



Complement activation on degraded polyethylene glycol-covered surface

Mitsuaki Toda^{a,b}, Yusuke Arima^a, Hiroo Iwata^{b,*}

^aAdvanced Software Technology & Mechatronics Research Institute of Kyoto, 134 Minamimachi Chudoji, Shimogyo-ku, Kyoto 600-8813, Japan

^bInstitute for Frontier Medical Sciences, Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:

Received 22 October 2009

Received in revised form 6 January 2010

Accepted 25 January 2010

Available online 1 February 2010

Keywords:

Complement activation

Polyethylene oxide

Protein adsorption

Surface modification

Surface analysis

ABSTRACT

Surface modification with polyethylene glycol (PEG) has been employed in the development of biomaterials to reduce unfavorable reactions. However, unanticipated body reactions have been reported, with activation of the complement system being suggested as having involvement in these responses. In this study, we prepared a PEG-modified surface on a gold surface using a monolayer of α -mercaptoethyl- ω -methoxy-polyoxyethylene. We observed neither protein adsorption nor activation of the complement system on the PEG-modified surface just after preparation. Storage of the PEG-modified surface in a desiccator under ambient light for several days or following ultraviolet irradiation, reflection-adsorption (FTIR-RAS) and X-ray photo spectrometry revealed deterioration of the PEG layer, which became a strong activator of the complement system through the alternative pathway.

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1. Introduction

Surface modification with polyethylene glycol (PEG) is one of the most promising strategies to prevent and/or reduce adsorption of proteins and the after-effects of the interaction of body fluids with biomaterials [1,2]. PEG has also been used for pharmaceutical applications to shield antigenicity of proteinaceous drugs and to prolong the circulating half-life of drug-loaded nanoparticles and liposomes [3–5]. However, unanticipated body reactions such as hypersensitivity caused by PEG-modified liposomes [6–9] and rapid clearance of PEG-modified liposomes from blood [10] have been reported. Activation of the complement system has been suggested as being associated with these body reactions [11]. The complement system, which plays important roles in the body's defense system against pathogenic xenobiotics, is an enzyme cascade system that consists of approximately 30 fluid-phase and cell-membrane bound proteins [12]. It is activated through three separate pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP) (Scheme 1A). Understanding the interaction of complement proteins with the PEG-modified surface may provide a basis for developing PEG-modified materials for biomedical and pharmaceutical use.

In a previous study [13], we examined complement activation behaviors on PEG-modified surfaces; however, there were uncertainties about the experimental setup. First, we used diluted (10%) normal human serum (NHS), and it has been reported that

activation of the alternative pathway may be diminished with use of diluted serum [14,15]. In addition, the PEG-modified surfaces were not sufficiently characterized. In the current study, we prepared a PEG-modified surface on a gold surface using a self-assembled monolayer of α -mercaptoethyl- ω -methoxy-polyoxyethylene (HS-mPEG). We then carried out detailed surface analyses of PEG-modified surfaces using the reflection-adsorption method (FTIR-RAS) and X-ray photo spectrometry (XPS) and examined activation of the complement system using undiluted NHS to obtain more detailed insight into the mechanisms of complement system activation by the PEG-modified surfaces.

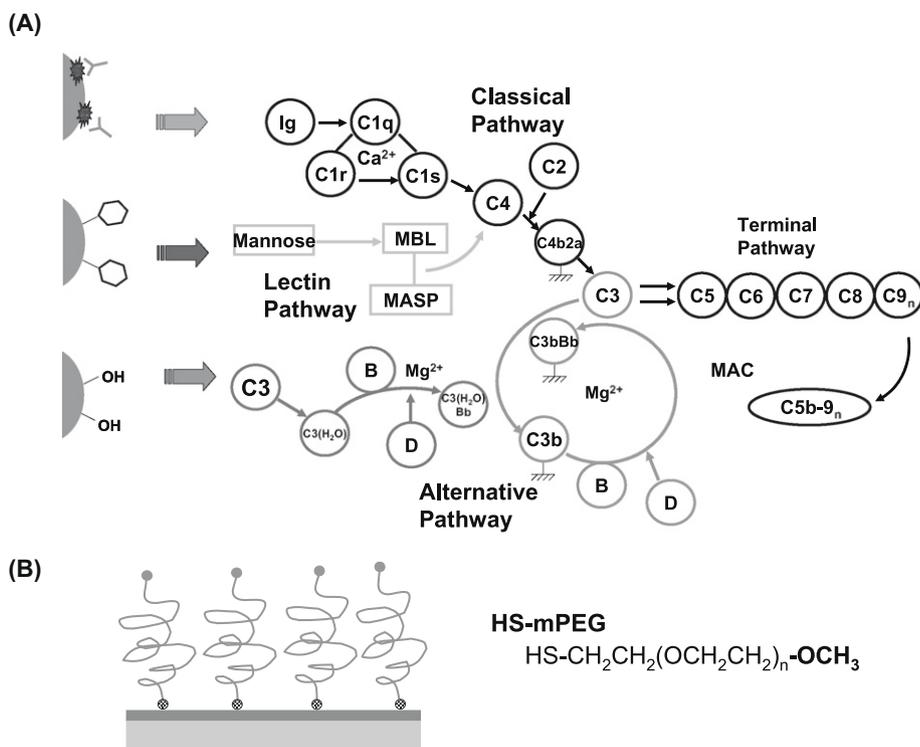
2. Materials and methods

2.1. Reagents and antibodies

HS-mPEG (SUNBRIGHT ME-050SH, Mn = 5000, NOF Corporation, Tokyo, Japan) was used as provided. Barbitol sodium, calcium chloride, magnesium chloride, and O,O'-bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (EGTA) (all purchased from Nacalai Tesque, Inc., Kyoto, Japan) and ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA; Dojindo Laboratories, Kumamoto, Japan) were of reagent grade. EDTA chelates Ca²⁺ and Mg²⁺ cations and inhibits all three activation pathways of the complement system. EGTA with abundant Mg²⁺ cation captures Ca²⁺ cation and thus inhibits classical and lectin pathways of the complement system. Ethanol (reagent grade, Nacalai Tesque) was deoxygenized with nitrogen gas bubbling before use. Water was purified with a Milli-Q system (Millipore Co.). Rabbit anti-human C3b antiserum (RAHu/C3b) was

* Corresponding author. Tel./fax: +81 75 751 4119.

E-mail address: iwata@frontier.kyoto-u.ac.jp (H. Iwata).



mPEG surfaces

mPEG surfaces examined after preparation procedure	: "naive mPEG"
mPEG surfaces stored in -20°C freezer for 12 days	: "F-mPEG"
mPEG surfaces stored in a dessicator under room light	: "R-mPEG"
mPEG surfaces irradiated with UV light for 60 min.	: "UV-mPEG"

Scheme 1. Schematic illustration of the complement activation pathways (A) and a surface modified with methoxy-capped PEG (B). Reprinted from Arima Y. et al., "Complement activation on surfaces modified with ethylene glycol units", *Biomaterials* 29(5), 552, Copyright (2008), with permission from Elsevier.

purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands) and its solution prepared and stored in accordance with supplier instructions.

2.2. Preparation of NHS and buffers

All participants enrolled in this research provided informed consent, which was approved and accepted by the ethics review board of the Institute for Frontier Medical Sciences, Kyoto University. Blood was donated from 10 healthy volunteers who had consumed a meal at least 4 h before the donation. The preparation method for the NHS has been described elsewhere [13]. Briefly, the collected blood was kept at ambient temperature for 30 min and centrifuged at 4°C . Supernatant was pooled and mixed and stored at -80°C until use. Veronal buffer (VB) was prepared referring to a protocol of CH50 measurement [16]. To prevent complement activation completely, $10\ \mu\text{l}$ of 0.5 M EDTA aqueous solution (pH 7.4) was added to 490 μl of NHS (final concentration of EDTA, 10 mM). To block the classical pathway of the complement system, $10\ \mu\text{l}$ of a mixture of 0.5 M EGTA and 0.1 M MgCl_2 aqueous solution (pH 7.4) was added to 490 μl of NHS (final concentration of EGTA, 10 mM; Mg^{2+} , 2 mM).

2.3. Preparation of self-assembled monolayer (SAM) of HS-mPEG

Glass plates were coated with gold as previously reported [13,17]. The gold-coated glass plate was immersed in a 4 mM

solution of HS-mPEG in a 1:6 mixture of Milli-Q water and ethanol at room temperature for at least 24 h to form the HS-mPEG-coated surface (Scheme 1B). Finally, the glass plate carrying a monolayer of HS-mPEG was sequentially washed with ethanol and Milli-Q water three times with each and then dried under a stream of dried nitrogen gas. The plate carrying a monolayer of HS-mPEG (naïve mPEG) was subjected to complement activation tests. The plates were stored in a dessicator under room light for a predetermined time to assess the effects of deterioration of the mPEG layer on complement activation (R-mPEG). To observe more directly the effects of ultraviolet (UV) oxidation, surfaces carrying mPEG were UV irradiated at 20 cm from a 15 W germicidal lamp (Matsushita Electric Industrial Co. Ltd, Osaka, Japan) in air at room temperature for 60 min (UV-mPEG) [13] (Scheme 1B). Plates carrying 11-mercaptoundecanol (Sigma-Aldrich Co., St. Louis, MO, USA) were formed as previously reported [17,18] and used as a positive control.

2.4. Surface analyses of modified surfaces carrying mPEG

Infrared (IR) adsorption spectra of sample surfaces were collected by the reflection-adsorption method (FTIR-RAS) using a Spectrum One (Perkin-Elmer, USA) spectrometer equipped with a Refractor™ (Harrick Sci. Co., NY, USA) in a chamber purged with dry nitrogen gas and a mercury-cadmium telluride detector cooled by liquid nitrogen. Gold-coated glass plates with a gold layer of 49 nm thickness were used for FTIR-RAS analysis. Spectra were

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