Lysozyme interaction with poly(HEMA)-based hydrogel

Megan S. Lord\textsuperscript{a,}\textsuperscript{*}, Martina H. Stenzel\textsuperscript{b}, Anne Simmons\textsuperscript{a}, Bruce K. Milthorpe\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a}Graduate School of Biomedical Engineering, The University of New South Wales, Sydney, NSW 2052 Australia
\textsuperscript{b}Centre for Advanced Macromolecular Design, School of Chemical Engineering and Industrial Chemistry, The University of New South Wales, Sydney, NSW 2052, Australia

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Abstract

Lysozyme interaction with an acrylic-based hydrogel, poly(2-hydroxyethyl methacrylate) co-methacrylic acid (P(HEMA-MAA)), was investigated using a combination of quartz crystal microbalance with dissipation (QCM-D), surface plasmon resonance (SPR) and dual polarisation interferometry (DPI). This combination of techniques demonstrated that lysozyme initially absorbed into the hydrogel matrix and displaced water from the hydrogel while subsequent lysozyme additions were adsorbed onto the surface of the hydrogel material. QCM-D, being sensitive to bound water, showed an overall decrease in mass and stiffening of the layer after lysozyme addition. SPR, a water insensitive technique, showed a net mass increase after addition of lysozyme and buffer rinses. DPI showed that the first exposure of lysozyme to P(HEMA-MAA) was consistent with lysozyme absorption while subsequent lysozyme exposures were consistent with lysozyme adsorption.

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1. Introduction

The interactions of proteins with hydrophilic materials is of importance in the contact lens industry due to their implication in allergic and inflammatory reactions as well as microbial contamination which may lead to infection. Hydrogel contact lenses with high water content tend to have an open matrix structure that may influence the uptake of proteins [1]. The molecular sizes of proteins are also thought to play a role in protein adsorption and absorption [2]. Lysozyme has been implicated as one of the major contributors to inflammation [3].

Lysozyme is one of the main components of tear fluid and the most commonly found protein on worn contact lenses [4]. The main role of lysozyme in tears is a defence mechanism. It can cause a breakdown of the bacterial cell membrane [5]. Hence it is not unexpected to find lysozyme deposits on contact lens materials that the body recognises as foreign. Lysozyme is a small, compact globular protein (14.5 kDa) with a net positive charge (pI 10.7) at physiological pH, thus its penetration into negatively charged hydrogels is electrostatically favourable [6,7].

Ionic monomers such as methacrylic acid (MAA) are commonly used in hydrogel contact lens materials to increase the water content of the lenses and thus increase the amount of oxygen that can reach the cornea [8]. Previous reports have shown that protein is extensively taken up by hydrogels containing MAA [9,10]. Lysozyme is electrostatically attracted to the negatively charged carboxyl groups of MAA, which are almost fully ionised at physiological pH, causing both surface adsorption and matrix penetration [8,11–13].

Studies by Leahy et al. [14] at wear times up to 8 h on poly(2-hydroxyethyl methacrylate), PHEMA-based hydrogel materials previously indicated that lysozyme was the predominant proteinaceous species adsorbed on the materials, while McArthur et al. [12] suggested that there was a variety of low molecular weight species present.
Proteins with a high internal stability, such as lysozyme, do not adsorb to hydrophilic surfaces unless there is electrostatic attraction [15]. A net decrease in hydrogel mass after exposure of poly(2-hydroxyethyl methacrylate) co-methacrylic acid (P(HEMA-MAA)) to lysozyme and subsequent rinsing has previously been reported [16].

It has previously been assumed that lysozyme does not change the hydration of the hydrogel upon diffusion into the matrix [8]. The in vitro absorption of lysozyme was investigated using a combination of quartz crystal microbalance with dissipation (QCM-D), surface plasmon resonance (SPR) and dual polarisation interferometry (DPI) to provide a better understanding of protein deposit formation, including the effect on material hydration, and assist in the development of deposit resistant hydrogel materials for contact lens applications.

2. Materials and methods

2.1. Synthesis of P(HEMA-MAA)

P(HEMA-MAA) was prepared using free radical polymerisation employing a Co(III) complex as catalytic chain transfer agent to control the molecular weight of the polymer to limit the occurrence of side reactions. The Co(III) complex was prepared according to the method described elsewhere [17]. The chain transfer to catalyst value was found to be 65 at 60°C for the HEMA/MAA (95/5 weight-%). Alternatively, the polymerisation can be carried out in the presence of Co(II) complexes [18]. The monomers used, HEMA and MAA, were purchased from Aldrich and inhibitor was removed using a column of basic alumina prior to use. A typical polymerisation was carried out by combining MAA (0.5 g, 5.81 × 10² mol), HEMA (9.5 g, 7.3 × 10⁻² mol), azo-bis(iso-butyronitrile) (7.1 mg, 4.35 × 10⁻³ mol), Co(III) complex (1.2 mg, 2.5 × 10⁻⁶ mol) and 10 ml N-methylpyrrolidone in a round bottom flask. The polymerisation was run at high conversion in order for the polymer composition to closely reflect the monomer proportion of 5 wt% MAA. The reaction vessel was sealed with a rubber septa, purged with nitrogen for 1 h and placed in a thermostated water bath (60°C) for 1 h followed by cooling down the polymerisation mixture with an icebath. The polymer was isolated by precipitation in diethylether. The polymer was filtered off and dried under reduced pressure at room temperature.

Molecular weight distribution was determined by size exclusion chromatography using a Shimadzu modular system, comprising an auto-injector, a Polymer Laboratories 5.0 µm bead-size guard column (50 × 7.5 mm), followed by three linear PL columns (10⁵, 10⁴, and 10¹ Å) and a differential refractive index detector. The eluent was N,N-Dimethylacetamide (0.5% LiBr) at 40°C with a flow rate of 1 ml/min. The system was calibrated using narrow polystyrene standards ranging from 500 to 10⁶ g/mol. Mₘ for P(HEMA-MAA) was determined to be 520,000 g/mol.

P(HEMA-MAA) was dissolved in ethanol (1 wt%) and spin coated onto QCM-D, SPR and DPI sensor chips at 2000 rpm for 30 s. The surface roughness of dry P(HEMA-MAA) on gold QCM-D crystals was found to be approximately 2 nm as determined by atomic force microscopy in tapping mode.

2.2. Preparation of lysozyme solutions

Lysozyme was prepared at various concentrations (50, 100, 200 and 1000 µg/ml) (Sigma) in Dulbecco’s phosphate buffered saline (PBS) (Sigma), pH 7.4. PBS was used in all experiments for buffer rinses. Low concentrations of lysozyme were used in order to test the hypothesis that lysozyme sorption is a process of both matrix penetration and surface adsorption. Previous work [16] revealed a net decrease in mass when P(HEMA-MAA) was exposed to lysozyme (1.9 mg/ml) at physiological conditions, however, at such a concentration it was not possible to show the two phases of protein deposition.

2.3. QCM-D measurements

QCM-D experiments were performed using an axial flow chamber (Q Sense AB, Sweden). The QCM-D, described in detail elsewhere [19], measures frequency (f) and dissipation (D) at the fundamental frequency (5 MHz) and three successive overtones (15, 25 and 35 MHz). A stable measure of bare gold sensor crystals, 5 MHz AT cut crystals (Q-Sense AB, Sweden), with buffer was established before spin coating as described above. Polymer-coated crystals were then exposed to buffer and a stable measurement established before the addition of lysozyme. Unpublished QCM-D measurements indicate stable polymer mass and thickness in PBS for the duration of the experiments.

To determine the effect of temperature on lysozyme sorption, 1 mg/ml lysozyme was exposed to polymer-coated crystals for 30 min and washed with buffer until consistent f and D measurements were obtained. Temperature was maintained at 20 ± 0.1, 25 ± 0.1 or 34 ± 0.1°C. Measurements at 34°C, eye temperature, have been included to demonstrate the validity of the lower temperatures examined. To determine the effect of protein concentration on protein sorption, lysozyme was added at a concentration of 50 µg/ml for 30 min and washed with buffer until consistent f and D measurements were obtained (usually 3 washes). Lysozyme addition and subsequent washing was repeated at 100, 200 and 1000 mg/ml with temperature maintained at 25 ± 0.1°C. f and D measurements quoted for the scaled third overtone (15 MHz), Df/3, unless otherwise stated. The noise of the instrument for the third overtone is approximately 0.29 Hz for frequency and 0.09 × 10⁻⁶ for dissipation. A minimum of three adsorption curves was recorded for each experiment. All solutions were passed through a 0.2 µm filter and degassed at room temperature before use.

2.4. SPR measurements

SPR experiments were performed using Biacore 2000 (Biacore AB, Sweden) optical biosensor with research-grade gold sensor chips. Once the P(HEMA-MAA)-coated sensor chip was stable in running buffer (PBS, flow rate of 20 µl/min), lysozyme was injected over the P(HEMA-MAA) surface at concentrations of 50, 100, 200 and 1000 µg/ml at a flow rate of 20 µl/min for 2 min. All solutions were passed through a 0.2 µm filter and degassed at room temperature before use. Experiments were performed at 25°C, with three separate adsorption curves recorded.

2.5. DPI measurements

DPI experiments were performed using an AnaLight® Bio200 (Farfield Sensors, United Kingdom) with amine-functionalised silicon oxynitride sensor chip. Once the P(HEMA-MAA)-coated sensor chip was stable in running buffer (PBS, flow rate of 100 µl/min), lysozyme (50 µg/ml) was added to the flow at 100 µl/min for 3 min. The system was then rinsed with PBS buffer for 6 min before lysozyme (50 µg/ml) was added for another 3 min at 100 µl/min. The system was then rinsed with PBS for a further 7 min before ending the experiment. Experiments were performed at 20°C, with three adsorption curves recorded. All solutions were passed through a 0.2 µm filter and degassed at room temperature before use.

3. Results and discussion

Table 1 shows the change in f and D due to the adsorption of lysozyme onto P(HEMA-MAA) at various temperatures. There is little difference between the values,
indicating that temperature does not affect the adsorption process within the temperature range tested. This allows comparison of the QCM-D and SPR data, measured at 25 °C, with the DPI data, measured at 20 °C. Table 2 shows a summary of lysozyme uptake by P(HEMA-MAA) as determined by each of the techniques. Expected lysozyme uptake values were determined by scaling the values reported by Garrett et al. [9] for the thickness of the hydrogel used in each of the techniques. It should be noted that the values obtained by Garrett et al. were determined after 24 h lysozyme adsorption at 34 °C. Experimentally determined lysozyme uptakes are comparable to the expected uptakes, except for the hydrated QCM-D measurements.

Fig. 1 shows a typical f and D response versus time for the adsorption of lysozyme onto P(HEMA-MAA) at 25 °C. After successive additions of lysozyme at increasing concentrations, with buffer rinses in between, there was a net increase in f (6.4 Hz) and a decrease in D (3.4 × 10⁻⁶) indicating a net decrease in mass as well as a stiffening of the layer. As the polymer, a hydrogel, naturally takes up water it is not possible to distinguish between the f and D response due to protein adsorption and due to changes in hydration of the polymer. However, measurement of the hydrogel-coated QCM-D crystals before and after lysozyme addition (in dry state) indicated a decrease in f (5.6 Hz) and increase in D (1.1 × 10⁻⁶), consistent with protein binding. Using the Sauerbrey relation [20], this equates to approximately 99 ng/cm² of bound lysozyme, which is a sub-monolayer level of lysozyme adsorption. The theoretical monolayer coverage of lysozyme is approximately 207–310 ng/cm² [21], or approximately 15 Hz frequency change for the QCM-D. This result suggests that, in the hydrated polymer, lysozyme addition displaced water from the hydrogel. Interestingly, lysozyme adsorption onto gold and poly(methylmethacrylate)-coated QCM-D crystals, examples of surfaces that permit lysozyme adsorption but not absorption, was found to be approximately 290 and 380 ng/cm² [16], respectively, when exposed to 1 mg/ml lysozyme at 25 °C. This indicates that the process of lysozyme interaction with P(HEMA-MAA) is quite different when compared with these surfaces.

The QCM-D response is quite different to the SPR and DPI techniques as it measures bound protein and water associated with the protein. The SPR technique complements QCM-D dry analysis as they both measure water-free bound mass. Hence any permanent response associated with the lysozyme addition step and buffer rinse must be caused by lysozyme binding, not its associated water. Fig. 2 shows a permanent lysozyme binding response which increases after each lysozyme addition and buffer rinse as measured by SPR. After four additions of lysozyme, successively increasing concentrations, there was a permanent binding response of 450 RU (approximately 45 ng/cm²). The thickness of polymer attached to the chip is approximately 15 nm. The SPR result shows that lysozyme was permanently bound to P(HEMA-MAA), hence the increase in f and decrease in D observed by QCM-D was caused by water loss from the hydrogel. Permanently bound lysozyme to a bare gold SPR chip was approximately 208 ng/cm² at 25 °C when exposed to 1 mg/ml lysozyme. The possibility of surface adsorption is not, however, excluded by SPR as SPR gives no indication of the location of the protein binding with respect to the hydrogel.

The DPI technique further adds to an understanding of the interaction of lysozyme with P(HEMA-MAA). Fig. 3 shows that the first injection of lysozyme (8–11 min) and subsequent buffer rinsing (11–17 min) caused a decrease in thickness of the polymer layer as well as an increase in mass.
and refractive index. As the density of lysozyme (1.38 g/cm³ [22]) is greater than the density of water it is expected that absorption of lysozyme into the hydrogel will increase mass and refractive index, or density, of the gel. The thickness measurement is calculated from changes in refractive index and mass. The lysozyme was permanently bound as mass, thickness and refractive index values did not return to pre-lysozyme levels after rinsing. The DPI result shows a net decrease in thickness of the gel after the first exposure to lysozyme, further supporting the observation that water was displaced from the hydrogel with the addition of lysozyme.

A second addition of lysozyme (17–21 min) and subsequent buffer rinsing (21–28 min) increased mass and thickness, while refractive index remained constant, indicating surface adsorption of the lysozyme. These two distinct binding steps are shown in the thickness plot in Fig. 3. While lysozyme is absorbing into the polymer the thickness of the layer decreases, however, when lysozyme is adsorbing onto the polymer the thickness increases.

Although it is possible to model the QCM-D f and D responses, models presently available assume a constant density throughout the adsorption process [23,24], which, in this case, would present misleading results as the DPI measurements show a change in refractive index, or density, throughout the adsorption process. For processes where changes in density occur, care must be taken in result interpretation.

4. Conclusion

Analysis using a combination of QCM-D, SPR and DPI demonstrated that the interaction of lysozyme with P(HEMA-MAA) is a process involving both matrix penetration and surface adsorption. Following initial lysozyme exposure, QCM-D results show that the mass of water displaced is greater than the mass of lysozyme absorbed. SPR confirmed the presence of lysozyme attached to the hydrogel, while DPI analysis demonstrated an increase in both mass and density consistent with protein absorption into the hydrogel matrix. The increase in hydrogel density caused by lysozyme absorption, shown by DPI, as well as the decrease in hydrogel hydration, shown by QCM-D, account for the decrease in thickness shown by DPI. Subsequent lysozyme additions appear to result in surface adsorption.

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