screening is against small molecules like the LMC targets examined in the present studies, which present the challenges discussed above.

4.18 Screening the libraries for antibodies against phytane and β-carotane

4.18.1.1 Naïve library screening

The screening of the naïve library for binders against phytane and β-carotane was composed of three panning rounds of increasing stringency, in the form of lowered concentration of coating antigen from round to round. At the end of the three rounds, binding ELISA were performed, and the unsatisfactory results that were obtained for both targets (Figure 4-), lead to no further rounds of panning. The titration results from each round of selection (Table 4-) did not appear unreasonable, but it became apparent from the Pan 3 titration results that they were by no means optimal. The titration results are viewed as a rough quality control of each step of the procedure, rather than a true indication of the success of the process, which can only be acquired from the binding ELISA data. The confluent growth which was observed in the post-PEG concentration titration plates in Pan 3 for both targets was also pointing at a possible over-selection pressure on the library, with the desired clones being overtaken by irrelevant clones of increased fitness.

These results were not unexpected; the initial small library size (10^7 clones) and the fact that it had not been raised against our particular target mixture lowered the chances of success. The fact that the library had been raised against different but similar size molecules did not cause a positive bias in the isolation of high-affinity clones against our own targets.

The outcome of the screening of the naïve library was an indication that, in the context of the development of the LMC instrument, with all the limitations in time and resources it involved, the use of naïve libraries is not recommended.

4.18.1.2 Immunised library screening

The immunised library which was used to generate antibodies against a mixture of LMC targets (including phytane from the beginning, with β-carotane being added at the final round of immunisation) was raised against own-synthesised conjugates of these hapten targets. This offered the possibility to design the immunogens based on a set of criteria for optimal target presentation to the immune system, using a carrier protein (KLH) and spacer structure and length that were considered appropriate. The polyclonal sera samples taken from the immunised sheep and analysed for recognition of phytane conjugate to BSA showed a definite increase in response between immunisation boosts (Figure 4-), and while it was not possible to examine the progress of the response to β-carotane due to its late addition, the results (titrations and post-screening binding ELISA) showed that this delay in its addition did not result to the absence of phage clones that could recognise it. A possible explanation for this result could be that the structural similarities between phytane and carotane meant that the early addition of phytane created a positive bias for carotane recognition.

More specifically, in the course of three panning rounds using the conjugated forms of both targets (increasing the selection stringency by lowering the concentration of the coating antigen) and one additional round with free antigen elution, it became possible to isolate a phage subpopulation that were displaying antibodies against the conjugated forms of phytane and β-carotane. This was obvious from the titration results during the biopanning procedure (Table 4-6), and even more so from the polyclonal binding ELISA performed upon completion of the screening (Figure 4-8). Both targets gave similar results; a notable difference was that the polyclonal ELISA for β-carotane showed recognition of both conjugates (BSA and KLH conjugate), while in the case of phytane there was no recognition of the BSA conjugate. The clones' ability to recognise one conjugate but not the other and none of the carriers alone may indicate that what they are actually binding to is an area composed of parts of KLH and the antigen (interface binders).

At a further step of the selection process, monoclonal ELISA was run for the two targets (the phytane-BSA conjugate was not used, as there was no recognition of it), and it was shown that a number of individual clones were able to recognise the conjugates but not

the carriers alone, providing an encouraging indication that the clones – especially where carotane was concerned - were recognising the hapten end of the conjugates, rather than the carrier end.

The matter of the clones' exact binding site within the conjugates was not resolved within the present studies, due to time restrictions. This means that there is no way to confirm that any of the clones that recognise the conjugated forms of phytane and carotane are indeed able to recognise free antigen. A number of experiments on different LMC targets that were run by other members of the LMC group after the completion of the present work did not lead to the isolation of clones that were able to recognise free antigen, so this issue remains to be clarified.

Although there is a need to advance this work by moving from the "phage level" to a soluble single-chain antibody (scAb) level, it appears that the use of immunised libraries can lead to the isolation of antibodies against LMC-relevant targets and in the context of recombinant antibody development within the time and resource constraints of the LMC programme it should be preferred over the use of naïve libraries.

4.19 Future work

4.19.1.1 Isolation of scAb against phytane and β-carotane

The phage clones that were shown to recognise the conjugated forms of phytane and β-carotane (but not the carrier proteins alone) with considerable sensitivity (Figure 4-9 to Figure 4-11) must be taken forward to competition ELISA, where recognition of free antigen can be confirmed, before moving ahead with the cloning of their responding display peptide sequences into appropriate vectors and expression of soluble antibody.

The results from the monoclonal phage ELISA are by no means final as to whether high affinity antibodies against phytane and β- carotane will be isolated from this library. It is possible that the phage clones identified are recognising the interface between carrier and hapten, and this will be determined by the competition ELISA using free antigen. Additionally, it is possible that a peptide displayed on the virion surface shows a strong

recognition of the target, but when "packaged" in a scAb format it shows less sensitivity.

4.19.1.2 Modification of the screening process

If the clones isolated at the end of the present work prove to be defective in the recognition of free target, there is always the option of screening the same immunised library once more, modifying the biopanning procedure. For instance, there is potential to remove interface binders by introducing free antigen elution earlier in the screening process, but the increase of stringency will result to reduced yield. This is not necessarily detrimental to the selection process, as long as every round is strictly monitored, preventing diversity loss from round to round.

In a different practice, it would be possible to maintain library diversity during the first rounds of panning by maintaining the concentration of the coating antigen high in the first rounds, but this would probably result to a screening process composed of many rounds of selection, and it would have the potential of leading to the amplification of interface or other irrelevant binders in the phage population.

Regardless of the modifications chosen and applied, the possibility to alter the selection process according to the requirements of a given project offers great flexibility and increases the chances of isolating high-affinity antibodies.

Another factor that can be introduced in the selection process before moving to the soluble scAb level is the affinity maturation of the relevant binders. In the context of the LMC, there is a requirement for antibodies that remain functional in harsh solvent mixtures. Additional rounds of panning, where particular solvent mixtures could be integrated into the phage elution step could lead to the isolation of clones whose performance would not be affected by the solvents.

As far as the use of naïve libraries is concerned, despite the poor results it gave in the present experiments, there is a possibility to modify the panning protocol and introduce different panning routes, in order to confirm whether the inability to isolate clones of

interest was due to the fact that they were not present in the library to begin with, or were present initially but unable to survive the selection stress.

4.19.1.3 The future of recombinant antibody generation within the LMC development

If the employment of immunised libraries results to the generation of high-affinity antibodies against the first set of targets, the method will be expanded to produce antibodies against more LMC-relevant molecules. The information collected at every step of the design, planning and screening of the present library can lead to a next round of antibody development which will be more time- and resource-efficient. That said, the need to approach every target (or set of similar targets) individually and modify the methods used accordingly will always be necessary. Additionally, the ability to produce antibodies against a group of haptens as extensive as the potential targets for the LMC instrument is a step forward in anti-hapten antibody development that could prove to have applications outside the development of the LMC.

Summary and Conclusions

Overall, the attempt to employ phage display technology for the generation of LMC candidate antibodies lead to interesting conclusions about the method's efficiency and limitations.

The first thing to note is the performance comparison between the naïve and the immunised phage antibody library; it was apparent that the former did not lead to the isolation of clones that were able to recognise the free or conjugated forms of phytane and β-carotane, while the latter resulted to a number of clones that can recognise the conjugated forms of both targets.

The naïve library's inability to produce clones of interest could be a result of the library's size and/or quality, or an indication that the panning route that was followed requires modifications in order to allow the enrichment in clones displaying antibodies against the two targets.

The immunised library responded well to the panning route that was followed and a number of clones that can recognise the conjugated forms of the two targets and not the carrier proteins alone were collected from it. The studies ended at this point and further analysis (competition ELISA) is required in order to determine whether they can recognise free antigen.

The clones that were isolated from the immunised library and were recognising the phytane-KLH conjugate were not recognising the phytane-BSA conjugate. This could mean that the clones are displaying interface binders, recognising a part of the conjugation site rather than the hapten end of the conjugate. Further analysis of said clones will be able to clarify this matter.

Similarly to the naïve library, should further analysis show that the clones isolated from the immunised library are not able to recognise free antigen, the panning protocols can be modified (less stringency/additional rounds of panning/earlier introduction of free antigen elution) to determine the library's potential to generate clones of interest.

In conclusion, phage display technology is undoubtedly a promising method of antibody production within the context of the development of the LMC instrument. It has proven quicker, more cost-efficient and more flexible than the traditional methods of antibody generation that had been employed in the past and had given low quality results. Whether it will be established as the standard method of LMC candidate antibody generation remains to be determined, after all the possibilities have been examined.

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Final discussion, conclusions and Future work

The results obtained from the experimental work that comprised the present thesis lead to useful conclusions about the use of antibodies in the core of biosensors like the Life Marker Chip instrument. Among the critical areas of the development of the LMC is the acquisition of antibodies against high-priority signatures of Life and the use of antibodies that can resist inactivation due to the combination of conditions associated with a planetary exploration mission like ExoMars. The research that was carried out within the present project helped clarify some aspects of these issues, although it was generally concluded that there is no "template", *i.e.* all antibodies tested do not behave identically, in the development and resistance behaviour of antibodies for the LMC; each potential target should be approached individually in order to obtain sensitive antibodies against it and each antibody's ability to remain active in extreme conditions should ideally be assessed prior to integration to the final instrument platform.

5.1 Ground-based radiation studies

Overall, the ground-based irradiations of antibodies at simulated radiation levels envisaged for the ExoMars mission to Mars, and also at much higher levels, have given valuable information and data input for the LMC development. It is apparent that the dominant effect on the loss of activity of five representative antibodies studied was the processing and packaging of the antibodies as freeze-dried preparations in glass fibre pads and their subsequent retrieval from the pads for use in ELISAs. The subsequent steps of international shipping of the samples to an irradiation facility and their irradiation typically contribute far less to the loss of antibody activity. Therefore, it is apparent that further studies of the freeze-drying, packaging and sample recovery process are required. Initial studies of varying the solutes co-freeze dried with the antibodies have displayed promise in improving the recovery efficiency. For the radiation effects, the important observation is that at a representative Mars mission dose, none of the antibodies studied exhibit any evidence of loss of activity due to the radiation. This therefore achieves a key de-risking step for the LMC instrument development process of demonstrating that the core antibody reagents will survived the radiation environment to be encountered on the ExoMars mission. Whilst this study de-risks the use of antibody-based assays in the exploration of the Red Planet, the

noticeable loss of antibody activity at higher radiation levels (x250 Mars mission dose) does suggest that further shielding or alterative radiation protection approaches will need to be considered for some other astrobiology targets such as future Europa missions.

5.2 Studies of the effect of space radiation in Low Earth Orbit (BIOPAN-6)

The experiment that was designed in order to be part of the BIOPAN-6 platform on board the FOTON-M3 mission lead to a number of conclusions concerning the ability of antibodies to form components of an instrument on a planetary exploration mission. Using this mission in LEO as an analogue of a mission to another planet, a variety of factors that could have a detrimental effect on antibody functionality were examined, including sample preparation, principally the lyophilisation of the antibodies into glass fibre pads, ground handling of the samples, orbital flight and sample recovery. The analysis that took place at the end of the mission revealed that, aside from the effect that lyophilisation has on the ability of antibodies to recognise and bind to their respective targets, the effect of all physical conditions during the mission and exposure to LEO level of radiation are not destructive to these antibodies.

Within the context of the development of the LMC, it is important to note that the radiation levels measured for the Foton M3 mission were considerably lower than the radiation levels envisaged for an actual mission to Mars; the radiation environment was also different (more heterogeneous) than the radiation used in the ground-based studies (composed exclusively of proton and neutron radiation). Nevertheless, the results from the BIOPAN-6 experiment agreed with the results of the ground-based experiments in that they showed that antibodies did not undergo inactivation due to exposure to radiation, even after exposure to a broad spectrum of radiation like the one expected in LEO. Additionally, the BIOPAN-6 results showed that the antibodies used in this experiment remained functional after exposure to the combination of physical factors associated with a space mission (launch and re-entry conditions), which is another encouraging step in their use within a planetary exploration mission context.

5.3 Development of recombinant antibodies for potential LMC targets via phage display technology

The screening of the naive library for binders against phytane and β-carotane was composed of three panning rounds of increasing stringency, with unsatisfactory results for both targets. These results were not unexpected; the initial library size (10^7 clones) and the fact that it had not been raised against our particular target mixture lowered the chances of success; additionally it was deemed that although the library had been raised against different but similar size molecules, there was no positive bias in the isolation of high-affinity clones against our own targets.

The outcome of the screening of the naive library was an indication that, in the context of the development of the LMC instrument, with all the limitations in time and resources it involves, the use of naive libraries is not recommended.

The immunised library which was used to generate antibodies against a mixture of LMC targets (including phytane from the beginning, with β-carotane being added at the final round of immunisation) was raised against synthesised conjugates of these hapten targets. In the course of three panning rounds using the conjugated forms of both targets (increasing the selection stringency by lowering the concentration of the coating antigen) and one additional round with free antigen elution, it became possible to isolate a phage subpopulation that were displaying antibodies against the conjugated forms of phytane and β-carotane. Both targets gave similar results, regardless of their structural differences.

Although there is a need to advance this work by moving from the "phage level" to a soluble single-chain antibody (scAb) level, it appears that the use of immunised libraries can lead to the isolation of antibodies against LMC-relevant targets and in the context of recombinant antibody development it should be preferred over the use of naive libraries.

If the employment of immunised libraries results to the generation of sensitive antibodies against the first set of targets, the method will be expanded to produce antibodies against more LMC-relevant molecules. The information collected at every step of the design, planning and screening of the present library can lead to a next round of antibody development which will be more time- and resource-efficient. That said, the

need to approach every target (or set of similar targets) individually and modify the methods used accordingly will always be necessary. Additionally, the ability to produce antibodies against a group of haptens as extensive as the potential targets for the LMC instrument is a step forward in anti-hapten antibody development that could prove to have applications outside the development of the LMC.

6 .

Research papers currently in preparation for publication

Effects of Simulated Space Radiation on Immunoassay Components for Life Detection Experiments in Future Planetary Exploration Missions

Mariliza Derveni[1], Alexander Hands,[2] Marjorie Allen[1], Mark R. Sims[3], David C. Cullen[1]

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Abstract

The Life Marker Chip instrument (LMC) is currently part of the payload on the European Space Agency's (ESA) ExoMars rover that is scheduled for launch in 2018. The LMC will use antibody-based assays to detect molecular signatures of life in the shallow subsurface of Mars. For all the antibodies at the heart of the LMC, the ability to resist inactivation due to space radiation will therefore be a prerequisite. The objective of the present work has been to expose a number of LMC-relevant antibodies to simulated Mars mission radiation in the form of proton and neutron radiation produced via a cyclotron source. The proton and neutron components of the mission radiation environment are those that are expected to have the dominant effect on the operation of the LMC. After irradiation, the antibodies were assessed for any loss of functionality. Modelling of the radiation environment for a mission to Mars led to the calculation of nominal mission fluences for proton radiation during the cruise phase of the mission (4 x 10^{10} protons/cm^2) and neutron radiation during Martian surface operations (2 x 10^7 neutrons/cm^2). Various combinations and multiples of these values have been used to demonstrate the effects of radiation on antibody activity both at the actual radiation levels envisaged for the ExoMars mission and at much higher levels (10x and 250x the mission dose). In total, five antibodies were freeze-dried in a variety of protective molecular matrices and were exposed to the various radiation conditions. After exposure, the antibodies' ability to bind to their respective antigens was assessed. At radiation levels equal to 1x times the mission dose, four antibodies out of the five maintained the majority of their binding activity, while at 10x and even 250x times the mission dose, the same four out of five antibodies maintained considerable amounts of their binding activity. It was also observed that the preparation and treatment (lyophilisation/rehydration) of the antibody samples had a detrimental impact on their performance, an effect that could be significantly reduced with the use of appropriate protective matrices and handling protocols. These experiments indicate that the expected radiation environment of a Mars mission does not pose a significant risk to antibodies packaged in the form anticipated for the LMC instrument.

Keywords

Immunoassay, antibodies, space radiation, Life detection, lyophilisation, ExoMars, Mars

Survivability of immunoassay reagents exposed to the radiation environment of Low Earth Orbit via the ESA BIOPAN-6 experimental platform as a simulated Mars planetary exploration mission

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Abstract

The Life Marker Chip (LMC) instrument is an antibody assay-based sensor which will attempt to detect molecular signatures of Life in the Martian subsurface as part of the payload onboard the ESA ExoMars mission, currently scheduled for launch in 2018. The molecular reagents at the core of the LMC have no heritage of interplanetary mission use, therefore the design of such an instrument must take into account a number of risk factors, among which the radiation environment that will be encountered en route to, and on the surface of, Mars is a key factor. In order to study the effects of space radiation on lyophilised immunoassay reagents, primarily antibodies, a number of ground-based and space studies were undertaken, the latter in the form of ESA's 2007 BIOPAN-6 Low-Earth Orbit (LEO) space exposure platform. Two representative LMC antibodies were used in these studies in the form of lyophilised samples integrated in glass fibre pads, loaded into a custom-made sample holder unit which was mounted on the BIOPAN-6 platform. The BIOPAN mission went into orbit for 12 days, after which all samples were recovered and their binding performance was measured via ELISA. The factors expected to affect antibody performance were the physical conditions of a space mission and the exposure to space conditions (i.e. the radiation environment in LEO). Both antibodies survived complete inactivation due to these factors. The data from the LMC on BIOPAN-6 experiment, in combination with data from ground-based irradiations of LMC components, are being used to de-risk the development of the LMC instrument.

Keywords

Antibodies, BIOPAN, Life Detection, lyophilisation, space radiation

Development of recombinant antibodies against haptens with biosignature properties with the use of naïve and immunised phage antibody libraries

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Abstract

The Life Marker Chip (LMC) instrument is an antibody assay-based system which will attempt to detect molecular signatures of Life in the Martian subsurface as part of the payload on board the ExoMars mission rover. One of the key stages in the development of the LMC is the selection of molecules that can serve as signatures of Life and the acquisition of high affinity antibodies against them. Phage display technology offers the possibility to generate high affinity recombinant antibodies against small molecules with poor immunogenic potential (haptens) which constitute a number of important prospective LMC target molecules. In this work, a naïve and an immunised phage antibody library have been employed in the production of recombinant antibodies against theexample LMC molecular targets phytane and β-carotane. The naïve library, which had been constructed for a non-related family of haptens, gave extremely poor results for both targets, as the panning did not result to the isolation of clones that could recognise either of the two molecules. The immunised library had been constructed using lymphocytes from sheep immunised with a mixture of LMC targets, and resulted to the identification of phage clones that appeared able to recognise target conjugated to a carrier protein, without presenting cross-reactivity with the carrier proteins alone. The present work is concluded at this point, but further analysis is required in order to determine whether these clones are able to recognise free hapten and proceed with the purification of soluble antibodies. The selection process involved in phage display allows isolation of antibody clones that maintain their functionality in a variety of non-standard conditions required by the LMC instrument. Therefore we consider phage display technology with the use of immunised libraries an appropriate method for obtaining antibodies for the LMC instrument.

Keywords

Phage display, hapten, antibody library, Life detection, Life Marker Chip, ExoMars

7 .

Poster Presentations

Cranfield Health

Cranfield UNIVERSITY

The effect of space radiation on immunoassay reagents: Implications for the Life Marker Chip experiment for ESA's ExoMars mission

Mariliza Derveni*, Marjorie Allen, Paul K. Wilson and David C. Cullen
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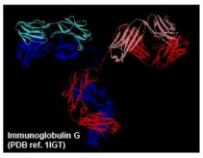
Launch of Foton-M3 in 2007 carrying ESA's Biopan-6 space exposure platform


Immunoglobulin G (PDB ref. 1IGT)


CGI representation of ESA's ExoMars rover

Introduction & Context

In recent years, the rise of interest in **planetary exploration** and the emergence of **astrobiology** as a promising field of research have lead to various programmes to develop sensitive instruments for Life detection in extreme environments.

An antibody assay-based Life detection instrument, called the Life Marker Chip (LMC), is currently under development by an international consortium led by Cranfield and Leicester universities for flight on ESA's ExoMars mission due for launch in 2013. The primary mission objective is to search for evidence of past or present Life on Mars.

The molecular reagents at the core of instruments such as the LMC have no heritage of interplanetary mission use. The radiation environment that will be encountered in missions posses a significant unknown risk to future missions such as ExoMars.

To this end, a ground and space based test campaign is on-going within the LMC consortium to de-risk and evaluate the affect of radiation environments relevant to the ExoMars mission on antibody-based assay reagents.

Aims & Objectives

- To study the effects of radiation on the survival and activity of antibody assay reagents including (i) antibodies, (ii) fluorescent labels and (iii) immunoassay microarrays
- To utilise a variety of ground-based radiation sources to simulate cumulative mission radiation exposure
- To validate antibody assay stability in a real space environment via the 2007 flight of the ESA Biopan-6 low-Earth orbit space exposure platform

Experimental

Sample preparation

- Antibodies and fluorescent assay reagents were freeze-dried from a variety of buffers (various conditions and cryo-protectants / re-solubilisation agents) into small glass-fibre pads (see glass fibre pads in fig. 1). This aided sample handling and is also representative of the proposed method of reagent integration into the ExoMars LMC instrument.
- Immunoassay reagents were immobilised as micorarrays onto glass or silicon supports using standard pin-based micro-arraying techniques.

LMC Experiment flight on the Foton-M3 / Biopan-6 mission

- Glass-fibre pads and immunoassay micro-arrays were mounted in a bespoke carrier (produced by DLR, Germany) – the LMC experiment (fig. 1 & 2) - under clean-room conditions within Cranfield University. The carrier was sealed with an atmosphere of inert argon gas.
- The experiment was delivered to ESA (NL), integrated into the Biopan-6 platform (fig 3.) and then flown to Baikonur Cosmodrome (Kazakhstan) for mounting onto the outside of the Foton-M3 orbital vehicle.
- Foton-M3 / Biopan-6 was launch on 14th Sept. 2007 and orbited the Earth for 12 days at an altitude of ~280km. In orbit, Biopan-6 opened exposing the LMC experiment directly to the space radiation environment (fig. 4). Just prior to re-entry, Biopan-6 closed and the Foton-M3 re-entry capsule landed in the Kazak Steppes on 26th Sept. 2007. Biopan-6 was de-mounted, returned to ESA (NL), opened and the LMC experiment removed and returned to Cranfield University.

Ground-based radiation exposure

- Samples were prepared similar to the Biopan-6 experiment but packaged in 384 microwell plates for glass fibre pads (fig. 5) and mounted with adhesive tape in disposable Petri dishes for other sample formats (*e.g.* micro-arrays).
- Samples were shipped to The Svedberg Laboratory (TSL) (Uppsala, Sweden) for exposure to proton and neutron beams produced by a cyclotron (fig. 6) to simulate various components of the space radiation. Samples were returned to Cranfield University. Note that previous iterations of the experiment have been studied with facilities at the University of Birmingham and The Paul Scherrer Institut (Switzerland).

Post-exposure analysis

- Samples were extracted from the glass-fibre pads and assayed appropriately for antibody function and fluorescent properties and the results compared to appropriate control (shipping and storage control samples sets).

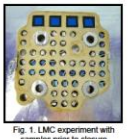


Fig. 1. LMC experiment with samples prior to closure

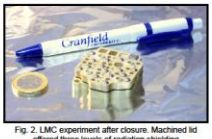


Fig. 2. LMC experiment after closure. Machined lid offered three levels of radiation shielding

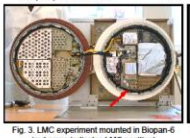


Fig. 3. LMC experiment mounted in Biopan-6 (red arrow indicates LMC position)


Fig. 4. Foton-M3 in orbit with Biopan-6 open (in red circle)


Fig. 5. 384 microwell plate with samples

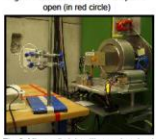


Fig. 6. Microwell plate with samples about to be exposed to neutron beam at TSL

Results

Biopan-6 flight

- Biopan-6 flight saw a combined radiation level of 0.4krad – this is significantly less than seen for the previous flight of Biopan-5 and approx one sixteenth of the modelled total radiation dose for the ExoMars mission (modelled as 6.6krad by QinetiQ, UK).
- The vibration and acoustic energy effects of launch and re-entry did not produce any visually observable degradation of (i) the glass-fibre pads or (ii) the silicon / glass micro-array supports.
- The antigen binding activity of recovered anti-GroEL antibodies after flight were assessed. No significant difference between (i) radiation levels and (ii) shipping and storage controls was observed (fig. 7). Loss of activity was seen during the lyophilisation of antibodies into the glass-fibre pads.
- The fluorescence intensity of AlexaFluor 633 fluorescent dye samples recovered after flight showed no significant effect of radiation but does show a 17% loss of intensity compared to storage controls (fig. 8).
- Further samples remain to be fully tested including immunoassay micro-arrays

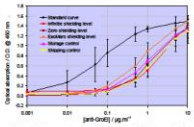


Fig. 7. Summary of anti-GroEL antibody activity survivability of Biopan-6 flight. Three different radiation shielding levels used (±1SD, n=3)

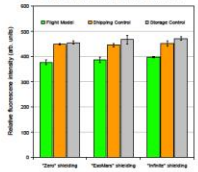


Fig. 8. Summary of Alexa Fluor 633 fluorescence intensity survivability of Biopan-6 flight. Three different radiation shielding levels used (±1SD, n=3)

Ground-based testing (TSL)

- Samples were exposed to x1 and x10 ExoMars equivalent dose of combined of proton and neutron radiation at The Svedberg Laboratory.
- Antibody samples were lyophilised into glass-fibre pads in various cocktails: (i) Blue Dextran and BSA cocktail, (ii) sucrose and BSA cocktail and (iii) as supplied native antibody preparations.
- The antigen binding activity of recovered anti-GroEL antibodies after exposure were assessed. No significant difference between x1 and x10 radiation levels and shipping control samples seen (figs. 9 & 10) and hence no affect of radiation on antibody observed for Blue Dextran and sucrose cocktails.
- Loss of antibody activity observed in storage and shipping control samples compared to non-lyophilised antibody samples (figs. 9 & 10). Total loss of antibody activity observed for lyophilised native antibody samples (data not shown).
- The fluorescence intensity of AlexaFluor 633 fluorescent dye samples recovered after exposure showed no significant effect of radiation (data not shown).

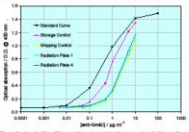


Fig. 9. Anti-GroEL antibody activity survivability after combined proton and neutron irradiation in "Blue Dextran cocktail". Radiation Plate 1 = x1 ExoMars dose, Radiation Plate 2 = x10 ExoMars dose

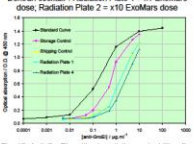


Fig. 10. Anti-GroEL antibody activity survivability after combined proton and neutron irradiation in "Sucrose cocktail". Radiation Plate 1 = x1 ExoMars dose; Radiation Plate 2 = x10 ExoMars dose

Conclusions and Future Work

- Biopan-6 LMC experiment showed antibodies and fluorescent assay reagents can survive at least one sixteenth ExoMars equivalent radiation levels AND the physical environment of spacecraft launch and atmosphere re-entry, both when packaged in ExoMars LMC intended formats.
- Ground-based radiation experiments show antibodies and fluorescent assay reagents can survive at least tens times ExoMars equivalent radiation levels when packaged in ExoMars LMC intended formats (antibodies and fluorescent dye labels freeze-dried into glass fibre-pads in suitable preservatives).
- The solute matrix in which antibodies are lyophilised into glass-fibre pads is critical to maintenance of antibody activity in samples eluted from glass-fibre pads.
- The combined space and ground based radiation testing campaign has demonstrated that the radiation environment envisaged for the ExoMars mission will not be an insurmountable problem for the antibody assay reagents.
- Further studies will include: a broadened range of samples tested (Sept 2008 TSL campaign), expanded micro-array testing and accelerated storage studies.

Acknowledgements: ESA and STFC for funding; Biopan-6 project in collaboration with DLR (Germany), Carnegie Institution of Washington (USA) & University of Leicester (UK); LMC ground based testing in collaboration with QinetiQ (UK), The Svedberg Laboratory (Sweden) & University of Leicester (UK).

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DETECTING MOLECULAR SIGNATURES OF LIFE ON MARS: THE LIFE MARKER CHIP (LMC) INSTRUMENT

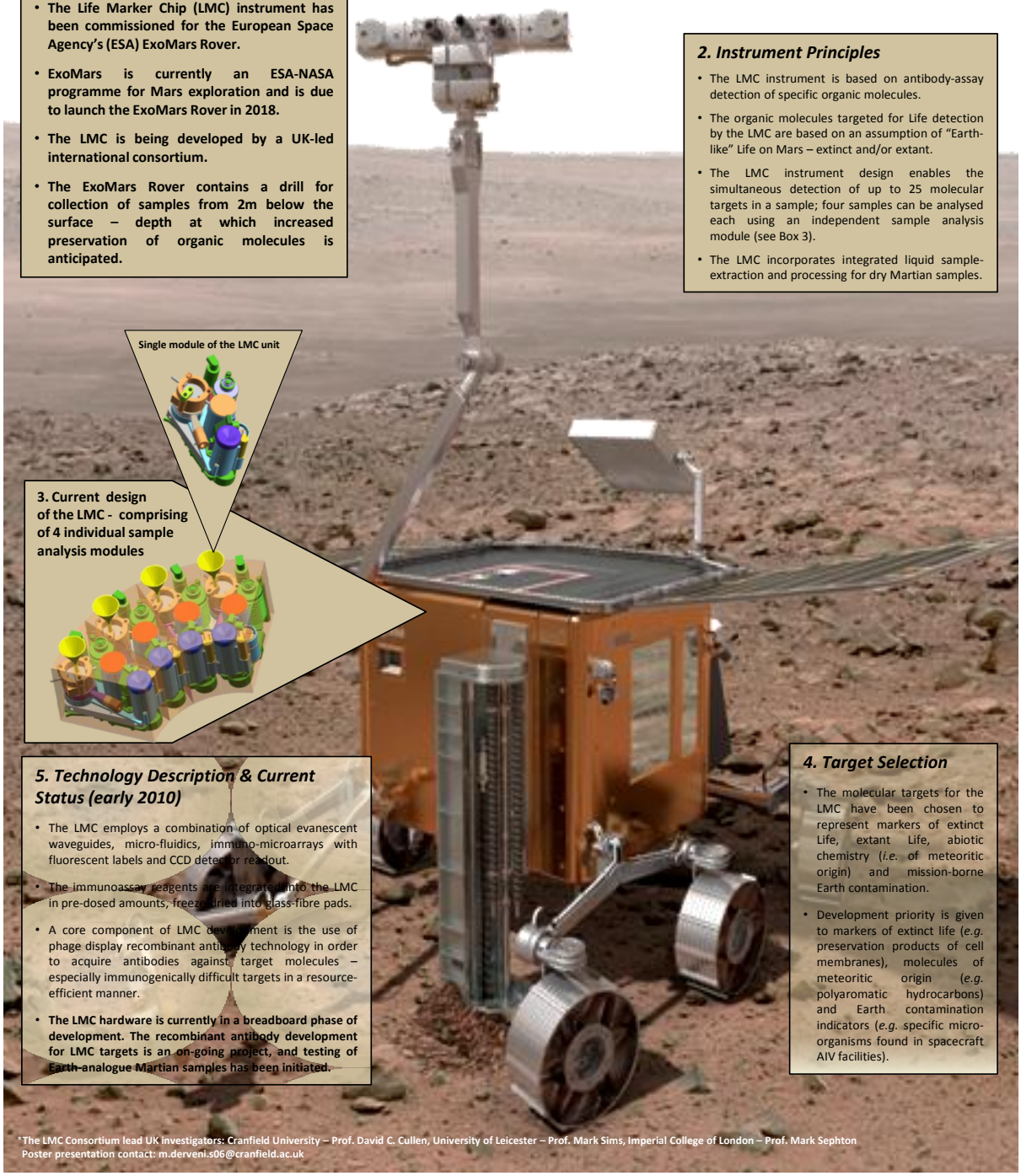
Derveni, M. and the LMC Consortium*

1. Instrument & Mission Introduction

- **The Life Marker Chip (LMC) instrument has been commissioned for the European Space Agency's (ESA) ExoMars Rover.**
- **ExoMars is currently an ESA-NASA programme for Mars exploration and is due to launch the ExoMars Rover in 2018.**
- **The LMC is being developed by a UK-led international consortium.**
- **The ExoMars Rover contains a drill for collection of samples from 2m below the surface – depth at which increased preservation of organic molecules is anticipated.**

2. Instrument Principles

- The LMC instrument is based on antibody-assay detection of specific organic molecules.
- The organic molecules targeted for Life detection by the LMC are based on an assumption of "Earth-like" Life on Mars – extinct and/or extant.
- The LMC instrument design enables the simultaneous detection of up to 25 molecular targets in a sample; four samples can be analysed each using an independent sample analysis module (see Box 3).
- The LMC incorporates integrated liquid sample-extraction and processing for dry Martian samples.



3. Current design of the LMC - comprising of 4 individual sample analysis modules

4. Target Selection

- The molecular targets for the LMC have been chosen to represent markers of extinct Life, extant Life, abiotic chemistry (*i.e.* of meteoritic origin) and mission-borne Earth contamination.
- Development priority is given to markers of extinct life (*e.g.* preservation products of cell membranes), molecules of meteoritic origin (*e.g.* polyaromatic hydrocarbons) and Earth contamination indicators (*e.g.* specific micro-organisms found in spacecraft AIV facilities).

5. Technology Description & Current Status (early 2010)

- The LMC employs a combination of optical evanescent waveguides, micro-fluidics, immuno-microarrays with fluorescent labels and CCD detector readout.
- The immunoassay reagents are integrated into the LMC in pre-dosed amounts, freeze-dried into glass-fibre pads.
- A core component of LMC development is the use of phage display recombinant antibody technology in order to acquire antibodies against target molecules – especially immunogenically difficult targets in a resource-efficient manner.
- **The LMC hardware is currently in a breadboard phase of development. The recombinant antibody development for LMC targets is an on-going project, and testing of Earth-analogue Martian samples has been initiated.**

*The LMC Consortium lead UK investigators: Cranfield University – Prof. David C. Cullen, University of Leicester – Prof. Mark Sims, Imperial College of London – Prof. Mark Sephton
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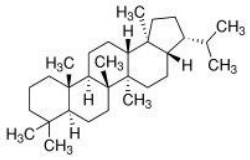
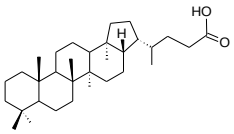
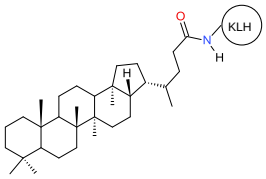
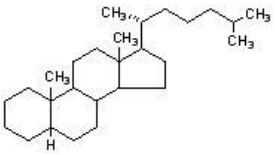
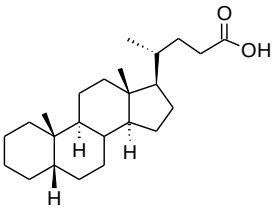
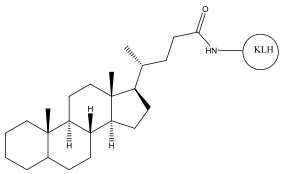
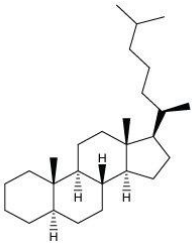
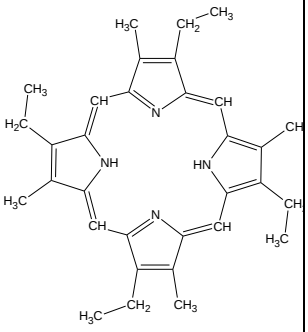
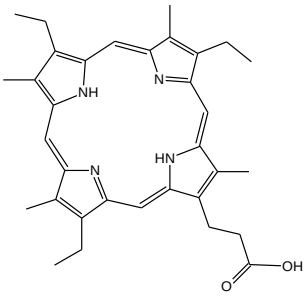
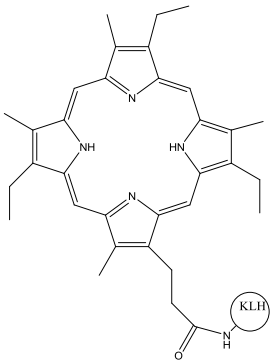
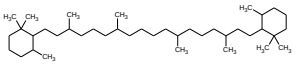
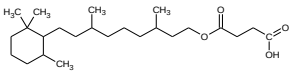
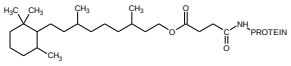
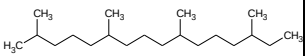
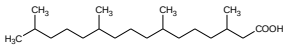
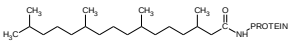
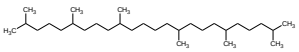
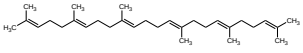
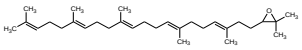
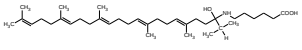
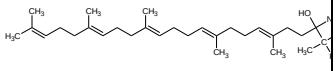
8.
Appendices

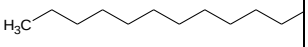
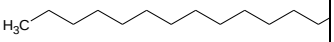
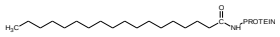
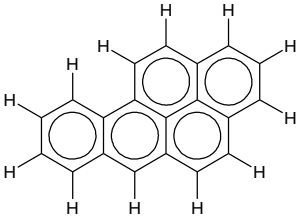
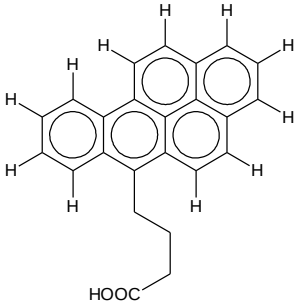
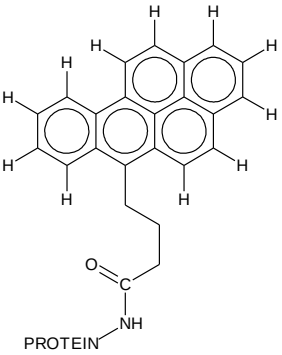
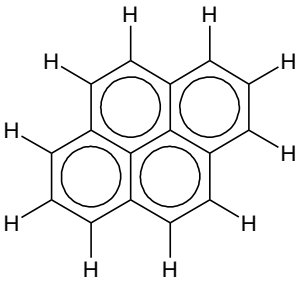
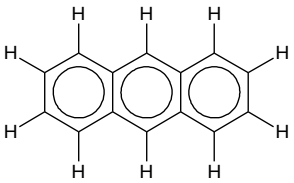
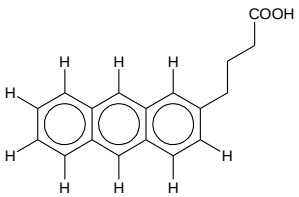
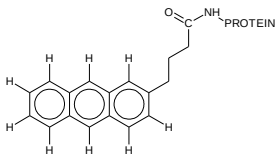
Appendix 1: Representative Molecular Targets for the Life Marker Chip Instrument

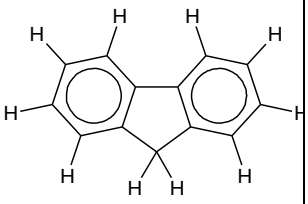
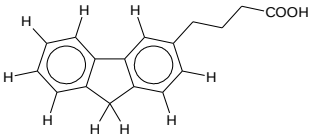
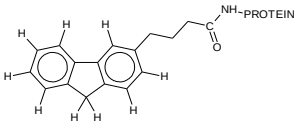
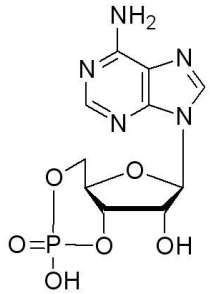
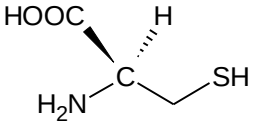
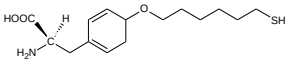
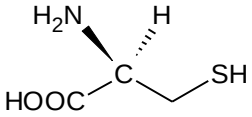
(Document developed by David C. Cullen, from original document produced by Catherine Rix, September 2010)

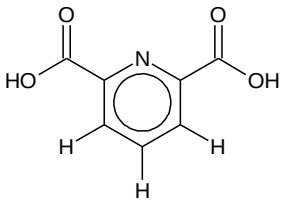
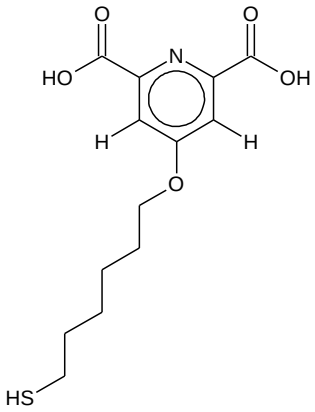
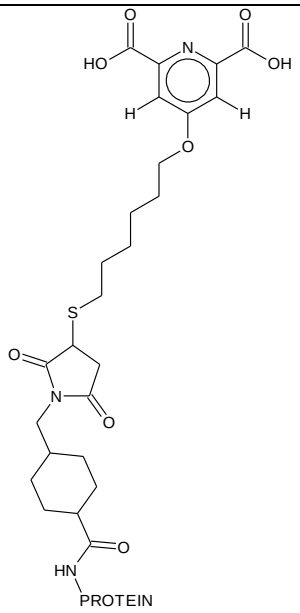
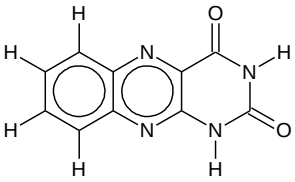
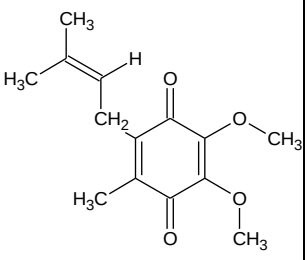
Appendix 1 shows a list of representative LC target molecules in their "free target" format, their derivatised forms and their synthesised (or envisaged) conjugated forms.

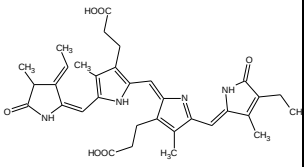
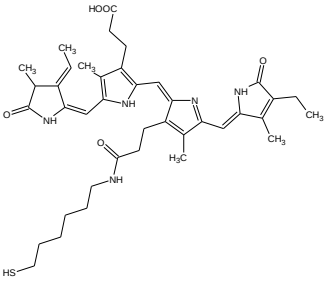
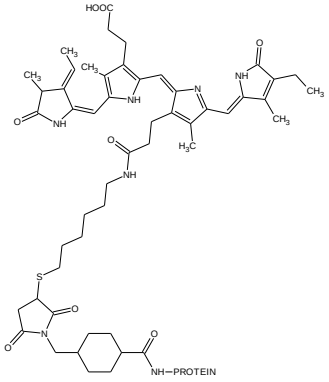
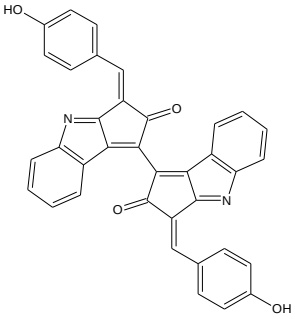
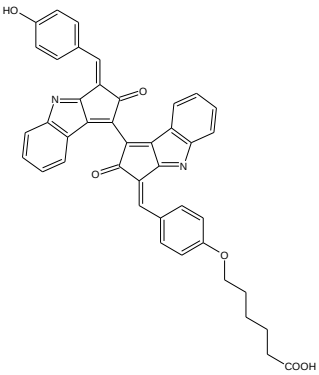
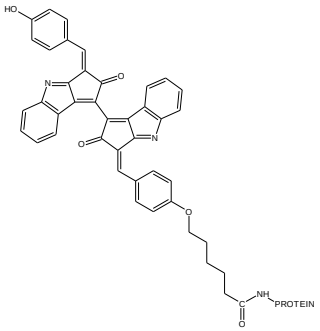
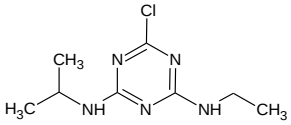
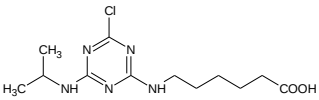
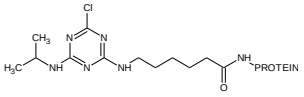
Assay Name/ target	Free Target	Target derivative	Structure for Immunisation
Hopane Hopanoid biomarkers, derived from components of microbial cell walls	17β(H),21β(H)-Hopane (Sigma 07562)  **MW = 412.73**		
5β-sterane Generic stearanes, derived from eukaryote and prokaryote membranes	5β-coprostane (Sigma C2005)  **MW = 372.67**		
5α-sterane Generic steranes, derived from eukaryote and prokaryote membranes	5α-cholestane (Sigma C8003)  **MW = 372.67**	TBD	TBD

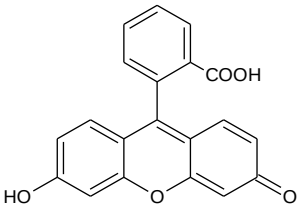
Porphyrin Generic porphyrins derived from chlorophylls	Etioporphyrin I (dihydrogenbromide) (Aldrich 252417)  **MW = 478.68 (for non bromide form)**		
Carotane Fossil carotenoids from pigments	β,β-carotane (Chiron, 0654.40-500-IO)  **MW = 559.07**	 **MW = 396.60**	 EDC/NHS conjugation
Generic isoprenoid Phytane Marker of exctinct Life	Phytane (Fluka 80165)  **MW = 282.55**	Phytanic acid (mixture of isomers) (Sigma P4060)  **MW = 312.53**	 EDC/NHS conjugation
Squalane / Squalene Marker of exctinct Life	Squalane (Aldrich 234311)  **MW = 422.81** Squalene (Sigma S3626)  **MW = 410.72**	2,3-Oxidosqualene (Sigma 41043)  **MW = 426.72** 2,3-Oxidosqualene (with C_6 carboxyl spacer)  **MW = 557.89**	 EDC/NHS conjugation

Generic straight chain alkane (Stearic acid) straight chain alkane derived from fatty acids	Octadecane (Sigma 74691)  **MW = 254.49**	Stearic acid (Octadecanoic acid) (Sigma S4751)  **MW = 284.48**	 EDC/NHS conjugation
Benzo[a]pyrene Meteoritic origin	Benzo[a]pyrene (Sigma, B1760)  **MW = 252.31**	4-(Benzo[a]pyren-6-yl)butanoic acid  **MW = 338.40**	 EDC/NHS conjugation
Pyrene Meteoritic origin	Pyrene (Aldrich 571245)  **MW = 202.25**	TBD	TBD
Anthracene Meteoritic origin	Anthracene (Aldrich 141062)  **MW = 178.23**	Anthracene (with C_4 carboxyl spacer)  **MW = 264.32**	 EDC/NHS conjugation

Fluorene Meteoritic origin	Fluorene (Aldrich 46880)  **MW = 166.22**	Fluorene (with C_4 carboxyl spacer)  **MW = 252.31**	 EDC/NHS conjugation
cAMP	Adenosine 3′,5′-cyclic monophosphate (Sigma A9501)  **MW = 329.21**	TBD	TBD
D-α amino acids (D-cysteine)	D-cysteine (Aldrich 30095)  **MW = 121.16**	D-cysteine (with C_6 ?? terminated spacer)  **MW = ??**	TBD
L-α amino acids (L-cysteine)	L-cysteine (Sigma C7352)  **MW = 121.16**	TBD	TBD

Dipicolinic acid Marker of extant Life	Dipicolinic acid (2,6-pyridinedicarboxylic acid) (Aldrich P63808)  **MW = 167.12**	Dipicolinic acid (with C_6 thiol terminated spacer)  **MW = 299.34**	 Conjugation to Sulfo-SMCC treated protein
Isoalloxazine (flavin) Marker of extant Life	Isoalloxazine (Alloxazine) (Aldrich A28651)  **MW = 214.18**	TBD	TBD
Ubiquinone-1 (quinones) Marker of extant Life	Ubiquinone-1 (Coenzyme Q1) (Chemical Book ref)  **MW = 250.29**	TBD	TBD

Phycocyanobilin (phycobilins) Marker of extant Life	Phycocyanobilin  **MW = 586.68**	Phycocyanobilin (with C_6 thiol terminated spacer)  **MW = 701.92**	 Conjugation to Sulfo-SMCC treated protein
Scytonemin (melanoidins) Marker of extant Life	Scytonemin (Santa Cruz Biotechnology sc-202337)  **MW = 544.56**	Scytonemin (with C_6 carboxyl terminated spacer)  **MW = 658.70**	 EDC/NHS conjugation
Atrazine; positive control for inhibition assay within LMC; positive control for scAb production	Atrazine (Sigma, 45330)  **MW = 215.68**	(with C_6 carboxyl spacer)  **MW = 301.77**	 EDC/NHS conjugation

Fluorescein positive control for inhibition assay within LMC; positive control for scAb production	Fluorescein (Fluka 28803)  **MW = 332.30 (free acid)**	TBD	TBD