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Absence of conformational change in complement factor 3 and factor XII adsorbed to acrylate polymers is related to a high degree of polymer backbone flexibility

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In previous investigations, the authors have examined the adsorption of albumin, immunoglobulin, and fibrinogen to a series of acrylate polymers with different backbone and side-group flexibility. The authors showed that protein adsorption to acrylates with high flexibility, such as poly(lauryl methacrylate) (PLMA), tends to preserve native conformation. In the present study, the authors have continued this work by examining the conformational changes that occur during the binding of complement factor 3 (C3) and coagulation factor XII (FXII). Native C3 adsorbed readily to all solid surfaces tested, including a series of acrylate surfaces of varying backbone flexibility. However, a monoclonal antibody recognizing a "hidden" epitope of C3 (only exposed during C3 activation or denaturation) bound to the C3 on the rigid acrylate surfaces or on polystyrene (also rigid), but not to C3 on the flexible PLMA, indicating that varying degrees of conformational change had occurred with binding to different surfaces. Similarly, FXII was activated only on the rigid poly(butyl methacrylate) surface, as assessed by the formation of FXIIa-antithrombin (AT) complexes; in contrast, it remained in its native form on the flexible PLMA surface. The authors also found that water wettability hysteresis, defined as the difference between the advancing and receding contact angles, was highest for the PLMA surface, indicating that a dynamic change in the interface polymer structure may help protect the adsorbed protein from conformational changes and denaturation. *© 2017 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).* [http://dx.doi.org/10.1116/1.4985698]

I. INTRODUCTION

Conformational changes occurring in proteins during adsorption have implications for a host's response to biomaterials,[1–3] but the processes that dictate the final protein conformation and corresponding cell responses are still relatively unknown. During spontaneous protein adsorption, energy is released according to the Gibbs law of free energy.[4] The combination of entropy and enthalpy changes in both the protein and substratum makes it difficult to predict the adsorption of proteins, and many models describing this phenomenon are based on empirically generated data using self-assembled monolayers[5,6] or silanized[7,8] surfaces. Using these model surfaces, the degree of water wettability, an important yet simple hydrodynamic aspect of protein adsorption, has been thoroughly investigated.[9–11] At low wettability (i.e., on hydrophobic surfaces), there is a risk that proteins may lose some of their original conformation as well as function, or even acquire new functions as a result of these changes in conformation.[12,13]

In our research, we have investigated the interactions of specific blood proteins with various solid surfaces, metals, and polymers and have also tried to relate the protein adsorption effects we have observed to the degree of wettability of the particular solid surface(s) involved. During the course of this work, we realized that other surface-associated parameters, e.g., the degree of polymer backbone or side group mobility, may also affect the conformation of the adsorbed proteins. The family of alkyl methacrylate polymers offers a unique opportunity to vary the molecular mobility of the polymers, usually expressed as the glass transition temperature (T_g), without significantly altering their surface chemical

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functional groups or wettability.[14] This makes this polymer series ideal when it comes to investigating the effect of surface dynamics on interfacial phenomena as suggested in the early eighties by Andrade and coworkers.[15] This was later explored in protein adsorption studies by Vandamme *et al.* in which they noticed that polymers with low (n = 1) and high (n = 18) number of carbons in the n-alkyl chain generally adsorbed less protein than the other polymers in the series.[16] We recently expanded these studies and investigated the effect on protein adsorption and conformational change of three different poly(alkyl methacrylates) with varying lengths and branchings of their side chain, i.e., poly(butyl methacrylate) (PBMA), poly(isobutyl methacrylate (PIBMA), and poly(lauryl methacrylate) (PLMA).[17] By using these polymers, control over polymer backbone chain mobility was achieved: they ranged from very flexible, with the polymer chains almost freely rotating along each monomeric unit and side group (PLMA); to polymers showing segmental flexibility, in which large parts of the polymer chain were mobile thanks to rotation at a few points along the polymeric chain (PBMA); and finally to inflexible polymers, in which very little movement of the polymer backbone or side groups occurred (PIBMA). The flexible PLMA polymer possessed enough rotational freedom to respond to interfacial changes, and it could reorient upon protein adsorption, minimizing the interfacial tension at the interface. The reduced interfacial tension has been hypothesized to prevent structural rearrangements in the protein, allowing it to retain a more native configuration upon adsorption. We explored this hypothesis with regard to the adsorption of IgG, albumin, and fibrinogen by combining Quartz crystal microbalance with dissipation (QCM-D) and surface plasmon resonance analyses. Our interpretation of the results is that fibrinogen was adsorbed in a less hydrated and more native conformation on the flexible PLMA than on its rigid counterpart. By comparison, IgG and albumin showed the same adsorption pattern regardless of the surface's degree of flexibility.

In the current investigation, we have continued our studies of the conformational status of proteins adsorbed to acrylate polymers of varying surface flexibility. As model proteins, we selected human complement factor 3 (C3) and human factor XII (FXII). Although biochemically quite different, the two proteins are similar in acting as known triggers of the cascade systems in the blood after its interaction with artificial interfaces.[18–21]

FXII is an 80-kDa protein that circulates in the blood at a concentration of ~25 μg/ml. However, upon contact with a foreign surface, the zymogen FXII becomes proteolytically autoactivated to the enzyme FXIIa, which is a key initiator of the intrinsic pathway of the coagulation cascade and the kallikrein pathway.[22,23] These reactions were initially attributed to occur on anionic surfaces, but recent studies demonstrate that that the autoactivation of FXII on surfaces is not merely associated with proteolytic cleavage, but rather with a conformational alteration. Nor is surface negative charge a prerequisite for activation.[24] The serine protease inhibitor (serpin) antithrombin (AT) binds specifically to activated FXIIa, but not to the zymogen FXII and can therefore be used to detect the formation of FXIIa after its adsorption to foreign surfaces.[25]

C3 is a 185-kDa protein that is present in human plasma at ~1 mg/ml and is a central component of the complement system. C3 can be activated by cleave by protease complexes termed C3-convertases, yielding C3a and C3b which becomes conformationally changed and may bind covalently to a surface via amine or carboxyl groups.[26,27] Alternatively, C3 may adsorb to a surface without establishing covalent bonds, but undergoing profound conformational changes, similar to those in C3b and thereby trigger the alternative pathway of complement activation, like C3b.[28] In an earlier study we have shown that C3 possesses hidden antibody binding epitopes that only are exposed when C3 is partially denatured or biologically activated. The conformational changes that occur during C3's activation are reflected in alterations in the exposure of such neo-epitopes on the surface of the protein. In an attempt to generate antibodies specifically directed against C3 fragments when in the bound conformation, we have produced monoclonal antibodies (mAbs) recognizing the reduced polypeptide chains of C3. The binding of these antibodies cannot be competed out by the corresponding soluble fragments and is therefore specific for the bound forms.[29,30]

In the present study, we used one of these antibodies, mAb 26.1 (anti-C3d, g), to detect differences in the conformational change in complement protein C3 upon adsorption to the various polymer surfaces. The aim of this investigation was to use adsorption of C3 and FXII, followed by the binding of antibody and AT, respectively, as a way to study adsorbed protein conformation at the flexible PLMA and the other acrylate surfaces. We also included "rigid" hydrophobic polystyrene and hydrophilic glass (SiO_2) in the experiments as relevant control surfaces. QCM-D monitoring was used to quantify FXII and C3 adsorption as well as antibody binding.

II. EXPERIMENT

A. Proteins and regents

FXII was purchased from Enzyme Research Laboratories, South Bend. AT and C3 were purified from human plasma as described previously.[31,32] The anti-C3 mAb 26.1 against a neo-epitope in C3d,g was produced and characterized according to Nilsson *et al.*,[33] and polyclonal anti-C3c was bought from Dako A/S, Glostrup, Denmark. The purified proteins were diluted in veronal-buffered saline (VB^{++}; 5 mM barbiturate, *p*H 7.4; 145 mM NaCl; 0.15 mM Ca^{2+}; 0.5 mM Mg^{2+}).

B. Polymers

Polystyrene (PS), PIBMA and PLMA were provided by Sigma-Aldrich, Sweden. PLMA was purchased dissolved in toluene (25% w/v) and was diluted in toluene to a final concentration of 0.5% (w/v) before use. PIBMA was obtained as pellets and dissolved in toluene at a concentration of 0.5%

(w/v). The PBMA was made in-house by radical polymerization using a protocol previously described.[34] The repeating units of the polymers used in this study are shown in Fig. 1.

The surface properties of the polymers have been investigated before, and a detailed description of the relevant methodology and results have been published.[34] In summary, x-ray photoelectron spectroscopy (XPS) was used to verify polymer composition. Dynamic contact angle analysis was used both to study molecular rearrangements and to calculate surface energy. Tapping mode atomic force microscopy (AFM) was used to investigate root mean square (RMS) roughness. The polymer flexibility, or glass transition temperature (T_g), was measured by differential scanning calorimetry (DSC).

The hardness or flexibility of the polymers was probed by indentation resistance Buchholz (IRB). Polymers (PIBMA, PBMA, and PLMA) were dissolved in toluene at 20% (w/v), and films were applied on aluminum panels using a wet film applicator set at 250 μm. Films were left to dry in a controlled atmosphere (23 °C, 50% relative humidity) for 2 days. The resulting dry film thickness was measured to be ~50 μm, as determined according to the method described in ISO 2808.[35] The indentation resistance was measured according to ISO 2815,[36] in which an indentation apparatus is used to yield an indentation on the experimental surface. The resulting length of the indentation crack is then measured, and the IRB value is calculated according to: IRB = 100/indentation length (mm).

C. QCM-D measurements

PIBMA, PBMA, PLMA, and PS were each dissolved in toluene to a final concentration of 0.5% (w/v). The gold-plated quartz crystals (Biolin Scientific AB, Sweden) were cleaned before use according to the following protocol: 10 min UV/ozone treatment, then immersion for 5 min in a 5:1:1 solution of MilliQ water, ammonia (25%), and hydrogen peroxide (30%) heated to 75 °C, rinsing in MilliQ water, and finally another UV/ozone treatment for 15 min. The polymeric coatings were then individually spin-coated onto the quartz crystal sensor surface by adding 50 μl of the polymer solution at 2000 rpm for 1 min. By the use of Sauerbrey relation,[37] the polymer thickness was estimated to be in the range of 20 nm when dry.

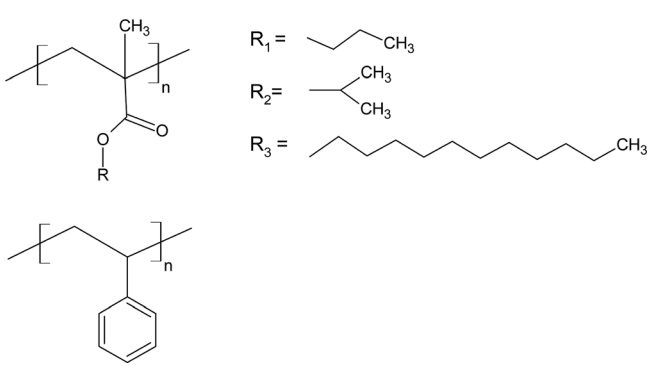

FIG. 1. Repeating units of the poly(alkyl methacrylates), in which R_1 = PBMA, R_2 = PIBMA, R_3 = PLMA, and PS.

Quartz crystal microbalance with dissipation monitoring was used to assess the structure and function of C3 after its adsorption to the three different polymeric substrates with varying flexibility/softness as well to a typical hydrophilic (SiO_2) surface and a hydrophobic (PS) surface. The theory behind the QCM-D technique has been described in detail elsewhere.[38] In short, the resonance frequency (f) of an oscillating quartz crystal is proportional to the mass of the crystal plus the assembled layer, and the mass can be calculated according to the Sauerbrey relation.[37] The damping or dissipation (D) of the oscillation reflects the viscoelastic properties of the attached material. This damping has to be taken into account when working with more extended and water-containing layers, since a soft and viscoelastic film will dampen the sensor's oscillation, and the mass as calculated by the Sauerbrey relation will be underestimated. By measuring the frequency shift (Δf) and dissipation (D), the gradual build-up of several layers on a surface can be followed in real time.

The QCM-D measurements were performed using a QCM-D Omega Pro from Biolin Scientific AB (Göteborg, Sweden). Changes in frequency and dissipation were recorded during the whole QCM-D experiment, and the mass uptake was calculated using the Sauerbrey equation (for rigid layers, $D < 1$) or from the modeling of data (soft layers, $D > 1$) according to Hernandez *et al.*[39] All QCM-D experiments were carried out at 22 °C. The purified proteins were diluted in Veronal buffer containing Mg^{2+} and Ca^{2+} (VB^{++}). The same buffer was used as running buffer for priming and rinsing the sensors. The sensors were rinsed for 5 min before, in between, and after addition of the proteins. The flow rate was set to 20 μl/min for the injection of protein as well as for rinsing with running buffer.

D. Determination of conformational changes of C3 and activation of FXII

The four different polymer-coated QCM-D sensors (PIBMA, PBMA, PLMA, and PS) and the SiO_2 sensor were mounted in the QCM-D Pro instrument. After priming with VB^{++} buffer, C3 (10 μg/ml) in VB^{++}-buffer was added and incubated for 50 min. Then, mAb 26.1 (10 μg/ml in VB^{++}) was added, and the samples were incubated for an additional 20 min, followed by the addition of anti-C3c antibody (10 μg/ml in VB^{++}) and incubation for 20 min.

For the determination of concentration dependent conformational change, solutions of C3 in VB^{++} buffer were prepared at four different concentrations (10, 20, 50, and 100 μg/ml) and adsorbed onto the semihard polymer surface (PBMA) and the soft polymer surface (PLMA) for 50 min at a flow rate of 20 μl/min. Thereafter, mAb26.1, the antibody recognizing the denatured form of C3 (10 μg/ml in VB^{++}), was added, followed by the anti-C3c antibody (10 μg/ml in VB^{++}). The sensor surface was rinsed with buffer before, in between, and after each component was added.

For the detection of FXII activation PBMA- and PLMA-coated sensors were mounted in the QCM-D Pro instrument and primed with VB^{++} buffer. FXII (25 μg/ml in VB^{++}) was added, and the samples were incubated for 20 min, followed by the addition of AT (0.125 mg/ml in VB^{++}) and incubation for 20 min. As in the experiment described above, the sensor surfaces were rinsed with buffer before, in between, and after incubation with each added component.

III. RESULTS AND DISCUSSION

A. Chemical and physical characterizations of polymers

The results of the chemical and physical characterizations are shown in Table I. In summary, XPS showed no deviation from the surface composition expected on the basis of the stoichiometry of the polymer's repeating units. Dynamic contact angle measurements and use of an equation of state were used to calculate the surface energy of the polymers. Receding contact angle reflects the configuration of the polymer surface in buffer (water) and indicated only minor differences in surface free energy between the polymers. Thus, any effects on conformational changes after adsorption were not dictated by differences in hydrophobicity. The contact angle was evaluated at 37 °C as well as at 22 °C. As expected, raising the temperature increased the flexibility, as manifested by an increase in contact angle hysteresis ($\theta_{\text{hysteresis}} = \theta_{\text{advancing}} - \theta_{\text{receding}}$). For example, the contact angle hysteresis increased from 28.8 to 30.1 in the case of PBMA and from 47.9 to 64.4 in the case of PLMA. Thus, at the higher temperature, the highly flexible PLMA polymer was able to reorient more hydrophilic ester functional groups toward the interface. The polymer flexibility, expressed as the glass transition temperature (T_g), ranged from −70 °C for PLMA to 17 °C in the case of PBMA and to 66 °C for PIBMA, as measured with DSC. In comparison, the T_g of PS was on the order of 100 °C. Finally, the hardness or flexibility on a more macroscopic level, expressed as the Buchholz hardness (IRB), ranged from 88 ± 1.3 for PLMA to 149 ± 2.0 for PBMA and 164 ± 2.5 for PIBMA. The polymers showed significantly different hardness ($p < 0.01$, t-test), but PBMA and PIBMA resembled each other more than the more flexible PLMA polymer. Altogether, the chemical and physical characterizations confirmed that the molecular flexibility is the major difference among these polymers.

B. Surface induced conformational change in C3 and activation of FXII

Native C3 was allowed to adsorb on sensor surfaces precoated with a rigid (PIBMA), semirigid (PBMA), or flexible (PLMA) polymer as well as hydrophobic PS and hydrophilic SiO_2 surfaces. After adsorption of C3 to the surface, a monoclonal antibody (mAb 26.1) recognizing the denatured form of C3 was added. This antibody binds to C3 only when C3 is activated: when the thioester of C3 is cleaved and C3 is hydrolyzed to $C3(H_2O)$. Finally, a polyclonal antibody recognizing C3c (anti-C3c) was added. This polyclonal antibody binds to C3 irrespective of its conformation and therefore reflects the total amount of C3 deposited at the surface. Representative QCM-D sensorgrams for the C3 deposition onto the three polymer (PIBMA, PBMA, and PLMA) surfaces, PS and SiO_2 was followed by the addition of the two antibodies for detection [Figs. 2(a) and 2(b)]; the results of at least three repeated measurements on each surface are summarized in Fig. 2(c).

As the figure shows, the surface concentration of C3 was lowest on the hard/semihard polymer surfaces PIBMA and PBMA (382 and 332 ng/cm^2, respectively), and a larger amount was adsorbed to the soft and flexible PLMA surface (593 ng/cm^2). A lower mass uptake, comparable to that for the hard methacrylates, was also measured on the hydrophobic hard PS surface (330 ng/cm^2). C3 adsorption to the hydrophilic SiO_2 surface was comparable to that to the soft PLMA (538 ng/cm^2). The binding of anti-C3c correlated well with the measured deposition of C3 on all the substrates and confirmed the differences in mass uptake at the various surfaces. This binding was also taken as an indication that C3 was not significantly desorbed during the experiments. However, monoclonal antibody mAb 26.1 only bound C3 adsorbed to the three hard surfaces (PIBMA, PBMA, and polystyrene), and there was no binding of this antibody to C3 at the soft PLMA surface. These results demonstrate that C3 had gone through a conformational change and formed $C3(H_2O)$ when it came into contact with the three rigid and hydrophobic surfaces, irrespective of the functional groups in their side chains (alkyl and ester in the case of the methacrylates and phenyl for polystyrene), while the absence of mAb binding to adsorbed C3 at the soft PLMA surface was interpreted as indicating that C3 remains more or less in its native form after adsorption to this soft surface, which also contains alkyl and ester functionalities.

Experiments were then performed to determine whether the conformational change from C3 to $C3(H_2O)$ that occurred

TABLE I. Characterizations of pristine acrylate polymers. Data except IRB hardness are reproduced from Ref. 34.

Polymer	C/O ratio (calculated)	C/O ratio (XPS)	RMS roughness (AFM, nm)	Surface energy γ_s (advancing/receding, mJ/m^2)	T_g (DSC, °C)	IRB hardness (1/mm)
PIBMA	4.0	4.2	0.88	28.3/36.5	66	164
PBMA	4.0	4.1	0.70	28.3/40.3	17	149
PLMA	8.0	8.6	0.70	19.1/40.5	−70	89

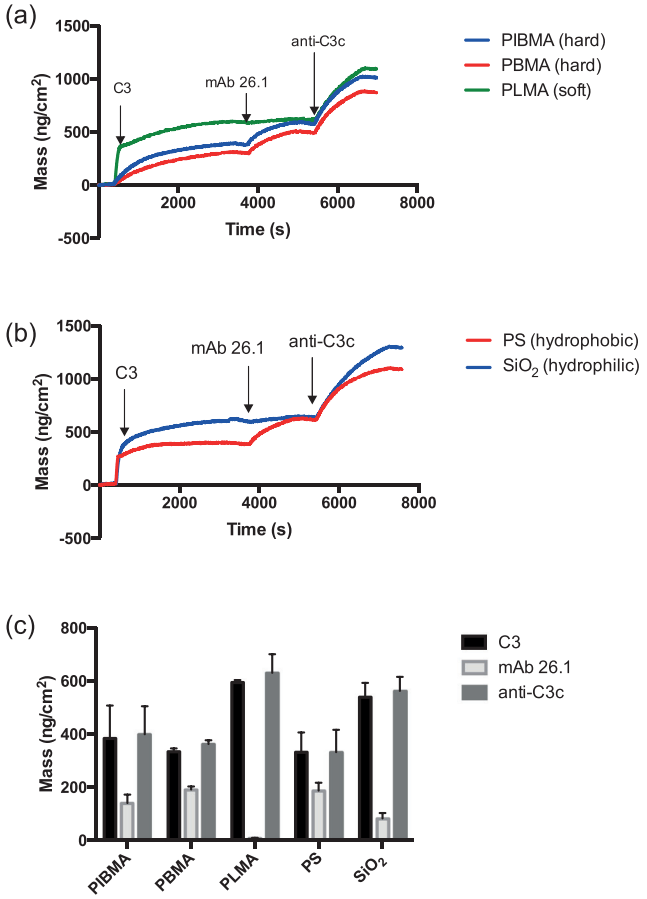

FIG. 2. Typical QCM-D sensorgrams showing the mass increase after the addition of C3, mAb 26.1, and pAb anti-C3c. (a) Sensorgrams for the rigid surface PIBMA (blue trace), the semirigid PBMA (red trace), and the flexible surface PLMA (green trace) and (b) sensorgrams for the hydrophilic SiO_2 (blue) and hydrophobic PS (red) surfaces. (c) Mass uptake of C3 (black bars), binding of monoclonal antibody 26.1 directed against the denatured form of C3 mAb (light gray bars), and polyclonal antibody against C3c (gray bars).

at the hard surfaces was dependent on the C3 concentration added. Four different concentrations of C3 in the range from 10 to 100 μg/ml were added to the hard PBMA polymer and the soft PLMA polymer coating. The degree of C3 denaturation was then followed by the addition of mAb 26.1, detecting epitopes that are only available for binding after the hydrolysis of the C3 thioester; the anti-C3c polyclonal antibody was added to confirm the total amount of C3 adsorbed to the surface. We found that the adsorption of C3 to the hard polymer PBMA surface was very sensitive to the surrounding C3 concentration [Fig. 3(a)]. At low concentration (10 μg/ml), C3 became denatured and spread out upon contact with the PBMA interface, and fewer C3 molecules were able to find space at the surface; increasing the concentration of C3 in the solution (to 20, 50, or 100 μg/ml) also increased the surface concentration of C3, and the molecules became more closely packed at the polymer surface. Figure 3(a) also shows that C3 was less prone to denaturation at the higher surface concentrations: decreasing amounts of mAb 26.1 binding occurred with increasing surface concentrations of C3, whereas the anti-C3c binding rose in accordance with

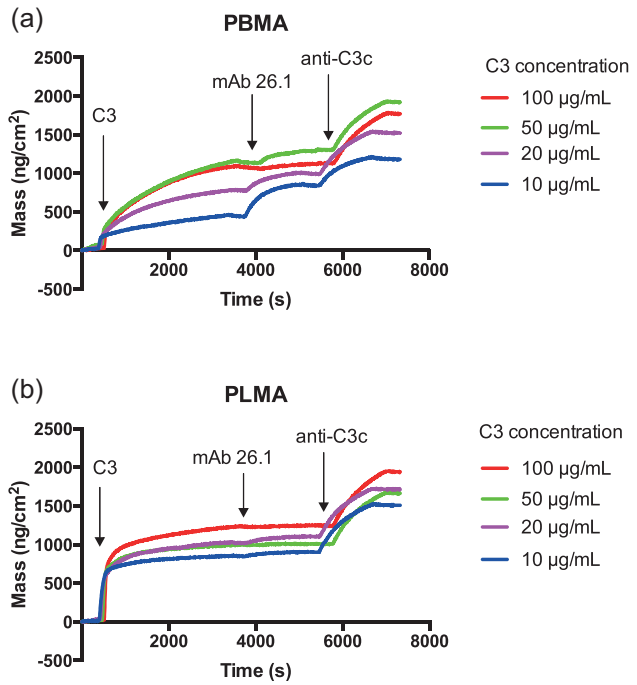

FIG. 3. QCM-D sensorgram showing the C3 adsorbed mass to rigid PBMA and flexible PLMA added at four different concentrations ranging from 10 to 100 μg/ml. Rigid PBMA (a) and flexible PLMA surface (b), and the subsequent addition of mAb 26.1 and anti-C3c.

the C3 surface density. In contrast, C3 adsorbed to the soft and flexible PLMA polymer surface was not affected by the C3 concentration in the solution. When the same concentrations of C3 in solution (10, 20, 50, and 100 μg/ml) were added to the PLMA surface, the C3 molecules became somewhat more densely packed on the surface as higher concentrations of C3 were added, Fig. 3(b), but the original C3 conformation remained intact, independent of the concentration of C3 added.

FXII was included in this study to validate if molecular flexibility of the substratum has effect on contact activated proteins stability in general or if the response of C3 was protein specific. FXII was allowed to interact with the PBMA- and PLMA-coated surfaces under constant flow while the mass uptake of FXII was recorded. After the surface was rinsed with buffer, the formation of activated FXIIa was detected by following the mass uptake upon addition of anti-thrombin (AT). Representative QCM-D sensorgrams are shown in Fig. 4. From these experiments, it was clear that FXII adsorbed to both the rigid PBMA polymer and to the soft PLMA. As we observed for C3, FXII adsorbed to the flexible PBMA polymer had preserved its native form, as indicated by the absence of AT binding. However, after contact with the hard PBMA surface, FXII was apparently converted to its activated form FXIIa, since a substantial amount of AT-FXIIa complex ($\sim$200 ng/cm^2) was detected at this surface.

C. Discussion

The majority of biomaterials activates coagulation via the contact pathway and complements via the alternative

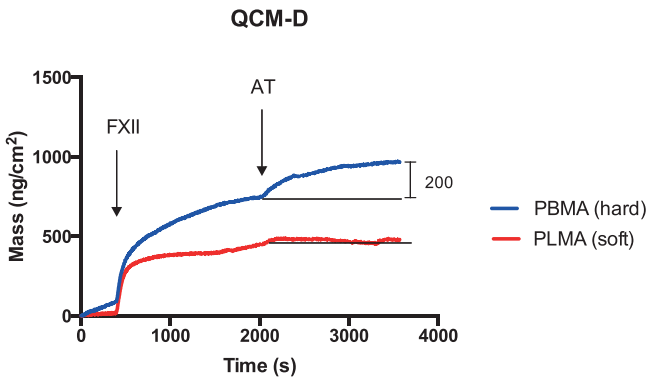

FIG. 4. QCM-D analysis of FXII adsorption to the rigid PBMA surface (blue line) and the flexible PLMA surface (red line), followed by AT binding to the activated form of FXII (FXIIa). The difference in mass before and after addition of AT is marked in the sensorgram.

pathway. In the present study, we show that polymers with almost identical chemical composition but with different degrees of alkyl side group chain length and branching, which result in varying degrees of rigidity/flexibility, are able to adsorb C3 and FXII and cause varying degrees of conformational change in the adsorbed molecules.

The conformational changes in C3 were detected by QCM-D combined with immunochemical assessment based on the anti-C3dg mAb 7D26.1. The results of the present study suggest that the initial C3(H_2O) generation may be induced by an interaction with surface interfaces, and this C3(H_2O) can then act as an initiator of the alternative pathway, provided that a conformational change exposing the neoepitope for mAb 7D.26.1 is induced in the molecule. This conformational change was evaluated by using four different polymers coated onto QCM-D sensors (PIBMA, PBMA, PLMA, and PS), in addition to a standard QCM-D SiO_2 surface. PIBMA, PBMA, and PLMA have the same chemical composition except for the length and branching of the polymer side group in each polymer, which are responsible for their differences in softness/hardness. PS, which is also a hard polymer, has a relatively high hydrophobicity, and SiO_2 is hydrophilic. mAb 7D26.1 bound to C3 adsorbed to the harder PIBMA and PBMA, but not to C3 adsorbed to the soft PLMA. Similarly, the epitope reactive with mAb 7D26.1 was exposed after C3 binding to PS, but not to SiO_2. These results indicate that a high degree of hardness and hydrophobicity induce an exposure of the relevant epitope.

In the experiments above, the kinetics of C3 adsorption carried out at 10 μg/ml was faster, and the final mass was somewhat higher on the flexible PLMA polymer compared with the rigid PBMA and PIBMA. The higher degree of packing observed on the PLMA might assist keeping the protein in its native configuration due to steric repulsion between neighboring C3 molecules.[40] This makes it difficult to assess to which degree the flexibility and/or the steric repulsion have on keeping the protein in native configuration on PLMA. However, it can be noted that the adsorbed mass obtained at 10 μg/ml to PLMA (860 ng/cm^2) and the adsorbed mass obtained at 20 μg/ml to PBMA (830 ng/cm^2) are approximately the same but mAb26.1 binding occurs only on the more rigid PBMA [Figs. 3(a) and 3(b)]. We interpret these results that C3 has gone through a conformational change to a higher degree on the rigid PBMA surface.

As discussed in the paper by Seigel *et al.*, the adsorbed proteins could exist in a number of different conformations, in which each of these conformations could decay to a range of unfolding by processes with complex, multistate kinetics with interactions between adsorbed and proteins in solution.[40] As the use of an antibody is an indirect detection of unfolding not able to detect differences in the range of unfolding other methods to further understand the process of protein unfolding on flexible/rigid surfaces could be employed. Recently, Latour and coworkers addressed conformational change upon adsorption by implementing an improved experimental circular dichroism set-up as well as mass spectroscopy.[41,42] By the use of these methods, eventual conformational change can be directly assessed and the degree of unfolding, and accessibility of the antibody binding site can be investigated.

Also, FXII was found to be notably affected by the surface flexibility. Activation of the zymogen FXII to the FXIIa enzyme upon contact with biomaterial surfaces is a well-known trigger of the coagulation cascade. The underlying mechanism is not completely understood, but the triggering is believed to be induced by a conformational change in the FXII molecule after it makes contact with the surface. In this study, we found that as for C3, FXII was activated after it made contact with the rigid PBMA surface, most likely as the result of a conformational change; in contrast, after adherence to the soft PLMA surface, FXII remained in its native and inactive form, as indicated by the absence of AT binding.

PBMA, which is a semirigid polymer and demonstrates some segmental movement, induced a similar degree of conformational change in C3 as did the hard PIBMA surface. Thus, in order to prevent conformational change, it is apparently important that the polymer have a sufficient amount of flexibility. It appears that the polymer must match the surface properties of the protein on a subnanometer scale, meaning that a high degree of rotational freedom is needed along the polymer backbone as well in the side groups (i.e., a low glass transition temperature). It should be stressed that flexibility is not the only important property. The alkyl methacrylates contain both hydrophilic (ester) and hydrophobic (alkyl) functionalities in their side groups, and this duality is believed to be important. A flexible polymer composed solely of hydrophobic groups, such as polyethylene, should not be able to suppress the conformational change resulting from protein adsorption, since there are no hydrophilic groups to counter the hydrophobic effect, resulting in higher interfacial tension between the polymer surface and hydrophilic domains of the protein.

The complement system is a critical component of innate immunity, and its normal function is to mediate a localized inflammatory response to a foreign material, in which C3 plays a central role. C3 is well known to undergo a conformational change upon adsorption and to trigger the alternative

pathway of complement activation via this conformational change. The present study corroborates our previous data indicating that C3 is contact-activated by adsorption to various hydrophobic biomaterial surfaces, such as metals, polymers, and gas bubbles (reviewed in Ref. 28), which might be critical for the generation of the alternative pathway convertases. Once C3 is activated, the local regulation of the alternative pathway will determine whether the alternative pathway amplification loop is engaged. Likewise, activated FXII is the key initiator of the contact activation pathway, which leads to a propagation of the coagulation cascade. It is therefore important to understand what types of surfaces induce this activation and which prevent it.

It can be noted that the polymers evaluated in this study all accumulate proteins, in contrast to nonadsorbing "stealth" surfaces based on neutral or zwitterionic polymers such as poly(ethylene oxide) and poly(carboxybetaine methacrylate).[43–47] Considering the *in vivo* situation, the soft PLMA surface will become covered with proteins, but as indicated in our study, in a rather native conformation. In contrast, a poly(ethylene glycol) modified surface will be free from proteins. One can easily anticipate that the biological responses (such as interactions with cells and foreign bodies) to these surfaces could be vastly different. Although this scenario is still very speculative, the PLMA surface could conceivably be sensed as "self" and trigger a minimum of downstream cascade reactions.

IV. SUMMARY AND CONCLUSIONS

In the current study, we show that FXII, an important contact activation initiator of the coagulation cascade, becomes activated after adsorption to a rigid polymer surface (PBMA) but remains in its native zymogen form after adsorption to a corresponding soft and flexible surface (PLMA). Similarly, we found that C3, a key player in the complement system, when adsorbed to hard polymer surfaces (PIBMA and PBMA), becomes denatured and forms C3(H_2O), whereas its native conformation is maintained after adsorption to the soft PLMA surface. The same relationship held true for hydrophobic versus hydrophilic surfaces, with C3 on the hydrophobic PS surface displaying a more open and denatured structure than C3 adsorbed to hydrophilic SiO_2. Our results indicate that surface flexibility is an important property to consider in the design on new biomaterials, and the modification of acrylate polymers may be a new way of developing polymer surfaces with improved blood compatibility.

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