Analytica Chimica Acta 708 (2011) 97–106

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

# Synthesis and characterisation of immunogens for the production of antibodies against small hydrophobic molecules with biosignature properties

Manisha Sathe[a,1], Mariliza Derveni[a], Gillian Broadbent[b], Anne Bodlenner[c], Keith Charlton[b], Bindu Ravi[b], Michel Rohmer[c], Mark R. Sims[d], David C. Cullen[a,*]

[a] Cranfield Health, Cranfield University, UK
[b] ImmunoSolv Ltd., Aberdeen, Scotland, UK
[c] University of Strasbourg, CNRS, Institut Le Bel, France
[d] Space Research Centre, University of Leicester, UK

ARTICLE INFO

*Article history:*
Received 6 June 2011
Received in revised form 9 September 2011
Accepted 15 September 2011
Available online 22 September 2011

*Keywords:*
Hapten
Life Marker Chip
Bioconjugation
Mars
Antibody generation
Hydrophobic molecules

ABSTRACT

In the present study, five different classes of small hydrophobic molecular targets, atypical for antibody generation, were structurally modified in order to introduce suitable reactive functionalities and/or spacers which allow covalent coupling to a carrier protein resulting in a stable carrier–hapten complex. These targets were chosen to serve as markers of extant and/or extinct life in the context of the development of the Life Marker Chip (LMC), an antibody-based instrument, which is being developed by a UK-led international consortium for flight to Mars on board the joint ESA/NASA Mars exploration ExoMars mission. The hapten–protein conjugates were designed to be used as immunogens for antibody generation and immunoassay reagents in subsequent stages of the LMC development. The extent of protein modification due to covalent attachment of hapten was determined by two independent methods, *i.e.* trinitrobenzenesulfonic acid (TNBSA) titrations of remaining protein reactive groups and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) of the resultant hapten–protein conjugates. In a further quality validation step, the conjugates were presented to an animal's immune system and polyclonal antibody titres with moderate specificity were obtained. These results suggest that conjugates synthesized as described herein can successfully be used in the generation of antibodies targeting small hydrophobic molecules.



## 1. Introduction

### 1.1. Life detection on Mars including LMC technology

The search for extraterrestrial life is still largely theoretical, but experiments designed to address questions regarding the detection of biomolecular components which would be unambiguous indicators of life at extraterrestrial locations are ongoing. Pertaining to Mars, the quest for evidence of both extant and extinct life is a basic goal of current planetary exploration missions [1–3]. In order to understand the distribution and evolution of organics within the Solar System using suitable planetary targets, like Mars, there is a need for *in situ* detection and characterisation of organic molecules. Whilst various hyphenated chromatography–mass spectrometry instruments have been the accepted standard for planetary exploration missions to detect organic molecule targets, recent advances in immunoassay-based biomarker detection systems [4], in antibody microarray technology for instance [5–8], are leading to their employment in planetary exploration [9–13]. Within this context, the immunoassay-based Life Marker Chip (LMC) [14] instrument is being developed by a UK-led international consortium to be part of the Pasteur instrument payload on board the ExoMars mission rover. The ExoMars mission is a collaboration between the European Space Agency (ESA) and NASA and is currently scheduled for launch in 2018.

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) 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. A key part of

* Corresponding author. Tel.: +44 01234 758340; fax: +44 01234 758380.
*E-mail address:* d.cullen@cranfield.ac.uk (D.C. Cullen).
[1] Current address: Discovery Center Discovery Center, Defence R&D Establishment, Ministry of Defence, Gwalior, India.

0003-2670/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2011.09.021

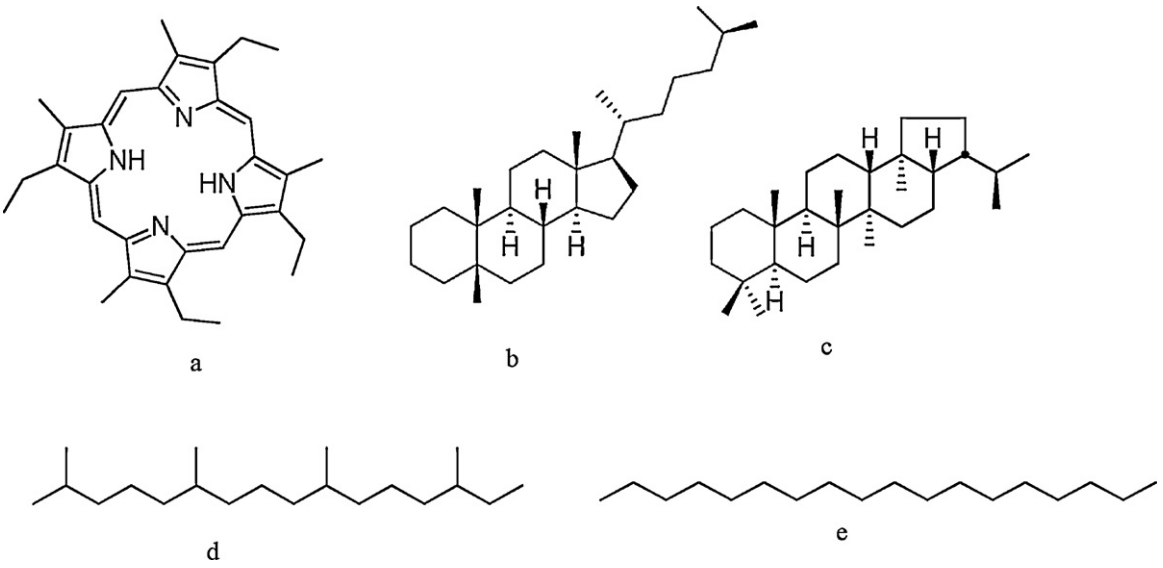


**Fig. 1.** Small hydrophobic biomarkers of extinct and extant life **1** (a–e).

the LMC development is focusing on the production of antibodies and the subsequent immunoassay development for use in the final instrument.

A range of biomarkers that can serve as LMC targets were identified during a workshop held in 2006 in support of the development of the Life Marker Chip for ESA's ExoMars mission [15]. The targets in question [16] are based upon an assumption of "Earth-like" life, with many of them being small hydrophobic molecules [17].

### 1.2. Immunoassays for small hydrophobic molecules

Simple, sensitive, selective, and field-deployable tools are highly desired for the detection of molecular signatures of life. Immunoassays such as enzyme-linked immunosorbent assays (ELISA) [24] have the potential to become an alternative or a complementary method to traditional techniques such as GC–MS or pyrolysis-MS, because they have proven to be fast, sensitive and cost-effective tools for detecting trace amounts of small hapten molecules, like a number of pesticides, in terrestrial applications. Several ELISA methods for the detection of organophosphorous (OP) pesticides like fenitrothion[25], diazinon [26], acephate [27], isofenphos [28], fenthion [29], triazophos [30], and pirimiphos-methyl [31], have been developed.

To the best of our knowledge, very few immunoassays for the detection of Mars relevant biomarkers of life have been reported [32]. The representative LMC molecular targets described in the present work are atypical for antibody generation given that they are apolar as well as chemically inert, due to the lack of functional groups. Five different classes of potential molecular targets, chosen to serve as markers of extant and extinct life, were employed for the first time in antibody production within this context. These compounds were porphyrin **1a** [18], coprostane **1b** [19], hopane **1c** [20], phytane 1d and straight chain alkane *e.g.* octadecane **1e** [21–23] (Fig. 1).

### 1.3. Protein carrier–hapten conjugates for antibody development

The initial step in the development of an immunoassay is the production of specific antibodies, but for small molecules that seldom stimulate an immune response (haptens) [33] like porphyrin, hopane, coprostane, phytane and straight chain alkane (*e.g.* octadecane) it is necessary to design appropriate chemical structures [34] which can be covalently coupled to a carrier protein and presented to the animal's immune system. The structure of the conjugate [35,36] that will be used as an immunogen can affect the characteristics of the resulting antibodies, as well as the sensitivity and specificity of the analytical method.

Two very common carrier proteins employed in such conjugations are bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). BSA is well suited as a carrier protein due to its high solubility in various aqueous buffers, moderate molecular weight ($66 \times 10^3$ Da), and high content of available primary amines (59 lysines plus the terminal amine) which facilitate the attachment of the hapten. KLH is widely used as a carrier protein to make conjugates more immunogenic for the purpose of antibody production. Because of its large mass and complexity, KLH elicits a stronger immune response than other carrier proteins. As a large protein, KLH has a large number of primary amines and carboxyl groups that can be targeted for conjugation. One disadvantage in the use of KLH is that its high molecular weight ($4.5 \times 10^5$ to $1.3 \times 10^7$ Da) compared to that of the hapten targets makes post-conjugation characterisation via MALDI-MS very difficult.

### 1.4. Aims and objectives

The aim of the present work has been to demonstrate that it is possible to extend the use of standard synthetic routes and conjugation protocols to the production of protein conjugates of small hydrophobic molecules that can successfully be employed to elicit an immune response to these challenging targets.

The targets used here were structurally modified (Fig. 2) prior to covalently coupling them to suitable carrier proteins. The verification of the coupling reaction and the determination of the hapten density of the resulting hapten–protein conjugates was performed spectrophotometrically [37], mainly by evaluating the available free amino groups before and after conjugation and by MALDI-MS [38], to determine the mass before and after conjugation. A mixture of hapten conjugates was used to hyper-immunise sheep for a preliminary round of polyclonal antibody production, with the intention of further validating the success of the conjugates both as immunogens and as screening reagents for subsequent ELISA.

## 2. Experimental

### 2.1. Reagents, materials and instrumentation

#### 2.1.1. Reagents and materials

Reagents were obtained from Sigma–Aldrich (Poole UK) unless stated otherwise, including bovine serum albumin (crystalline,