

Complement activation on degraded polyethylene glycol-covered surface

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Abstract

Surface modification with polyethylene glycol (PEG) has been employed in the development of biomaterials to reduce unfavorable reactions. Unanticipated body reactions, however, have been reported, with activation of the complement system 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 (HS-mPEG). We observed neither protein adsorption nor activation of the complement system on the PEG-modified surface just after preparation. After storage of the PEG-modified surface in a dessicator under ambient light for several days or following UV irradiation, reflection-adsorption (FTIR-RAS) and X-ray photo spectrometry both revealed deterioration of the PEG layer, which became a strong activator of the complement system through the alternative pathway.

Keywords;

Complement activation; Polyethylene oxide; Protein adsorption; Surface modification;

Surface analysis

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 aftereffects 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 MilliQ system (Millipore Co.). Rabbit anti-human C3b antiserum (RAHu/C3b) was 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 µl 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 µl of a mixture of 0.5 M EGTA and 0.1 M MgCl₂ aqueous solution (pH 7.4) was added to 490 µl of NHS (final concentration of EGTA, 10 mM; Mg²⁺, 2 mM).

2.3. Preparation 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 desiccator 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 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 RefractorTM (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 obtained using the p-polarized IR laser beam at an incident angle of 75 degrees for 128 scans at 4 cm⁻¹ resolution from 4000 to 750 cm⁻¹.

Surfaces were also analyzed by using XPS. The XPS spectra of the surfaces were collected by an ESCA-850V (Shimadzu Co., Kyoto, Japan), with a magnesium target and an electric current through the filament of 30 mA at 8 kV. The pressure of the analysis chamber was less than 1×10⁻⁵ Pa. All spectra shown in the figures were corrected by reference to the peak of Au 4f_{7/2} to 83.8 eV.

2.5. Total protein and C3b deposition observed by surface plasmon resonance (SPR)

We used an SPR apparatus assembled in our laboratory [18]. Gold-coated glass plates with a gold layer of 49 nm thickness were used. Undiluted NHS or NHS supplemented with EDTA or EGTA was injected into a flow cell, and change in the SPR angle was monitored as a function of time for 90 min. After the NHS was washed out with VB, a solution of rabbit anti-human C3b antiserum diluted to 1% with VB was applied to detect C3b and C3b degraded products, and the change of the SPR angle was estimated to determine antibody binding on the surface.

Protein layer thickness was calculated from the SPR angle shift using Fresnel fits for the system BK7/Cr/Au/SAM/protein/water [19,20]. Both refractive indices of SAM and protein layers were assumed to be 1.45 [19,20], and the density of the protein layer was assumed to be 1.0. From these values, the amounts of proteins adsorbed onto the surfaces could be estimated from the following simple relation [18]: 1.0 degree SPR angle shift (DA) → 0.5 µg of protein on 1 cm² of the surface.

2.6. Release of the soluble form of the membrane attack complex, SC5b-9, and anaphylatoxins C5a and C3a

We used a lab-made incubation chamber to examine release of C3a-desArg, SC5b-9, and C5a-desArg when NHS samples were exposed to different surfaces. The chamber was composed of two glass plates carrying the same sample surfaces and a silicone gasket of 1 mm thickness with a hole of 20 mm in diameter. After NHS samples were incubated in the chamber for 1.5 h at 37 °C, they were collected and EDTA was immediately added to a final concentration of 10 mM to stop further activation of the complement system. Commercial enzyme-linked immunosorbent assay (ELISA) kits (for C3a-desArg detection: BD OptEIA Human C3a ELISA, BD Biosciences Pharmingen, CA, USA; for C5a-desArg detection: BD OptEIA Human C5a ELISA, BD Biosciences Pharmingen, CA, USA; for the soluble form of membrane attack complex - SC5b-9: Quidel SC5b-9(TCC) EIA kit, Quidel Corp., CA, USA) were used to determine the fragments, C3a-desArg and C5a-desArg, and the complex, SC5b-9, in the collected NHS samples. The measurement procedure was performed in accordance with supplier instructions.

2.7. Statistical analysis

Data from the experiments are expressed as the mean \pm standard deviation. A one-way analysis of variance (ANOVA) was used to identify the statistical significance of the data. ANOVA was followed by post-hoc pair-wise t-tests adjusted using Holm's method and employing R language environment ver. 2.9.1 [21].

3. Results

3.1. Analyses of mPEG surfaces

FTIR adsorption spectra and XPS spectra were obtained to study HS-mPEG SAM formation and its deterioration during storage under ambient conditions and in a -20 °C freezer (F-mPEG). Fig. 1A shows the C-O-C stretch region of the IR spectra and Fig. 1B shows the C1s region of the XPS spectra for naïve mPEG, F-mPEG, R-mPEG, and UV-mPEG. Signal intensities obtained from R-mPEG and UV-mPEG surfaces significantly decreased in the region of C-O-C stretch of the IR spectra. For XPS spectra, the intensity ratios of C1s over Au 4f signals also decreased with storage under the ambient conditions or treatment by UV irradiation. These results suggest that the mPEG layers became thinner by degradation of mPEG chains or that an mPEG surface became heterogeneous by removal of some part of the mPEG surface during storage and UV irradiation. Fig. 1 also includes an mPEG surface that was stored in a -20 °C freezer for 12 days (F-mPEG). The F-mPEG surfaces did not demonstrate such changes in either the FTIR adsorption spectra or XPS spectra. Therefore, the mPEG surface could be stored without damage in a -20 °C freezer for at least 12 days.

3.2. Protein adsorption and C3b deposition onto mPEG-HS surfaces

When mPEG surfaces were exposed to NHS, we used SPR to follow protein adsorption and activation of the complement system. Fig. 2 shows SPR sensorgrams for the naïve mPEG, F-mPEG, and R-mPEG surfaces. Initially sharp increases in SPR angle shifts were the result of a larger refractive index of NHS than VB (Fig. 2A). For the cases of naïve mPEG and/or F-mPEG surfaces, no additional increase in SPR angles was observed during exposure to NHS. When NHS was washed out by infusion of VB, the SPR signals rapidly decreased to the low pre-exposure levels. The SPR angle shifts indicate that small amounts ($0.04 \mu\text{g}/\text{cm}^2$) of proteins adsorbed onto naïve mPEG and F-mPEG. On the other hand, the SPR angle shift continuously increased during exposure to NHS on the R-mPEG surface. After the surface was exposed to NHS for 90 min, NHS was washed out by infusion of VB. A large shift in SPR angle (1600 mDA) was still observed. The amount of adsorbed proteins estimated from the SPR angle shift was $0.8 \mu\text{g}/\text{cm}^2$.

When the complement system is activated through any of the three pathways, C3b is immobilized on surfaces, as Scheme 1A illustrates. After NHS was washed out, a solution of anti-C3b antiserum was applied to examine the existence of C3b and C3b degraded products in the protein layers formed on the mPEG surfaces (Fig. 2B). The SPR angles gradually increased with time. Only 0.17 and $0.19 \mu\text{g}/\text{cm}^2$ of anti-C3b antibody were immobilized on the mPEG and F-mPEG surfaces, respectively. On the other hand, a much larger amount of anti-C3b antibody ($0.95 \mu\text{g}/\text{cm}^2$) was bound on the protein adsorbed layer formed on the R-

mPEG surface. These results indicate that the naïve mPEG surface deteriorated during storage under ambient conditions and acquired the ability to activate the complement system.

Fig. 2 also includes behaviors of protein adsorption and anti-C3b antibody deposition on the UV-mPEG surface. They were the same on the UV-mPEG surfaces as on the R-mPEG. Irradiation with UV light could accelerate the deterioration of the naïve mPEG surface, and the mPEG layer gained the ability to activate the complement system.

3.3. Release of C3a, SC5b-9, and C5a

C3 is cleaved into C3a and C3b in the contact activation of the complement system on artificial materials. The C3 convertase, C3bBb, which forms on artificial materials, cleaves the α -chain of C3, generating C3a and C3b, and then the smaller 9-kDa anaphylatoxin C3a is released into the fluid phase and rapidly cleaved to form C3a-desArg. Thus, the presence of C3a/C3a-desArg in the fluid phase indicates activation of the complement system. Fig. 3A shows the results for C3a/C3a-desArg levels in NHS samples applied to naïve mPEG, R-mPEG, and UV-mPEG. Amounts of C3a-desArg released into NHS samples on the R-mPEG and UV-mPEG surfaces were significantly greater than those on naïve mPEG surfaces.

A terminal complement complex, C5b-9, is generated by the assembly of C5 through C9 as a consequence of activation of the complement system. In the absence of a target cell membrane (that is, activation on artificial materials), it binds to regulatory proteins, like the S

protein, and is released to serum as a non-lytic SC5b-9 complex. When the complement system is activated on mPEG surfaces, we expect release of SC5b-9 into serum. Fig. 3B summarizes the released amounts of SC5b-9 identified when NHS samples were incubated with naïve mPEG, R-mPEG, and UV-mPEG. Although a small amount of SC5b-9 was found when naïve mPEG surfaces were examined, large amounts of SC5b-9 were observed in NHS samples exposed to the R-mPEG and UV-mPEG surfaces. Those amounts were much larger than those observed in NHS samples exposed to OH-SAM, which has been reported to be a strong complement activator [18].

The C5 convertases (C4b2a3b : the classical and the lectin pathways, C3bBb : the alternative pathway) cleaves the α -chain of C5, generating C5a and C5b, and then the smaller 11-kDa anaphylatoxin C5a is released into the fluid phase and rapidly cleaved to form C5a-desArg. Thus, the presence of C5a/C5a-desArg in the fluid phase also indicates activation of the complement system. The generated amounts of C5a-desArg were also determined, showing a tendency that was almost the same as that for SC5b-9 measurements (Fig. 3C).

3.4. Activation pathway

Whereas addition of EDTA to NHS inhibited all three activation pathways, addition of EGTA-Mg²⁺ can inhibit the classical and lectin pathways, but not the alternative pathway.

Fig. 4 and Fig. 5 show effects of addition of EDTA and EGTA-Mg²⁺ on the complement

activation. When an R-mPEG and UV-mPEG surface was exposed to NHS supplemented with 10 mM EDTA, the amounts of adsorbed serum proteins and anti-C3b antibody bound were greatly reduced. Practically no protein adsorbed onto the surface in the presence of EDTA. In NHS samples supplemented with 10 mM EGTA-Mg²⁺, protein adsorption was retarded but gradually increased at 20 min after exposure. Using anti-C3b antibody, we detected a large amount of C3b/C3bBb and C3b degraded products in the protein-adsorbed layer. These results indicate that the complement system was activated through the alternative pathway on the R-mPEG and UV-mPEG surfaces.

4. Discussion

Although PEG has been widely used for preparation of biomedical devices and drug carriers to reduce interaction of artificial materials with proteins and cells, unanticipated body reactions such as hypersensitivity reactions caused by PEG-modified surfaces have been reported [6-9]. Body reactions against PEG-modified surfaces should be carefully examined. In our previous study [13], naïve mPEG, and R-mPEG and UV-mPEG surfaces were exposed to 10% diluted NHS and then anti-C3b antiserum was applied to see immobilization of C3b on these surfaces. A large amount of anti-C3b antibody was found on R-mPEG and UV-mPEG surfaces, but not naïve mPEG. These results suggest that naïve mPEG is not an activator of the complement system, but R-mPEG and UV-mPEG surfaces are strong

activators. We need more relevant supporting data to conclude it and to identify activation pathways. In this study, complement activation by the latter two surfaces was further confirmed by releases of C3a-desArg, C5a-desArg and SC5b-9 into liquid phase NHS. Even with blocking of the classical and lectin pathways by the addition of EGTA-Mg²⁺ to NHS, R-mPEG and UV-mPEG surfaces can activate the complement system. We concluded from all of these data that R- and UV-mPEG surfaces activate the complement system through the alternative pathway.

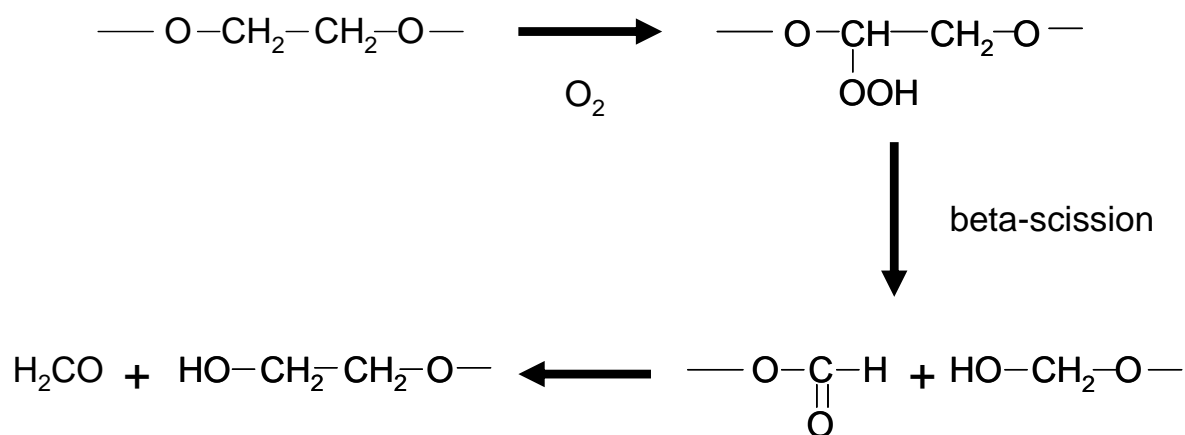
In our previous study [13], we also examined a surface carrying tri(ethylene glycol)-terminated alkanethiol (HS-TEGOH) as an activator of the complement system. When the classical pathway was blocked with EGTA-Mg²⁺, deposition of proteins, including C3b, on it was delayed from 0 min and 40 min by addition of EGTA-Mg²⁺ to 10% NHS, respectively. Several groups have reported that a protein layer containing IgG formed on artificial materials starts the activation of the complement system through the classical pathway and then an amplification loop of the alternative pathway follows [22-25]. Complement activation should be carefully examined under more physiologically relevant conditions. We expected that amounts of proteins (including IgG and IgM) deposited on naïve mPEG surface increase with increasing serum concentration and thus it becomes an activator. No proteins adsorption, however, was detected on naïve mPEG surface when it was exposed to undiluted NHS. It

indicates that naïve mPEG neither activates complement system through the classical nor alternative pathway even in undiluted NHS.

Kiwada's group reported that PEG-modified liposomes activate the complement system, resulting in opsonization of liposomes [26-28]. When the spleen of a recipient was removed, no opsonization was observed [28]. From this fact, they inferred that anti-PEG IgM [26,27] secreted from the spleen [28] activated the complement system on PEG liposomes through the classical pathways. Our serum donors had not been exposed to PEG-modified substances; thus, it is difficult to predict whether they carried anti-PEG IgM or IgG antibodies.

R-mPEG and UV-mPEG surfaces showed a 64% and 43% decrease in C-O-C peak area of IR spectra and a 43% and 36% decrease in C/Au ratios of XPS spectra, respectively. These decreases might arise from (1) the heterogeneous removal of whole HS-mPEG chains from gold surfaces or (2) removal of low molecular weight substances produced by degradation of PEG chains on the specimens. We examined protein adsorption onto a bare Au surface, a large amount of proteins, $0.49 \mu\text{g}/\text{cm}^2$ and $0.89 \mu\text{g}/\text{cm}^2$, was found on it when it was exposed to NHS and 1% γ -globulin/VB solution, respectively. In the former situation, proteins should be adsorbed onto the bare area of the surface with surface exposure to NHS supplemented with EDTA. As Fig. 4 shows, however, we observed no protein adsorption. This finding indicates that the latter situation is more likely.

It has been reported that PEG chains are degraded through mechanical shocks, heating, and irradiation with light [29,30]. Mkhathresh et al. [29] proposed a degradation mechanism of PEG chains under light irradiation as follows:



The resulting fragments of PEG chains carry OH groups at the chain ends. The nucleophilic OH groups have been reported to strongly activate the complement system through the alternative pathway. As mentioned previously, complement activation on the mPEG surfaces robustly correlates with decreased PEG chain length on the surfaces. These facts indicate that the PEG layer easily deteriorated, resulting in formation of a surface carrying nucleophilic OH groups, and thus gained the ability to activate the complement system through the alternative pathway after storage in a desiccator under ambient light for several days or after UV irradiation.

Conclusion

A PEG-modified surface strongly activates the complement system through the alternative pathway after its storage under ambient conditions for several days. PEG-modified artificial materials, proteins, and liposomes should be carefully stored.

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REFERENCES

1. Harris J M. Poly(ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications. New York: Plenum Press; 1992
2. Kingshott P, Griesser H J. Surfaces that resist bioadhesion. *Curr Opin Solid State Mater Sci* 1999;4:403-12
3. Nucci M L, Shorr R, Abuchowski A. The therapeutic value of poly(ethylene glycol)-modified proteins. *Adv Drug Deliv Rev* 1991;6:133-51
4. Harris J M, Chess R B. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2003;2:214-21
5. Duncan R. The dawning era of polymer therapeutics. *Nat Rev Drug Discov* 2003;2:347-60
6. Uziely B, Jeffers S, Isacson R, Kutsch K, Wei-Tsao D, Yehoshua Z, Libson E et al. Liposomal doxorubicin: Antitumor activity and unique toxicities during two complementary phase I studies. *J Clin Oncol* 1995;13:1777-85
7. de Marie S. Liposomal and lipid-based formulations of amphotericin B. *Leukemia* 1996;10:93-6
8. Alberts D S, Garcia D J. Safety aspects of pegylated liposomal doxorubicin in patients with cancer. *Drugs* 1997;54:30-5
9. Skubitz K M, Skubitz A P N. Mechanism of transient dyspnea induced by pegylated-

- liposomal doxorubicin (Doxil(TM)). *AntiCancer Drugs* 1998;9:45-50
10. Laverman P, Brouwers A H, Dams E T M, Oyen W J G, Storm G, Van Rooijen N, Corstens F H M et al. Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose. *J Pharmacol Exp Ther* 2000;293:996-1001
 11. Moghimi S M, Szebeni J. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog Lipid Res* 2003;42:463-78
 12. Walport M J. Complement. First of two parts. *N Engl J Med* 2001;344:1058-66
 13. Arima Y, Toda M, Iwata H. Complement activation on surfaces modified with ethylene glycol units. *Biomaterials* 2008;29:551-60
 14. Andersson J, Ekdahl K N, Lambris J D, Nilsson B. Binding of C3 fragments on top of adsorbed plasma proteins during complement activation on a model biomaterial surface. *Biomaterials* 2005;26:1477-85
 15. Tengvall P, Askendal A, Lundström I. Complement activation by 3-mercapto-1,2-propanediol immobilized on gold surfaces. *Biomaterials* 1996;17:1001-7
 16. Whaley K, North J. Haemolytic assays for whole complement activity and individual components. In: Sim R B, Dodds A W, editors. *Complement*. Walton Street, Oxford: Oxford University Press; 1997.

17. Toda M, Kitazawa T, Hirata I, Hirano Y, Iwata H. Complement activation on surfaces carrying amino groups.. *Biomaterials* 2008;29:407-17
18. Hirata I, Morimoto Y, Murakami Y, Iwata H, Kitano E, Kitamura H, Ikada Y. Study of complement activation on well-defined surfaces using surface plasmon resonance. *Colloids Surf B Biointerfaces* 2000;18:285-92
19. Azzam RMA, Bashara NM. Reflection and Transmission of Polarized Light by Stratified Planar Structures. In: *Ellipsometry and Polarized Light*. Amsterdam: Elsevier; 1987. pp. 269-363
20. Knoll W. Polymer thin films and interfaces characterized with evanescent light. *Makromol Chem* 1991;192:2827-56
21. R Development Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing; 2009. ISBN 3-900051-07-0, URL: <http://www.R-project.org>
22. Pangburn M K. Alternative Pathway: Activation and Regulation. In: Rother K, Till G O, Hänsch G M, editors. *The Complement System, Second Revised Edition*. Berlin: Springer-Verlag; 1998. pp. 93-115
23. Lhotta K, Wurzner R, Kronenberg F, Oppermann M, König P. Rapid activation of the complement system by cuprophane depends on complement component C4. *Kidney Int* 1998;53:1044-51

24. Nilsson U R. Deposition of C3b/iC3b leads to the concealment of antigens, immunoglobulins and bound C1q in complement-activating immune complexes. *Mol Immunol* 2001;38:151-60
25. Tengvall P, Askendal A, Lundström I. Studies on protein adsorption and activation of complement on hydrated aluminium surfaces in vitro. *Biomaterials* 1998;19:935-40
26. Ishida T, Ichihara M, Wang XY, Yamamoto K, Kimura J, Majima E, Kiwada H. Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. *J Control Release* 2006;112:15-25
27. Wang XY, Ishida T, Kiwada H. Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. *J Control Release* 2007;119:236-44
28. Ishida T, Ichihara M, Wang XY, Kiwada H. Spleen plays an important role in the induction of accelerated blood clearance of PEGylated liposomes. *J Control Release* 2006;115:243-50
29. Mkhathresh O A, Heatley F. A ¹³C NMR Study of the Products and Mechanism of the Thermal Oxidative Degradation of Poly(ethylene oxide). *Macromol Chem Phys* 2002;203:2273-80
30. Morlat S, Gardette J-L. Phototransformation of water-soluble polymers. I: photo- and

thermooxidation of poly(ethylene oxide) in solid state. Polymer 2001;42:6071-9

Figure legends

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

Fig. 1. Analyses of HS-mPEG-carrying surfaces. FTIR-RAS spectra and peak areas of C-O-C stretch region (A); error bars represent \pm SD (n=8). XPS spectra of carbon atoms and intensity ratios of C1s over Au 4f signals of HS-mPEG carrying surfaces (B); error bars represent \pm SD (n=9). (—): naïve HS-mPEG, (- . - .): F-mPEG, (- - -): R-mPEG, (.....): UV-mPEG surfaces.

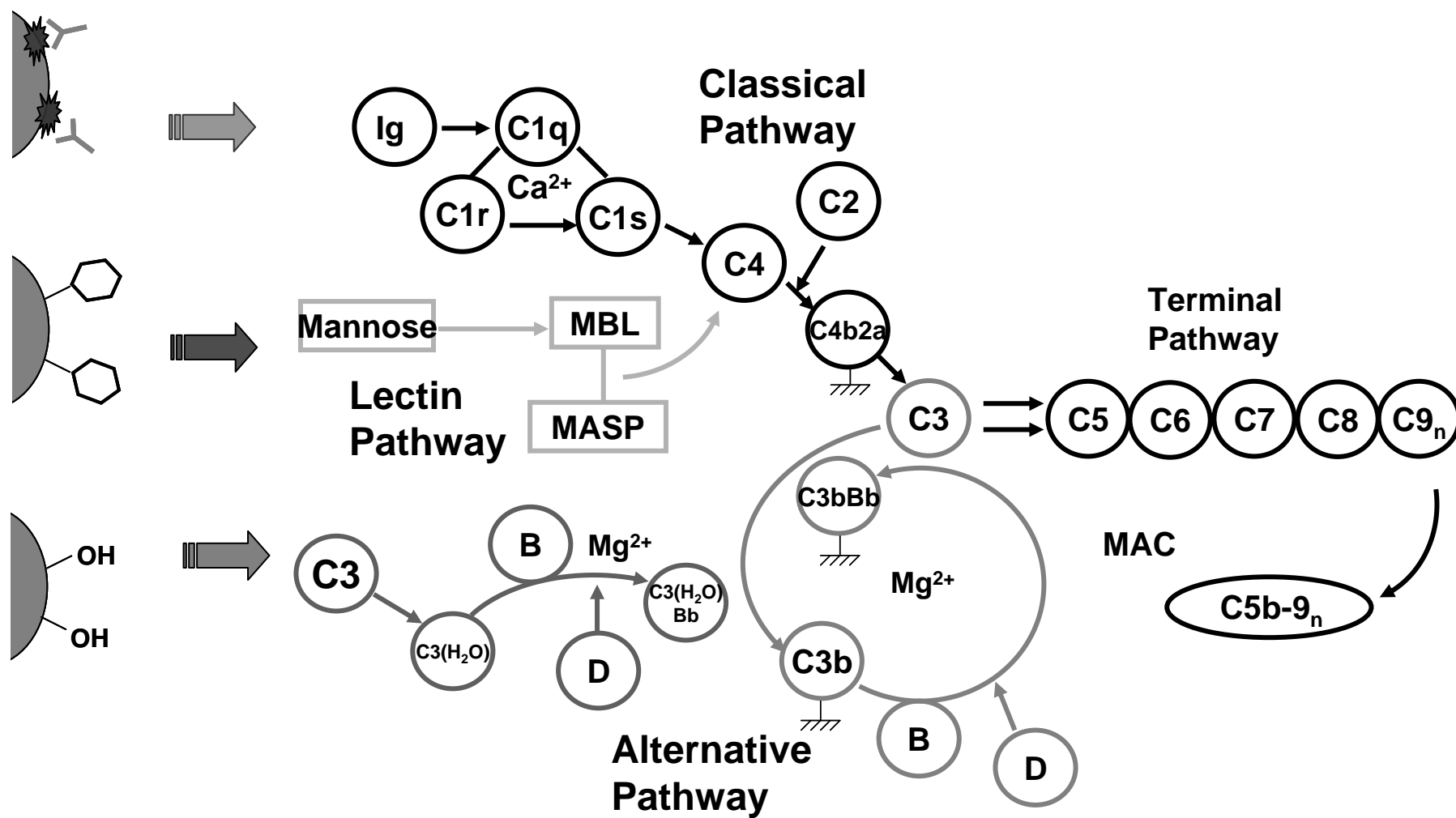
Fig. 2. SPR sensorgrams. During exposure of undiluted normal human serum (NHS) to HS-mPEG surfaces (A). Sequential exposure of 1% anti-C3b antiserum to the surfaces (B). (—): naïve HS-mPEG, (- . - .): F-mPEG, (- - -): R-mPEG, (.....): UV-mPEG surfaces. DA: degrees of angle.

Fig. 3. Release of C3a-desArg, SC5b-9, and C5a-desArg into NHS. Undiluted serum was exposed to CH₃-, OH-SAM, and naïve mPEG, R-mPEG and UV-mPEG surfaces. CH₃- and OH-SAM were used as negative- and positive-controlled surfaces, respectively, for complement activation, and zymosan was used to fully activate complement. Experiments were repeated 3 times for each surface. C3a-desArg (A): Amounts of C3a-desArg in naïve undiluted NHS and fully activated by incubation with zymosan were $3.3 \pm 0.6 \mu\text{g}/\text{cm}^3$ (n=3) and $56.2 \pm 4.5 \mu\text{g}/\text{cm}^3$ (n=3), respectively; error bars represent \pm SD. SC5b-9 (B): amounts of SC5b-9 in naïve undiluted NHS and fully activated by incubation with zymosan were $0.3 \pm 0.03 \mu\text{g}/\text{cm}^3$ (n=3) and $310 \pm 34 \mu\text{g}/\text{cm}^3$ (n=3), respectively; error bars represent \pm SD. C5a-desArg (C): amounts of C5a-desArg in naïve undiluted NHS and fully activated by incubation with zymosan were $0.010 \pm 0.007 \mu\text{g}/\text{cm}^3$ (n=3) and $3.0 \pm 0.2 \mu\text{g}/\text{cm}^3$ (n=3), respectively; error bars represent \pm SD.

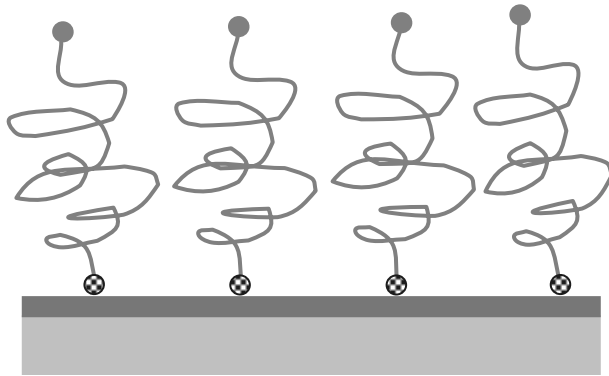
Fig 4. Effect of EDTA and EGTA added to NHS on complement activation on R-mPEG surfaces. SPR sensorgrams during exposure of NHS (A), and sequential exposure of 1% anti-C3b antiserum to the surfaces (B). (— — —): NHS (same data in Fig. 2), (————): NHS supplemented with 10 mM EDTA, (— . — .): NHS supplemented with 10 mM EGTA (with 2 mM Mg²⁺). DA: degrees of angle.

Fig 5. Effect of EDTA and EGTA added to NHS on complement activation on UV-mPEG surfaces. SPR sensorgrams during exposure of NHS (A), and sequential exposure of 1% anti-C3b antiserum to the surfaces (B). (.....): NHS (same data in Fig. 2), (————): NHS supplemented with 10 mM EDTA, (— · — ·): NHS supplemented with 10 mM EGTA (with 2 mM Mg^{2+}). DA: degrees of angle.

(A)



(B)



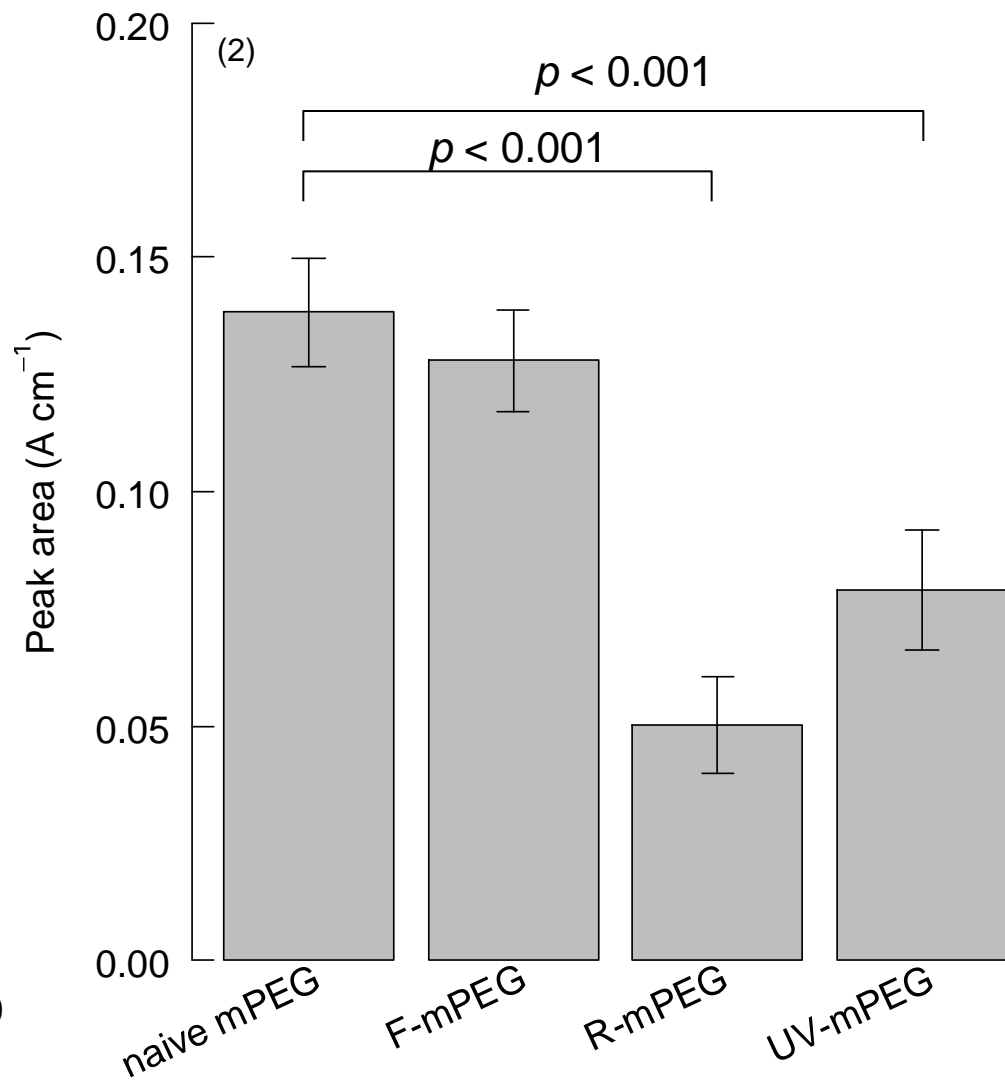
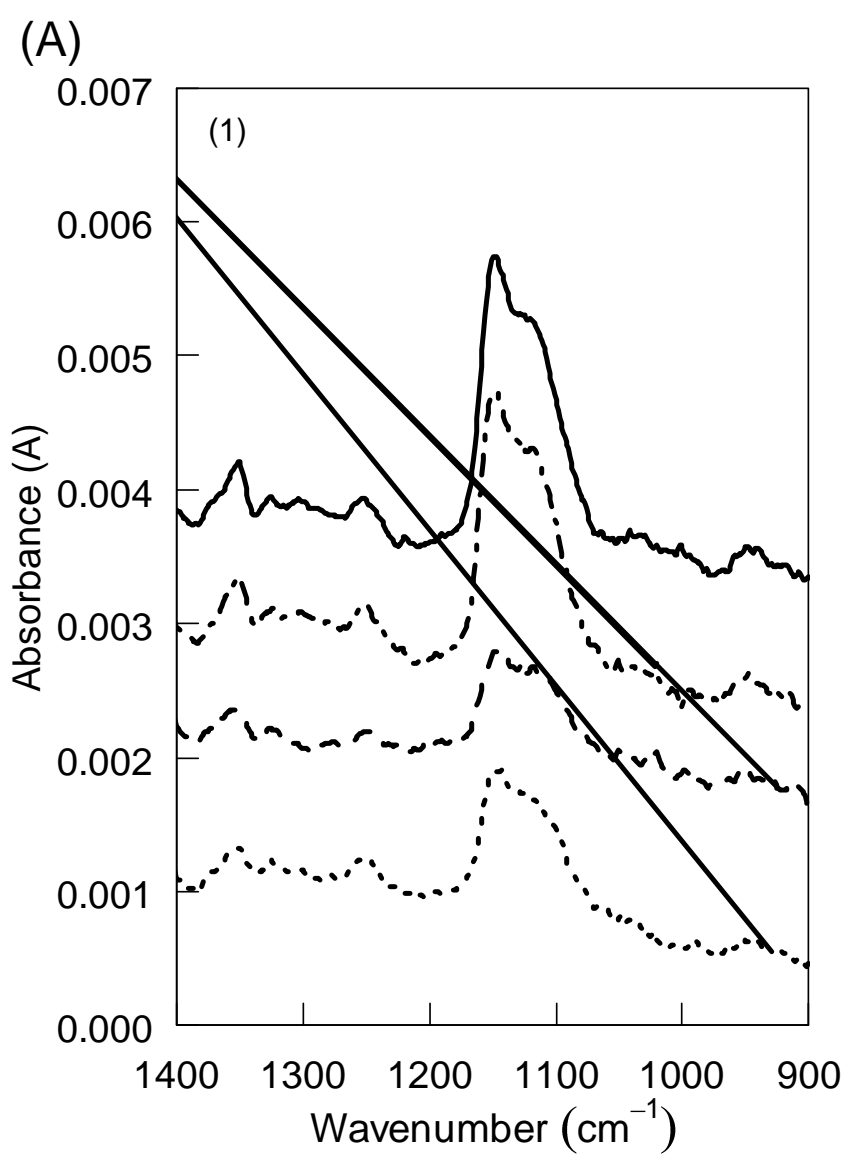
HS-mPEG

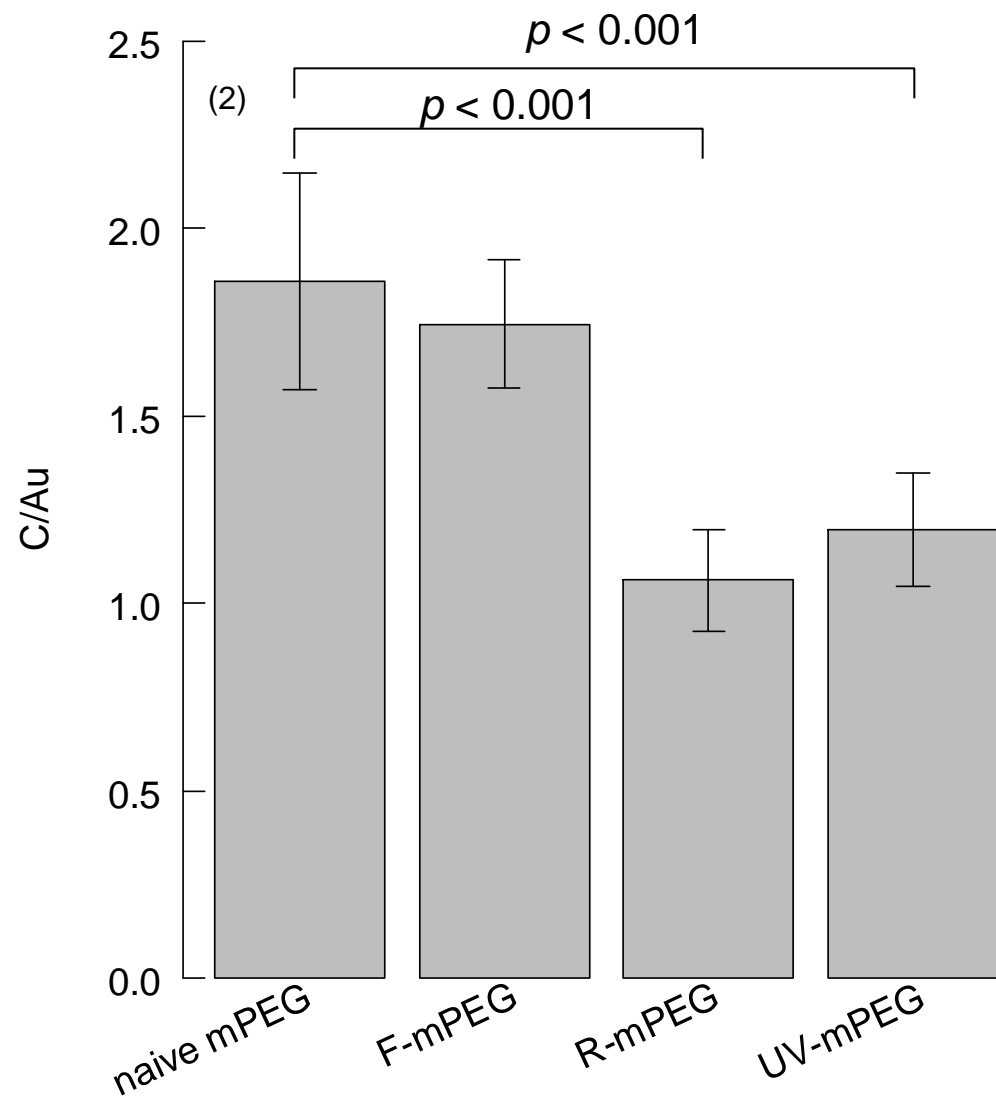
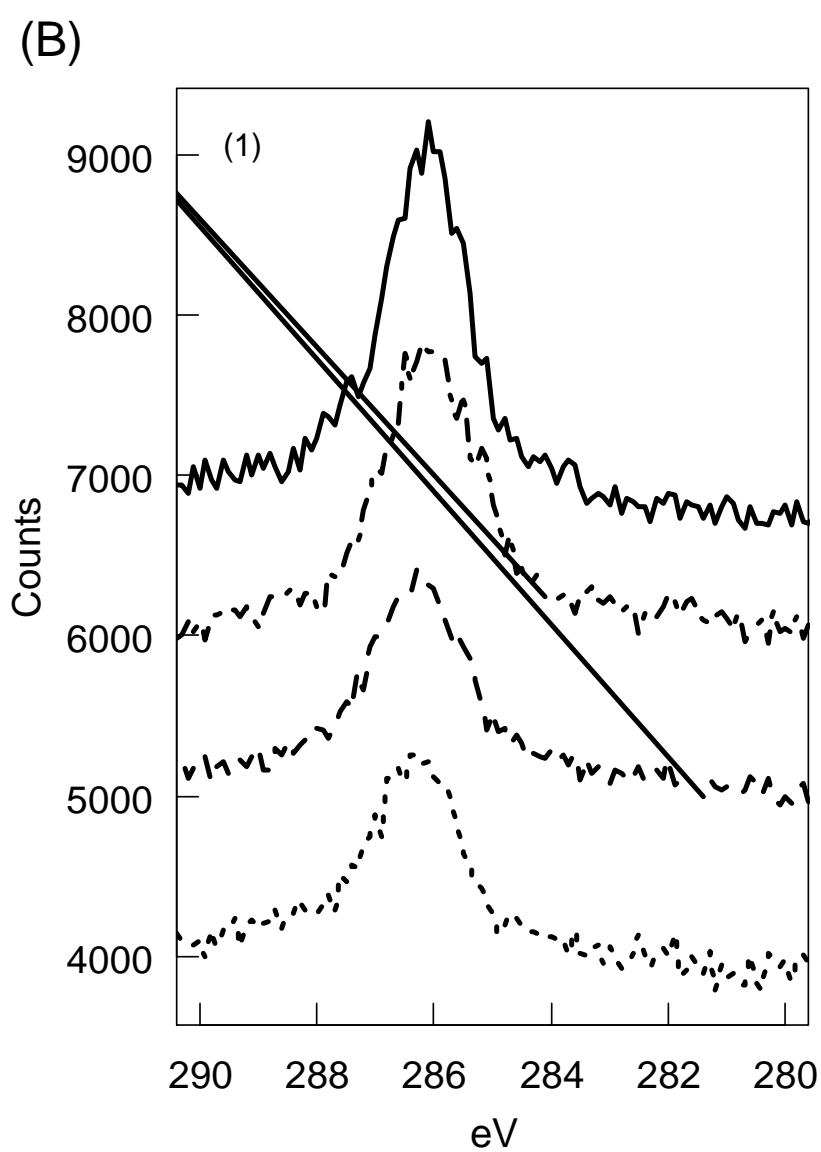


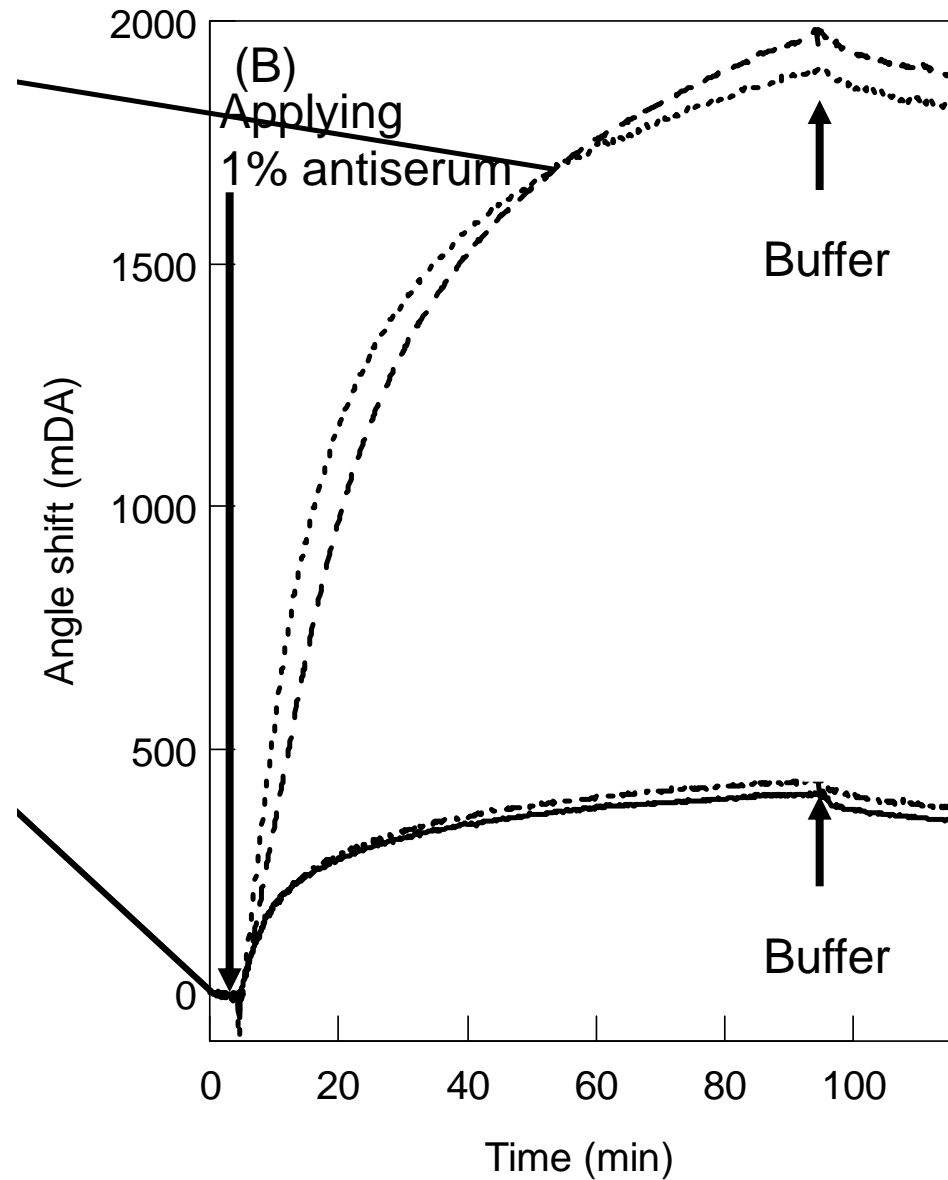
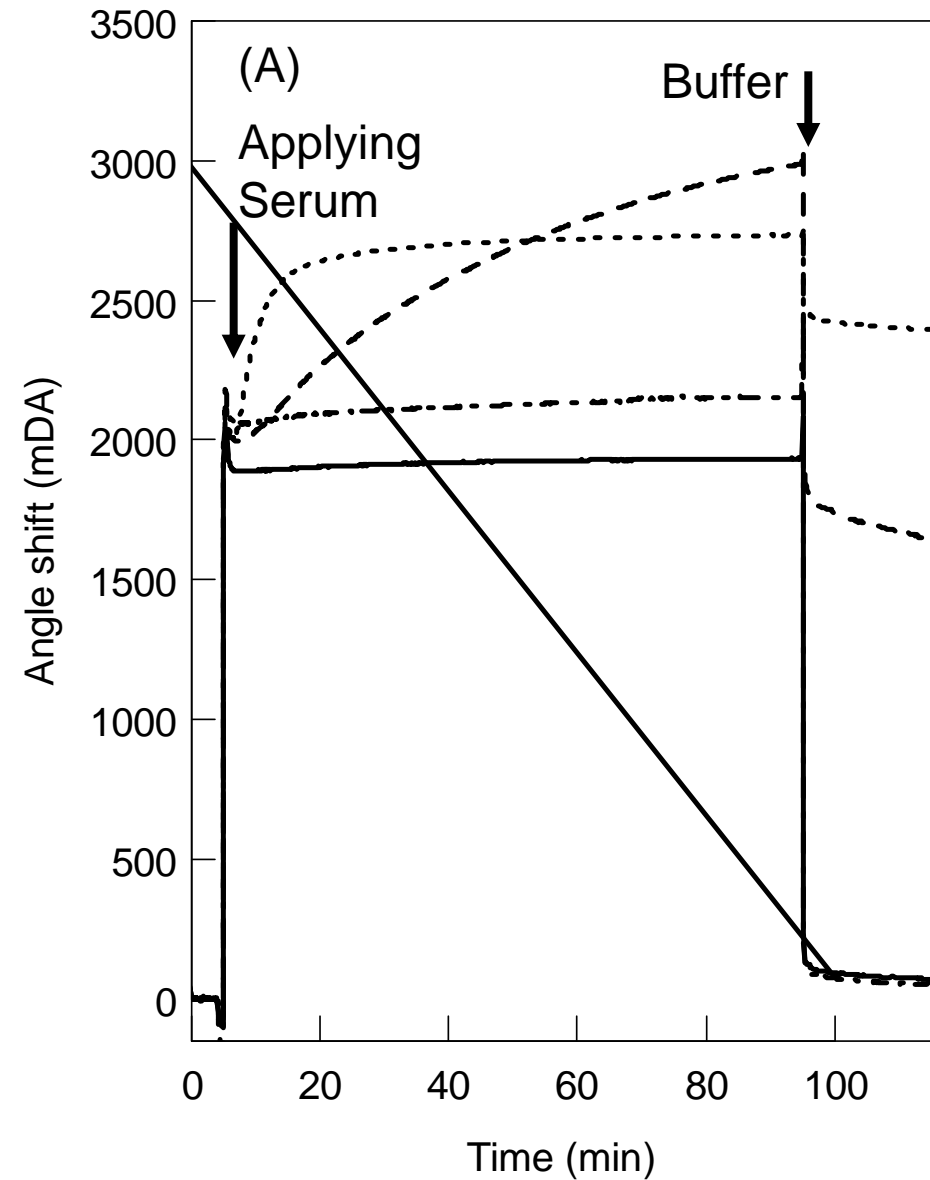
mPEG surfaces

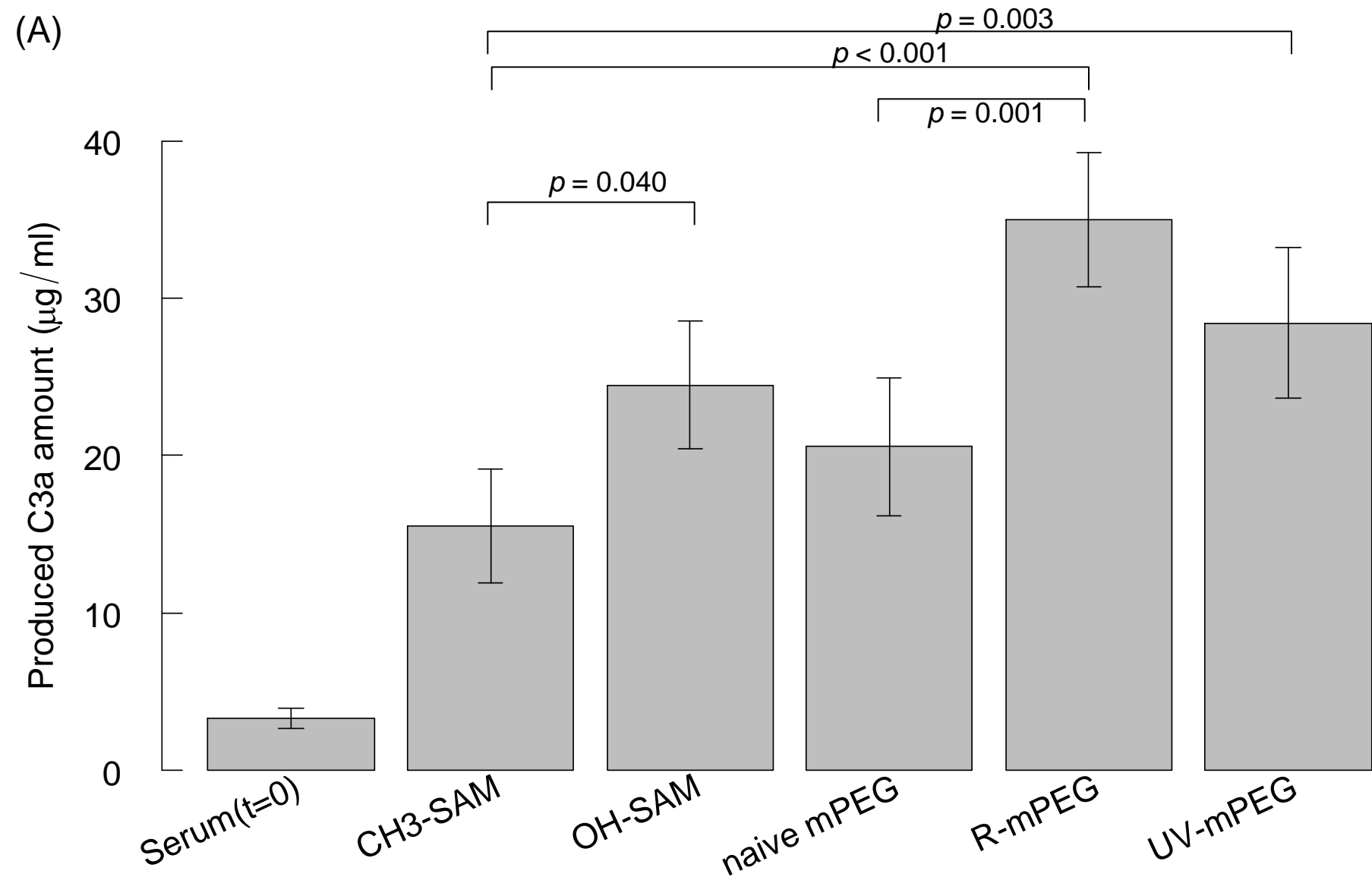
mPEG surfaces examined after preparation procedure
mPEG surfaces stored in -20 °C freezer for 12 days
mPEG surfaces stored in a dessicator under room light
mPEG surfaces irradiated with UV light for 60 min.

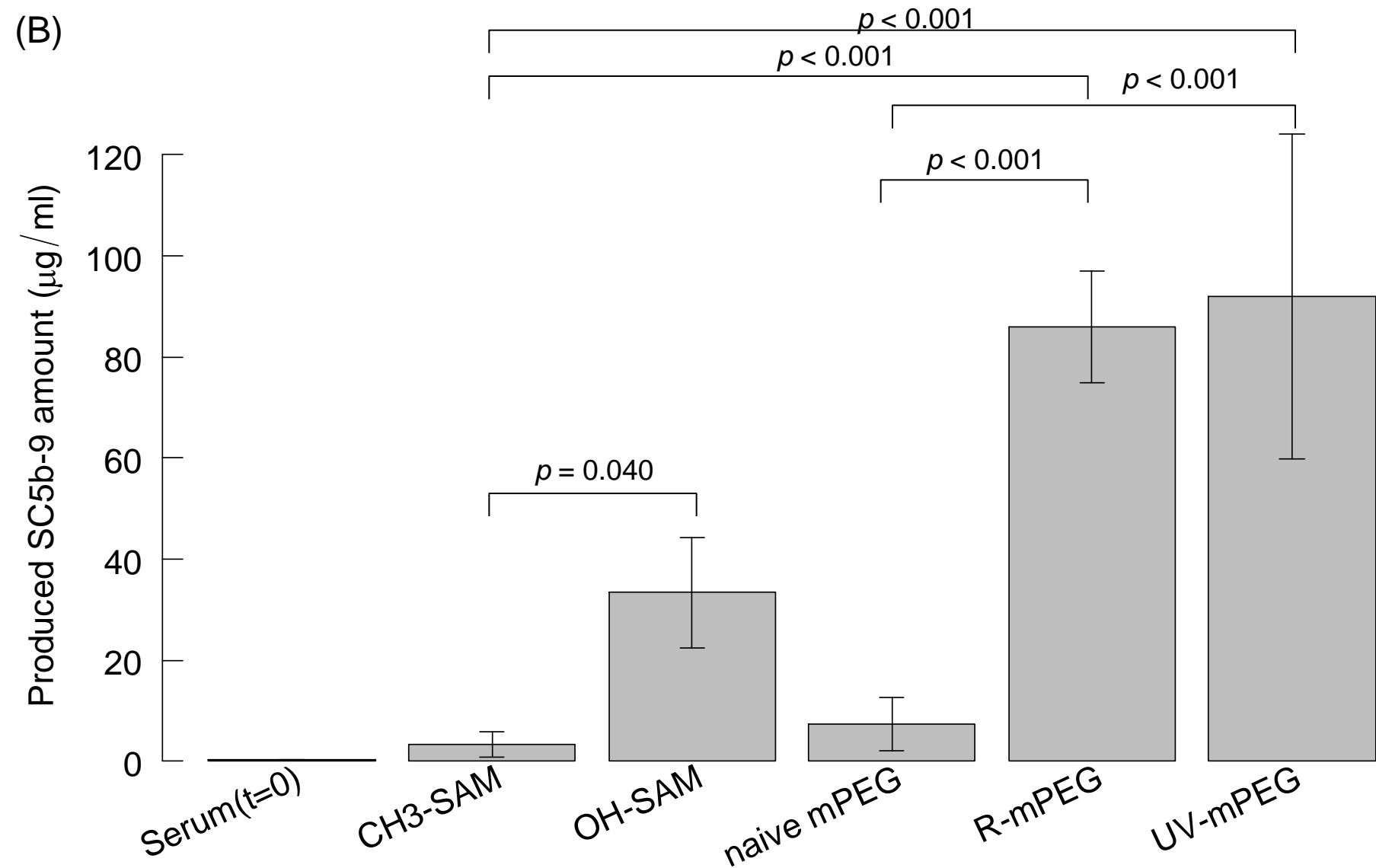
: "naive mPEG"
: "F-mPEG"
: "R-mPEG"
: "UV-mPEG"











(C)

