Lysozyme Sorption in Hydrogel Contact Lenses

Qian Garrett, 1 R. Wayne Garrett, 2 and Bruce K. Milthorpe 1

PURPOSE. To examine the processes involved in formation of protein deposits on hydrogel contact lenses.

METHODS. The adsorption and/or penetration of lysozyme on or into three types of contact lenses, etafilcon A, vifilcon A, and tefilcon, were investigated in vitro using a radiolabel-tracer technique, x-ray photoelectron spectroscopy, and laser scanning confocal microscopy.

RESULTS. Binding of lysozyme to high-water-content, ionic contact lenses (etafilcon A and vifilcon A) was dominated by a penetration process. The extent of this penetration was a function of charge density of the lenses, so that there was a higher degree of penetration of lysozyme in etafilcon A than in vifilcon A lenses. In contrast, the binding of lysozyme to tefilcon lenses was a surface adsorption process. The adsorption and desorption kinetics showed similar trends to those found in human serum albumin (HSA) adsorption on lens surfaces. However, the extent of lysozyme adsorption on tefilcon is much higher than HSA adsorption, probably because of the self-association of lysozyme on the tefilcon lens surface. Furthermore, either penetration or adsorption of lysozyme involved reversible and irreversible processes and were both time dependent.

CONCLUSIONS. Binding of lysozyme to hydrogel lenses involves surface adsorption or matrix penetration. These processes may be reversible or irreversible. The properties of the lens materials, such as charge density (ionicity) and porosity (water content) of the lenses, determine the type and rates of these processes. (Invest Ophthalmol Vis Sci. 1999;40:897-903)

Surface properties such as hydrophobicity and electrical charge of a variety of materials used in biomedical applications have a substantial effect on the rate, the extent, and the mechanism of protein adsorption. 1-3 In the case of hydrogel contact lenses with considerable water content, the relatively open matrix may also influence the take-up and release of protein independent of inherent protein-surface interactions. 4 Sizes of protein molecules, in this case, are also thought to play a role in controlling the sorption process.

In a previous study of human serum albumin (HSA) adsorption to hydrogel contact lenses, 5 we did not observe the penetration of HSA into hydrogel lens materials; surface adsorption of HSA on lenses was indicated. This was probably because of the relatively large size of the HSA molecule. However, lysozyme, a major protein constituent in tears, is a much smaller molecule. At pH 7.4, it carries a net positive charge and has strong internal coherence. Rearrangement of the protein structure, which is suggested with HSA adsorption, 5 may be limited. Small molecules with strong internal coherence ('hard' molecules) are likely to have different adsorption characteristics. Thus, the in vitro sorption of lysozyme (with the same commercial hydrogel contact lenses as used previously for HSA) was investigated, to provide a better understanding of protein deposit formation and assist in development of deposit-minimized hydrogel contact lens materials.

MATERIALS AND METHODS

Materials

The three commercial hydrogel contact lenses studied were etafilcon A (2-hydroxyethyl methacrylate [HEMA] polymer with sodium methacrylate and 2-ethyl-2-hydroxymethyl-1,3-propanediol trimethacrylate; Johnson & Johnson, Jacksonville, FL), vifilcon A (methacrylic acid polymer with ethylene glycol dimethacrylate, HEMA, and N-vinyl pyrrolidone; Ciba Vision, Duluth, GA), and tefilcon (poly[HEMA] cross-linked and copolymerized with ethylene glycol dimethacrylate; Ciba Vision). Hen egg lysozyme was obtained from Sigma (Sydney, Australia). All chemicals used were of analytical grade.

Radiolabel-tracer Technique

The procedures for radiiodination of protein and purification of radiiodinated protein have been described previously. 6 Adsorption experiments were carried out at 34°C, the typical surface temperature of a human eye, in polypropylene tubes with 2-cm diameters. Each lens (total surface area of approximately 3.0 cm²) was soaked statically in 1.5 ml protein solution with the lens resting on its convex face. The concentration of protein solution for adsorption was 1 mg/ml. After adsorption, the lenses were placed gently into phosphate-buffered saline (PBS; pH 7.4) for 10 seconds to remove any excess adsorption solution adhering to the lenses. The lenses were then placed in the gamma counter to read the activities of the totally adsorbed protein. After the initial reading, further static rinsing (without
agitation) of the protein-adsorbed lenses in 40 ml PBS was performed at room temperature over various time periods, and the radioactivity of the protein remaining on or in the lenses was measured. A gamma counter (Cobra II Auto-Gamma Counter; Packard Instruments, Meriden, CT) was used to read the radioactivity of the samples. Samples were measured in triplets.

**X-ray Photoelectron Spectroscopy**

X-ray photoelectron spectroscopic (XPS) analysis of the amount of lysozyme adsorbed on hydrogel lens surfaces was performed using a spectrometer (Vacuum Generators Escalab U; Vacuum Generators, East Sussex, UK). With photoelectron collection normal to the macroscopic surface plane of the sample under analysis, the probe depth of XPS, from which 99% of the signal originates, is close to 10 nm. The technique is thus ideally suited to the study of adsorbed protein monolayers and submonolayers. In contrast to the radio-label-tracer technique, however, XPS does not detect proteins that have diffused deeper than approximately 10 nm into the hydrogel matrices.

Nonmonochromatic Mg Kα radiation at 200 W (10 kV × 20 mA) was used for excitation. The pressure in the analysis chamber was typically between 1 × 10⁻¹¹ and 8 × 10⁻¹² mbar. The protein adsorbed lens samples were mounted on stainless steel sample stubs using double-sided adhesive tape. Survey spectra were collected for identification of the elements present (e.g., C, N, O) at 1001 eV pass energy. Individual elements were quantified by integrating the high-resolution spectra.

Based on an analog of the Beer-Lambert equation and assuming a uniform overlayer of adsorbed protein material on the lens surfaces, the effective thickness of the adsorbed protein layer can be determined from the intensity of the photoelectrons originating from nitrogen. The relationship between intensity and the thickness of the adsorbed protein layer is given in Equation 1:

\[
I/I_0 = 1 - e^{-\frac{\lambda x}{d}}
\]

where \( I \) represents the percentage of nitrogen determined for the adsorbed protein overlayer, \( I_0 \) is the %N in a pure protein layer considerably thicker than the XPS probe depth, \( d \) is the thickness of the adsorbed protein layer, \( \lambda \) is the mean free path of the N 1s photoelectrons (2.5 nm), and \( x \) is the photoelectron emission angle. This equation was used to determine the effective thickness of the adsorbed protein layer. The thicknesses of the adsorbed protein layers were measured by a Sephadex column (PD-10 G25; Pharmacia Biotech, Sydney, Australia) equilibrated with PBS. Further dialysis of FITC-labeled protein solutions was accomplished in PBS at 4°C for a total of 48 hours using cellulose dialysis tubing with a molecular weight cutoff of 1000 D (M. B. Selby, Sydney, Australia). Three changes of PBS solution during dialysis were made, and no free FITC was detected in the last dialyzed PBS solution.

The adsorption and desorption of FITC-labeled lysozyme was performed at 34°C in polypropylene tubes. Hydrogel contact lenses were cut at their centers into 2 × 4 mm sections to minimize the lens curvature. Each lens section was soaked in FITC-labeled lysozyme solution for 24 hours. The concentration of FITC-labeled lysozyme solutions for adsorption was 1 mg/ml. After adsorption, each lens section was gently placed into PBS solution for 10 seconds, gently removed from solution, and subjected to 24 hours' static rinsing in PBS at room temperature.

The FITC-lysozyme-adsorbed lens sections were mounted onto microscopy slides using an excess of PBS solution. Coverslips were carefully placed on the lens sections without entrapping any air bubbles. The lens sections were examined with a laser scanning confocal microscope (Olympus FluoView FV 300). A total of 48 hours using cellulose dialysis tubing with a molecular weight cutoff of 1000 D (M. B. Selby, Sydney, Australia). Three changes of PBS solution during dialysis were made, and no free FITC was detected in the last dialyzed PBS solution.

The adsorption and desorption of FITC-labeled lysozyme was performed at 34°C in polypropylene tubes. Hydrogel contact lenses were cut at their centers into 2 × 4 mm sections to minimize the lens curvature. Each lens section was soaked in FITC-labeled lysozyme solution for 24 hours. The concentration of FITC-labeled lysozyme solutions for adsorption was 1 mg/ml. After adsorption, each lens section was gently placed into PBS solution for 10 seconds, gently removed from solution, and subjected to 24 hours' static rinsing in PBS at room temperature.

The FITC-lysozyme-adsorbed lens sections were mounted onto microscopy slides using an excess of PBS solution. Coverslips were carefully placed on the lens sections without entrapping any air bubbles. The lens sections were examined with a laser scanning confocal microscope (Olympus FluoView FV 300). A total of 48 hours using cellulose dialysis tubing with a molecular weight cutoff of 1000 D (M. B. Selby, Sydney, Australia). Three changes of PBS solution during dialysis were made, and no free FITC was detected in the last dialyzed PBS solution.

The FITC-lysozyme-adsorbed lens sections were mounted onto microscopy slides using an excess of PBS solution. Coverslips were carefully placed on the lens sections without entrapping any air bubbles. The lens sections were examined with a laser scanning confocal microscope (Olympus FluoView FV 300). A total of 48 hours using cellulose dialysis tubing with a molecular weight cutoff of 1000 D (M. B. Selby, Sydney, Australia). Three changes of PBS solution during dialysis were made, and no free FITC was detected in the last dialyzed PBS solution.

The FITC-lysozyme-adsorbed lens sections were mounted onto microscopy slides using an excess of PBS solution. Coverslips were carefully placed on the lens sections without entrapping any air bubbles. The lens sections were examined with a laser scanning confocal microscope (Olympus FluoView FV 300). A total of 48 hours using cellulose dialysis tubing with a molecular weight cutoff of 1000 D (M. B. Selby, Sydney, Australia). Three changes of PBS solution during dialysis were made, and no free FITC was detected in the last dialyzed PBS solution.
drophilic monomers such as methacrylic acid, N-vinyl pyrrolidone, or both and have relatively high water contents (58% and 55%, respectively). In aqueous media, such hydrogel contact lenses become porous, and when they are in contact with a protein solution, penetration of relatively small molecules such as lysozyme into the lens matrices is likely to occur.

The amount of protein adsorbed could also be high if there is no penetration but a thick multilayer of protein is adsorbed on the lens surfaces, as suggested by Gachon et al. However, a multilayer of approximately 100 and 1000 molecules thick would be necessary to produce the observed result for vifilcon A and etafilcon A, respectively. This is, however, not observed with XPS, with which only the surface existence of protein is detected. The surface concentration of the adsorbed lysozyme on etafilcon A lens surfaces measured by XPS is 32 ng/cm² (Table 1), which is an order of magnitude below the monolayer coverage (207-310 ng/cm²). This indicates that most of the lysozyme absorbed was in the lens matrix, with little on the lens surface. The absorption of lysozyme in etafilcon A lenses seems to be dominated by a penetration process. This protein penetration, however, cannot be confirmed for vifilcon A lenses by XPS; the XPS data show a higher percentage of nitrogen on the clean lens surface before protein adsorption (because of N-vinyl pyrrolidone content in the lens materials) than after protein adsorption.

**Table 1. Percentage of Nitrogen and Surface Concentration of Lysozyme Adsorbed on Etafilcon A, Vifilcon A, and Tefilcon Lens Surfaces**

<table>
<thead>
<tr>
<th></th>
<th>Percentage Nitrogen</th>
<th>After Adsorption</th>
<th>Surface Concentration (ng/cm²)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etafilcon A</td>
<td>0*</td>
<td>7.1</td>
<td>32</td>
</tr>
<tr>
<td>Vifilcon A</td>
<td>7.1</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>Tefilcon</td>
<td>0</td>
<td>0.2</td>
<td>4</td>
</tr>
</tbody>
</table>

Adsorption was for 24 hours in 1 mg/ml lysozyme solution followed by initial rinsing in PBS for 10 seconds and further static rinsing in PBS for 24 hours.

* Concentration measured by XPS, using Equation 1.

In comparison, tefilcon lenses do not contain extra hydrophilic monomers and consist almost exclusively of poly-(HEMA). The porosity of these lenses is so low that they may allow lysozyme to adsorb only onto the lens surfaces. However, this was not supported by the XPS data. Instead, an extremely small amount of lysozyme was adsorbed on tefilcon lens surfaces (far below the monolayer coverage; Table 1). This level of lysozyme adsorbed on tefilcon lenses may not be sufficient for a definitive measure of the surface concentration of the adsorbed protein using XPS.

Earlier, the penetration of proteins into hydrogels was visualized using light microscopy followed later by a two-dimensional electrophoretic system. However, the former technique was limited in resolution, and the latter risks perturbation of the protein absorption by the application of an electric field. In this work, laser scanning confocal microscopy of fluorescently labeled proteins with higher resolution (~0.3 μm) should enable study of the depth distributions of the proteins in the hydrogels and thus the efficiency of diffusive penetration within the duration of the experiments with minimal disturbance to the system. The average fluorescence intensities for each image plane were plotted as a function of depth to create the profiles of the protein's adsorption or penetration on or into hydrogel lenses (Fig. 2). Lysozyme penetrates into etafilcon A and vifilcon A lenses, whereas almost no penetration of lysozyme into tefilcon lenses was observed within the resolution of the confocal microscopy. That there was no penetration of lysozyme into tefilcon lenses is in contrast to findings in earlier work of Refojo and Leong, in which penetration of lysozyme into the pure poly(HEMA) hydrogels was observed. However, the pore size for the pure poly(HEMA) hydrogels is larger than that for tefilcon lenses in which poly(HEMA) has been cross-linked and copolymerized with ethylene glycol dimethacrylate, resulting in a smaller pore size. It should also be noted that the degree of lysozyme penetration is related directly to the charge density of the lenses with maximum penetration in etafilcon A. Because the optical density of the hydrogels was very low, potential complications due to fluorescence quenching could be neglected.

**Irreversibility of Lysozyme Sorption—Desorption Kinetics**

Irreversible absorption of lysozyme in etafilcon A, vifilcon A, and tefilcon lenses was observed. The remaining lysozyme on or in the lenses became nearly constant after static elution in PBS for approximately 140 hours (Fig. 3). However, in contrast...
FIGURE 2. Profile of FITC-labeled lysozyme penetration into etafilcon A (A), vifilcon A (B), and tefilcon (C) contact lenses, after soaking in 1 mg/ml FITC-labeled lysozyme solution for 24 hours, followed by initial rinsing in PBS for 10 seconds and further static rinsing in PBS for 24 hours.

to HSA desorption kinetics, the "plateau" value of the absorbed lysozyme was much higher than that of HSA in all the lenses except tefilcon. In addition, lysozyme desorption took considerably longer to reach a plateau than did HSA. Thus, there may be different processes involved in the absorption and desorption of lysozyme and HSA. The type of lens material may influence the lysozyme adsorption and desorption process.

Desorption of lysozyme from the etafilcon A and vifilcon A lenses is best fitted to a single exponential process with an "offset" as shown in Figures 3A and 3B, respectively (TableCurve for Windows, ver. 1.12; Jandel Scientific, Corte Madera, CA). Correlation coefficients are approximately 0.99 for both lenses. This single exponential process is described in Equation 2:

$$C_s = C_{in} + C_{rev}e^{-kt}$$  \hspace{1cm} (2)

where $C_s$ is the apparent surface concentration of protein remaining on the lenses after time $t$ of desorption in PBS, $C_{rev}$ is a constant representing the initial apparent surface concentration of reversibly adsorbed protein, $C_{in}$ is a constant that is assumed to be the apparent surface concentration of protein that cannot desorb from the hydrogels in PBS, and $k$ is the desorption rate constant for reversibly adsorbed protein.

However, desorption of lysozyme from tefilcon lenses is best treated as a double exponential process (Fig. 3C; correlation coefficient, 0.99). This double exponential is described in Equation 3:

$$C_s = C_Ae^{-k_At} + C_Be^{-k_BT}$$  \hspace{1cm} (3)

where $C_s$ is the apparent surface concentration of protein remaining on lenses after time $t$ of desorption in PBS, $C_A$ and $C_B$ are constants representing the initial apparent surface concentrations of adsorbed protein that can be considered loosely bound in layers somewhat remote from the surface where surface forces may be considerably weaker ($A$) and more tightly bound protein in layers closer to the surface and/or on the surface in a reversible state but where surface effects are not diluted ($B$), and $k_A$ and $k_B$ are the rate constants for the two types of protein desorption from the hydrogel lenses, respectively.

The single exponential desorption kinetics for lysozyme sorption in etafilcon A and vifilcon A lenses suggests that only one dominant desorption process is occurring. Whereas the "irreversibly" bound lysozyme resists desorption, desorption of the remaining lysozyme is diffusion limited. $C_{rev}$ and $C_{irr}$ are higher for etafilcon A than those for vifilcon A lenses (Table 2). The desorption rate ($k$) value of the reversibly absorbed lysozyme is not significantly different for etafilcon A (47 X 10^{-5} minute^{-1}) and vifilcon A lenses (40 X 10^{-5} minute^{-1}).

The double exponential desorption kinetics for lysozyme adsorbed on tefilcon lenses suggests two desorption processes with very different rates. The desorption rate $k_A$ (Table 3), which represents the desorption of loosely and reversibly bound lysozyme on tefilcon lenses is higher (100 X 10^{-5} minute^{-1}) than the value of $k$ for etafilcon A and vifilcon A lenses (47 X 10^{-5} and 40 X 10^{-5} minute^{-1}, respectively). The rate of desorption depends on the concentration of lysozyme in the hydrogel matrix and/or on the surfaces, and/or on the hydrogel porosity. It is thus not surprising that the desorption is a constant representing the initial apparent surface concentration of reversibly adsorbed protein, $C_{rev}$ is a constant that is assumed to be the apparent surface concentration of protein that cannot desorb from the hydrogels in PBS, and $k$ is the desorption rate constant for reversibly adsorbed protein.

FIGURE 3. A comparison of experimental data (symbols) with the fitted data (lines) for the apparent surface concentration of protein remaining on lenses, based on Equation 2 for lysozyme desorption from etafilcon A (A) and vifilcon A (B) lenses into PBS and on Equation 3 for lysozyme desorption from tefilcon lenses (C) into PBS. The lenses were presoaked in 1 mg/ml lysozyme solution for 24 hours and then were gently rinsed in PBS for 10 seconds followed by static elution in PBS for different times. Error bars, SD ($n = 3$).
Lysozyme Sorption in Hydrogel Contact Lenses

Lysozyme is a relatively "hard" protein molecule that is less likely than HSA to change its conformation. However, whether electrostatic attraction causes some conformational changes of the bound lysozyme had not been determined in this work. Alternatively, the irreversibility of bound protein in the lens matrices may also be attributed to protein self-association. Self-association may result from the relatively high concentration of lysozyme accumulated inside the lens matrix, because self-association of lysozyme is favored at relatively high local concentrations. The concentration of lysozyme inside the lenses, in the case of the negatively charged etafilcon A and vifilcon A lens material, is probably caused by electrostatic attraction forces.

The kinetics of total and irreversible adsorption in teflon lenses show different characteristics from those for etafilcon A and vifilcon A (Figs. 4A3, 4B3). Total adsorption occurs rapidly and reaches a plateau within a relatively short time of adsorption, with irreversible adsorption developing simultaneously but at a relatively slower rate. These patterns are relatively similar to those of HSA adsorption on the lenses. However, the mechanisms for the slow onset of the irreversibility may not be the same as those for HSA, in which the irreversible adsorption of HSA is assumed to be governed by the kinetics of protein conformational change. This conformational change may be less likely for the "hard" lysozyme. Nevertheless, it requires further examination. Another possibility is that after the initial high rate of adsorption, conditions on the surface of the lens favor self-association of lysozyme. This self-association may be kinetically controlled, leading to the observation of a slow build-up of irreversibly adsorbed protein.

In conclusion, absorption of lysozyme in hydrogel contact lenses can be a process of surface adsorption or a combined process of surface adsorption and matrix penetration, depending on the lens material. For high-water-content, ionic contact lenses such as etafilcon A and vifilcon A, absorption of lysozyme is dominated by a penetration process. This penetration process may be initially driven and further enhanced by electrostatic attraction forces between positively charged lysozyme and negatively charged lenses. Therefore, the extent of the penetration of lysozyme is higher in etafilcon A than in vifilcon A lenses. In contrast, the adsorption of lysozyme to low-water-content, nonionic teflon lenses is a surface adsorption process with some degree of irreversibility. The adsorption and desorption kinetics show trends similar to those found in HSA adsorption on lenses surfaces, except that the extent of lysozyme adsorption on teflon is much higher than that of HSA adsorption. This may be because of the self-association of lysozyme on the teflon lens surfaces.

Table 2. Parameters of Lysozyme Desorption Kinetics from Etafilcon A and Vifilcon A Lenses into PBS, with 95% Confidence Limits

<table>
<thead>
<tr>
<th>Parameters*</th>
<th>Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{REV}$†</td>
</tr>
<tr>
<td>Etafilcon A</td>
<td>160,557</td>
</tr>
<tr>
<td>Vifilcon A</td>
<td>44,960</td>
</tr>
</tbody>
</table>

* Estimated from a single exponential equation fitted to the experimental data (Eq. 2).
† Data are in nanograms per square centimeter.

Table 3. Parameters of Lysozyme Desorption Kinetics from Tefilcon Lenses into PBS, with 95% Confidence Limits

<table>
<thead>
<tr>
<th>Parameters*</th>
<th>Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_X$†</td>
</tr>
<tr>
<td>Tefilcon</td>
<td>455</td>
</tr>
</tbody>
</table>

* Estimated from a double exponential equation fitted to the experimental data (Eq. 3).
† Data are in nanograms per square centimeter.
FIGURE 4. Adsorption time profile for the apparent surface concentration of totally adsorbed lysozyme on etafilcon A (A1), vifilcon A (A2), and tefilcon (A3) lenses, and adsorption time profile for the apparent surface concentration of irreversibly adsorbed lysozyme on etafilcon A (B1), vifilcon A (B2), and tefilcon (B3) lenses. The irreversibly adsorbed lysozyme on lenses was obtained after the lenses were soaked in 3 mg/ml lysozyme solution for 24 hours followed by static rinsing in PBS for 140 hours. Error bars, SD (n = 3).

Acknowledgments
The authors thank Peter Kingshott for assistance in the x-ray photoelectron spectroscopic analysis and Paul Halasz for assistance with confocal microscopy.

References


