FEATURE ARTICLE ON LINE

Confocal Microscopy and Albumin Penetration into Contact Lenses

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ABSTRACT

Purpose. To develop a novel in vitro method to detect the depth of penetration of the tear film protein albumin into contact lens materials using confocal laser scanning microscopy (CLSM).
Methods. A poly-HEMA-based hydrogel (etafilcon A) and a silicone hydrogel material (lotrafilcon B) were examined. In vitro, bovine serum albumin (BSA) was labeled with 5-(4,6-dichloro-s-triazin-2-ylamino) fluorescein hydrochloride (DTAF). The lenses were incubated in this protein solution (0.5 mg/ml) at 37°C. After 1 and 7 days incubation, the lenses were examined using CLSM (Zeiss 510, config. META 18) and the location of the fluorescently labeled BSA was identified.
Results. BSA adsorption on the surface and penetration into the lens matrix occurred at a higher concentration for etafilcon compared to lotrafilcon ($p < 0.001$). For both materials, BSA was detected on the surface after 1 day of incubation. Significant levels of BSA were detected within the matrix of etafilcon after as little as 1 day ($p < 0.001$), but no BSA was detected in the matrix of lotrafilcon at any time ($p > 0.05$).
Conclusion. CLSM can be successfully used to examine the depth of penetration of fluorescently labeled proteins into various hydrogel polymers. Our results show that etafilcon lenses both adsorb BSA on the surface and absorb BSA within the matrix, whereas lotrafilcon B adsorbs small amounts of BSA on the surface only.
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Key Words: confocal laser scanning microscopy, bovine serum albumin, contact lens, etafilcon A, lotrafilcon B, protein penetration

Deposition of tear film components such as proteins, mucins, and lipids on contact lenses can cause discomfort and inflammatory complications such as giant papillary conjunctivitis (GPC),[1–6] and these problems can appear with any type of daily or extended wear lenses.[7] The recently introduced silicone hydrogel (SH) materials have different deposition profiles to that seen with conventional hydrogel lenses (CH) based on poly-HEMA, with lower levels of protein deposition and higher levels of lipid deposition being measured.[8–12]

Of the tear film proteins that deposit on contact lenses, most of the literature to date has concentrated on the deposition of the positively charged protein lysozyme, which is the most abundant protein in the tear film, with a concentration of approximately 3 mg/ml,[13–15] and a molecular weight of 14.4 kDa. Another protein of interest is the larger protein serum albumin, with a molecular weight of 66 kDa, which is negatively charged and has a lower concentration in the tear film of approximately 0.04 mg/ml during the daytime.[14] This amount in-

creases to approximately 0.2 mg/ml during sleep and may rise as high as 0.5 mg/ml after wear of orthokeratology lenses.[14,15]

Work to date on conventional poly-HEMA-based lens materials has shown that the deposition of lysozyme and albumin depends upon the polymer composition,[16] charge,[17–19] and water content,[20] with lysozyme being mainly deposited on negatively charged substrates and albumin being deposited on neutral and/or positively charged materials. Thus far, although the deposition of lysozyme on SH materials has been determined,[9–12] less information is available describing the deposition of albumin on these new materials.[21]

A variety of methods can be used to examine deposition on contact lenses, including visible clinical grading, surface imaging and analytical methods that require the deposits of interest be removed.[8,22–25] Major disadvantages for clinical grading include substantial intersubject variability and a lack of biochemical analysis of the deposits. Imaging techniques such as microscopy do not allow for quantification of the species of interest. Methods requir-

ing removal lack certainty both in terms of the removal process and the exact location of the deposited substance on or within the lens material.

The purpose of this study was to develop a novel method to investigate the spatial and temporal penetration profile of serum albumin labeled with a fluorescent marker on and into various hydrogel contact lens materials and to compare the differences between a representative poly-HEMA-based hydrogel and a novel siloxane hydrogel.

METHODS

Bovine serum albumin (BSA) with a purity of 99% (agarose gel electrophoresis) and a molecular weight of 66 kDa was purchased from Sigma-Aldrich (St. Louis, MO). BSA was chosen for this study, because it has very similar properties to albumin from human serum (HSA).[26] BSA was labeled with the fluorescent dye 5-(4,6-dichloro-s-triazin-2-ylamino) fluorescein hydrochloride (DTAF) from Sigma-Aldrich. This dye was chosen, as it does not significantly change the molecular weight and size of BSA.[27,28] For the labeling procedure, BSA (180 mg) was dissolved in 0.05M borate buffer (pH = 8.5) containing 0.04M NaCl (18 ml). DTAF (10 mg) was dissolved in dimethylsulfoxide (DMSO; 1 ml; Sigma-Aldrich) and was added drop wise, while stirring the solution. The BSA-DTAF was stirred for 2 h at room temperature before separating the conjugate from unreacted labeling agent using PD10 desalting columns (Amersham Biosciences, Piscataway, NJ). Further elimination of unreacted DTAF was done by dialysis against phosphate buffered saline (PBS, pH = 7.4) (5×4 l). The dialysis cassettes were purchased from Pierce (Rockford, IL) and the membrane, with pore sizes of 7000 MW, filtered all particles out of the protein solution that were small enough to diffuse through the pores, including free dye and small protein fractions. Subsequent measurements with a fluorescence spectrophotometer (Hitachi F-4500, Tokyo, Japan) verified a continuous decrease of the unbound dye. The calculated labeling ratio was two molecules of dye per molecule of BSA, and this solution was diluted with PBS to obtain a final BSA concentration of 0.5 mg/ml. To verify the purity and molecular size of the BSA before and after the labeling process a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. A prestained standard with molecular markers from 10 to 250 kDa was used on a PhastGel Gradient 10 to 15 (Amersham Bioscience, Uppsala, Sweden).

The lens materials examined were etafilcon A (Acuvue 2; Johnson & Johnson, Jacksonville, FL) and lotrafilcon B (O₂Optix; CIBA Vision, Duluth, GA), details of which can be seen in Table 1.

All lenses examined had powers of −3.00 D. They were individually soaked for 30 min in 10 ml of sterilized PBS, before they were incubated in the protein solution for 1 and 7 days, with four replicates for each condition. The labeled BSA solution was sterilized with syringe filters (Pall Corporation, Ann Arbor, MI), and in total, eight lenses of each type were incubated in individual amber vials, which were filled with 1 ml of the protein solutions and kept in an oven at 37° on a gently rotating plate. Negative controls consisted of eight further lenses for each lens type, incubated for equivalent periods of time in PBS. Thus, 32 lenses in total were examined (two lens types, two doping solutions, two incubation times, and four replicates of each). After the defined incubation

TABLE 1.

Hydrogel lens materials

Proprietary name	O2 Optix	Acuvue 2
United States adopted name	Lotrafilcon B	Etafilcon A
Manufacturer	CIBA Vision	Johnson & Johnson
Center thickness (at −3.00 D) mm	0.08	0.084
Water content (%)	33	58
Oxygen permeability (10^{-11})	110	17
Oxygen transmissibility (10^{-9})	138	21
Surface treatment	25 nm plasma coating with high refractive index	No surface treatment
FDA group	I	IV
Principal monomers	DMA + TRIS + siloxane macromer	HEMA + MA

DMA, *N,N*-dimethylacrylamide; HEMA, poly-2-hydroxyethyl methacrylate; MA, methacrylic acid; TRIS, trimethylsiloxy silane.

time, lenses were rinsed for 5 s with PBS, and a punch press was used to remove a circle of 4 mm diameter from the middle of the lens, which was then placed on a microscope slide (Fisher Scientific, Pittsburgh, PA) using PBS as mounting solution. Samples were covered with cover slides (VWR, Bridgeport, NJ), sealed with nail polish.

Samples were analyzed using a confocal laser scanning microscope (CLSM) Zeiss 510, config. META 18 equipped with an inverted motorized microscope Axiovert 200M, (Zeiss Inc. Toronto, Canada). The Argon laser was set to an output of 50% to obtain a stable laser beam. The beam pathway was assigned to channel 3, and the main (HFT 488 nm) and secondary (NFT 490 nm) dichroic mirrors were chosen according to the dye specific excitation wavelength. The long pass filter LP 505 nm was used to detect the emission wavelength. The water-immersion C-Apochromat objective (numeric aperture 1.2) was chosen to achieve an optimized image quality, and the pinhole size was set to 1 Airy unit to eliminate out of focus rays. Settings for the scan control were 625 for the detector gain, −0.025 for the amplifier offset and 1 for the amplifier gain. A laser transmission of 5% at 488 nm was chosen to minimize photobleaching of the fluorescent dye. For the image settings a frame size of 512 by 512 pixels, maximum scan speed, a pixel depth of 8 bit and the returning scan direction was used for collection of all images. All described microscope settings remained the same for the duration of the study.

To detect the contact lens surface of the sample under the microscope, a small area on the lens was marked with a pen that was visible using 2% transmitted light. A suitable position on the lens surface was chosen and using the z-stack, which is the module to measure through the sample, the first and last positions on the sample were determined. With a constant step size of 1 μm, continuous images were captured from the front to the back surface of the sample.
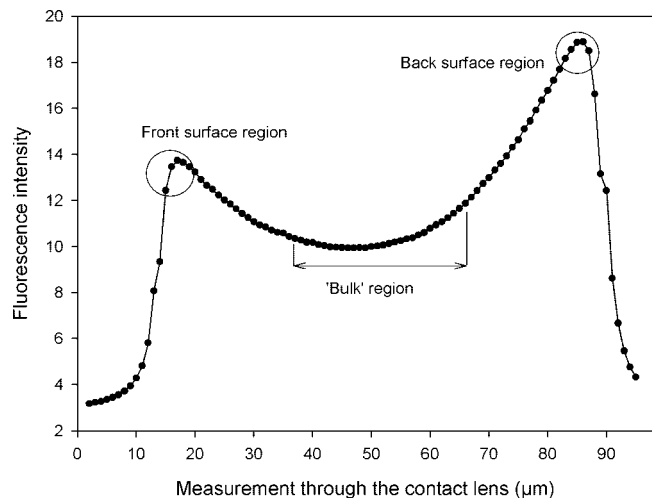
FIGURE 1.

Definition of front, back and "bulk" regions displayed for an etafilcon A lens, which was incubated in labeled BSA. The "front surface region" was defined as the average of the front fluorescence peak $\pm$ 2 μm, the "back surface region" was defined as the average of the rearmost peak $\pm$ 2 μm and a "central region" or "bulk" was defined as the average of the 30 central images, using the front and back peaks as borders. The x-axis shows the measurement through the thickness of the central lens material (μm) and the y-axis shows the relative fluorescence intensity.

Six scans of each sample were obtained. To investigate the influence of any potential photobleaching effects, two measurements at identical central locations were taken (scan numbers 1 and 6), with a scan size of 190 by 190 μm. After scan 1 and before scan 6, four other readings (scans 2 to 5) were obtained in the four corners of the sample (115 by 65 μm) in a randomized fashion to investigate differences in penetration profiles over the lens. Scans 1 and 6 were measured using 400× resolution, and the other locations were measured using 800× resolution. ImageJ (Bethesda, MD) was used to calculate the fluorescence signal of BSA-DTAF for each single image along the vertical axis.

The cross-section through the lens material was divided into three regions of interest (Fig. 1). The "front surface region" was defined as the average of the front fluorescence peak $\pm$ 2 μm, the "back surface region" was defined as the average of the rearmost peak $\pm$ 2 μm and a "central region" or "bulk" was defined as the average of the 30 central images, using the front and back peaks as borders.

One factor to consider when conducting studies using dye-tagged proteins is whether the data obtained could be due to the absorption of unbound dye and that the results obtained are more indicative of dye-binding rather than protein uptake. To reduce this, the labeled protein solution was extensively dialysed until only very minor amounts of fluorescent signal were detectable in the protein solution. In addition, lenses were incubated in a control PBS-DTAF solution without the addition of BSA, at a dye concentration approximately 200 times lower than the study solution.

For analysis of the protein uptake on the front, back, and "bulk" regions, a repeated measures ANOVA (analysis of equal variance) was applied (significance level $p < 0.05$), with the factors being solution (labeled BSA and PBS solution), contact lens type (lotrafilcon B and etafilcon A), incubation time (1 and 7 days), and regions (front, back, bulk). To determine if any photobleaching

had occurred during the exposure to the confocal laser beam, the Limits of Agreement (LOA) between scans 1 and 6 were examined, where LOA = d $\pm$ 1.96 by SD on the three defined regions (front, back, bulk). The value d is the mean difference between the two central locations (1 and 6) and SD is the calculated standard deviation. Additionally, the Correlation Coefficient of Concordance (CCC) was calculated to describe the concordance between the repeated scans (1 and 6).[29] CCC describes the deviation between the scans from a perfect 45° line and therefore the repeatability. (CCC = 1 = perfect correlation and perfect repeatability; CCC = 0 = no correlation and no repeatability).

RESULTS

SDS-PAGE was used to verify purity and final molecular weight (approximately 66 kDa) for the unlabeled, labeled and sterilized BSA solutions, as seen in Fig. 2. The gel also shows that no smaller BSA fractions appear below the standard of 50 MW but some proteins aggregated and therefore weaker bands with higher molecular weights were found. These results are of importance, as it may be expected that smaller proteins or protein fractions would penetrate more easily into hydrogels polymers than the original BSA of 66 kDa. This was not the case in this study.

The fluorescent signals of the labeled BSA on the lens surfaces and inside the matrix were different for the two contact lens materials ($p < 0.001$). Fig. 3A and 3B demonstrate the typical pattern of the fluorescent signal on both surfaces and inside the matrix of etafilcon and lotrafilcon B materials after 7 days incubation with labeled BSA. The image galleries were plotted in a step size of 1 μm through the thickness of the lens materials. The brighter the image, the more fluorescent signal was detected, representing a greater degree of albumin deposition. For the etafilcon material (Fig. 3A), an almost equally distributed fluorescent intensity was found on the surface regions and inside the matrix, indicating that the surface of the etafilcon lens was not a barrier for penetration of the BSA molecules. This was contrary to the results seen with the plasma-coated lotrafilcon B material (Fig. 3B), where a weak fluorescence signal was found on the surfaces and no penetration into the matrix was detected.
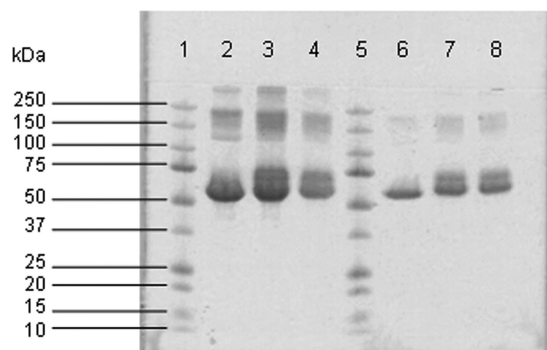

FIGURE 2.

SDS-PAGE for different BSA-PBS solutions to verify no proteins are smaller than the expected MW of 66 kDa. Column 1: Molecular marker; Column 2: 1.5 mg/ml BSA; Column 3: 1.5 mg/ml labeled BSA; Column 4: 0.5 mg/ml labeled BSA; Column 5: Molecular marker; Column 6: 0.25 mg/ml BSA; Column 7: 0.25 mg/ml labeled BSA; Column 8: 0.25 mg/ml labeled and sterilized BSA.
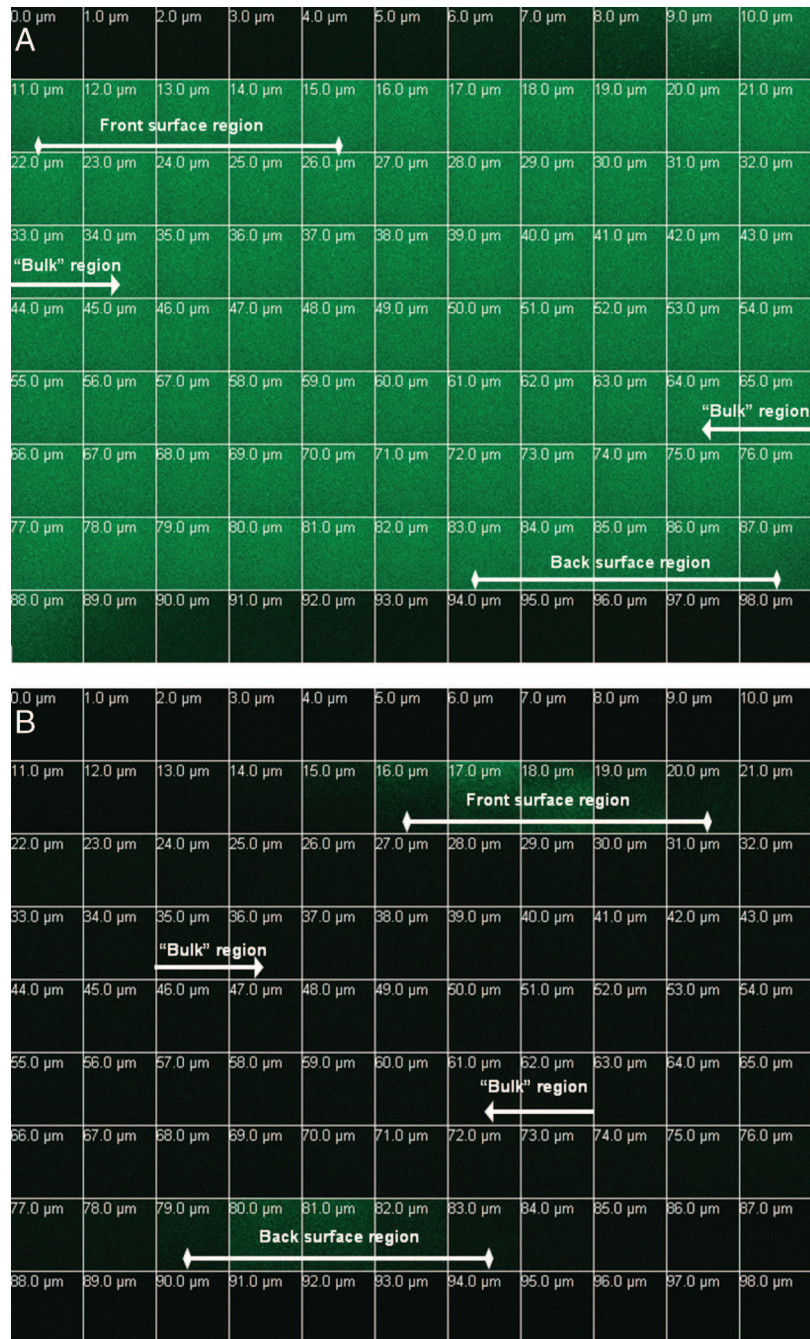
FIGURE 3.

Image galleries of typical x-y-confocal scans along the vertical axis in 1 μm z-steps for etafilcon A (A) and lotrafilcon B (B) after 7 days of incubation in labeled BSA. Brighter colors indicate an increased fluorescent signal and therefore a higher BSA concentration.

Fluorescence intensity profiles for each lens material for each incubation time is plotted in Fig. 4A, B. These scans of individual replicates clearly reveal differences between materials in terms of fluorescence intensity on the lens surface and within the matrix, as well as the impact of time on protein accumulation.

Figs. 5 to 7 demonstrate the differences in fluorescence intensity over time for each of the three regions of interest (front, back surface, and "bulk"), by taking all replicates into consideration.

Fig. 5 illustrates the average fluorescence intensity for all four replicates for the etafilcon A material, for lenses incubated in both the test and control solutions, for the surface regions only,

for both time periods. There was a significant difference between the control (PBS only) and labeled BSA solution at all times ($p < 0.001$), indicating that BSA adsorbed in significant quantities even after 1 day of incubation. The amount of adsorbed BSA increased significantly between days 1 and 7 ($p < 0.001$), with no such change being seen for the PBS control group ($p > 0.05$). There was no significant difference in the degree of albumin deposition between the front and back surfaces ($p > 0.05$).

Fig. 6 illustrates the average fluorescence intensity for all four replicates for the lotrafilcon B material, for lenses incubated in
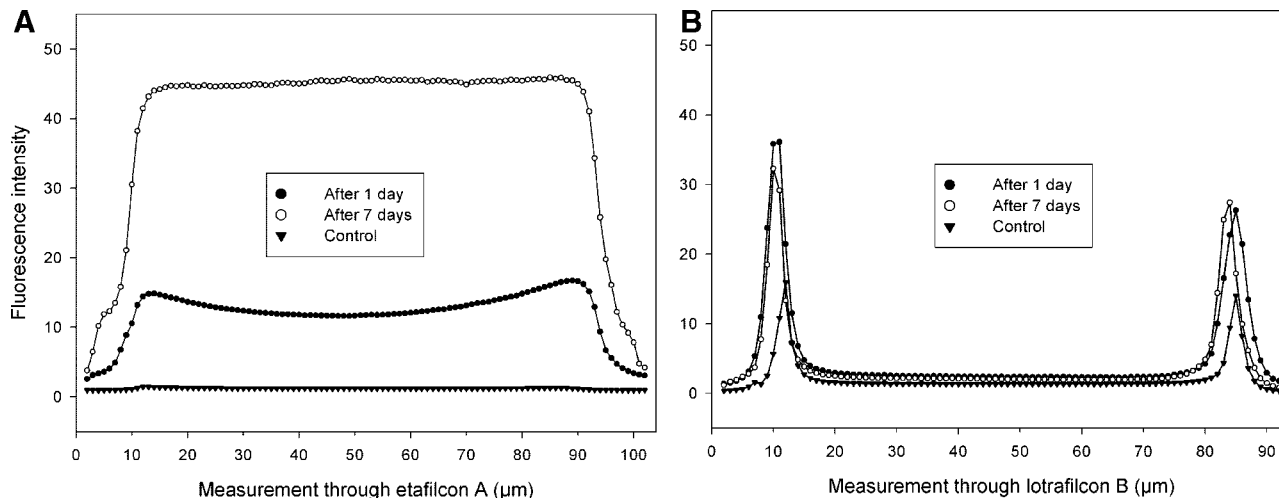
FIGURE 4.

Typical pattern for BSA-DTAF penetration into etafilcon A (A) and lotrafilcon B (B) plotted from a single scan after 1 and 7 days of incubation. The x-axis shows the measurement through the thickness of the central lens material (μm) and the y-axis shows the relative fluorescence intensity.
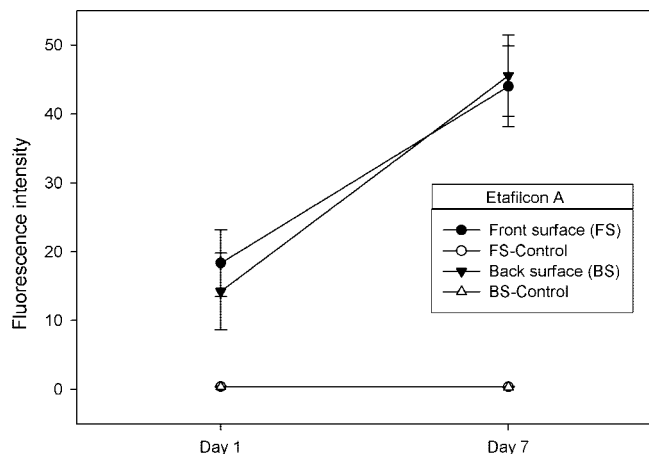

FIGURE 5.

Average fluorescence intensity for all four replicates for the etafilcon A material, for lenses incubated in both the test and control solutions, for the surface regions only, for both time periods.
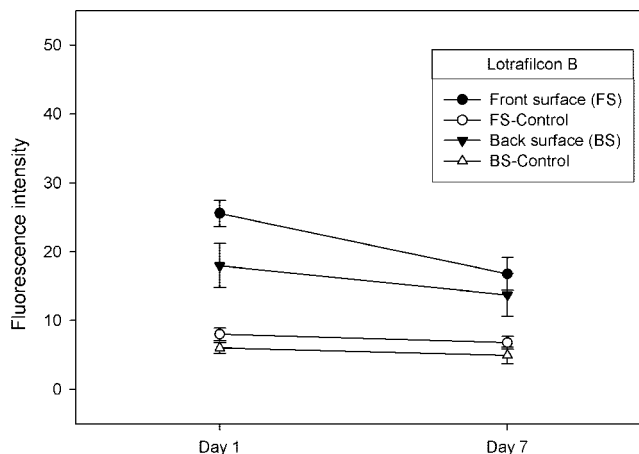

FIGURE 6.

Average fluorescence intensity for all four replicates for the lotrafilcon B material, for lenses incubated in both the test and control solutions, for the surface regions only, for both time periods.

both the test and control solutions, for the surface regions only, for both time periods. There was a significant difference between the lenses incubated in the control and labeled BSA solution at all times (p < 0.001), indicating that BSA adsorbed in significant quantities even after 1 day of exposure. Examination of Fig. 6 indicates that the amount of adsorbed BSA apparently decreased over time on both the front and back surfaces (p = 0.05), but at both points in time the fluorescence intensity was greater than that seen in the PBS-doped control lenses (p < 0.001). A significant difference between the front and back surfaces were found for day 1 (p < 0.001), but no significant difference was found for day 7 (p > 0.05).

Fig. 7 illustrates the average fluorescence intensity for all four replicates for both materials, for lenses incubated in both the test and control solutions, for the bulk region only, for both time periods. For etafilcon, the fluorescent intensity for the lenses incubated in the labeled protein solution was significantly higher than

the lenses incubated in the PBS-control solution at all times (p < 0.001), indicating that BSA penetrated into the material in significant quantities even after 1 day of incubation. In addition, the amount of absorbed BSA increased significantly between days 1 and 7 (p < 0.001), as compared with the PBS-doped control lenses, which did not alter over time (p > 0.05). For the lotrafilcon B matrix, there was no significant difference in signal comparing the lenses incubated in labeled BSA to the control solution at any time point (p > 0.05), indicating that no detectable BSA penetrated into the bulk of the material over the 7 days. Fig. 7 also shows that there was a significant difference in absorbed BSA between the materials at both points in time (p < 0.001).

Comparison of Figs. 5 to 7 indicates that the fluorescence intensity of the labeled protein recorded on day 1 was higher on the surfaces compared to the "bulk" region for both materials, suggesting that after 1 day the amount of protein adsorbed onto the surface of both materials is greater than that absorbed into the
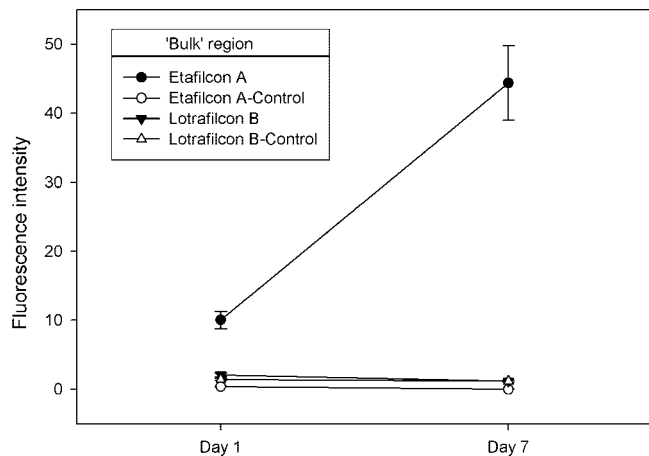
FIGURE 7.

Average fluorescence intensity for all four replicates for both materials, for lenses incubated in both the test and control solutions, for the bulk region only, for both time periods.
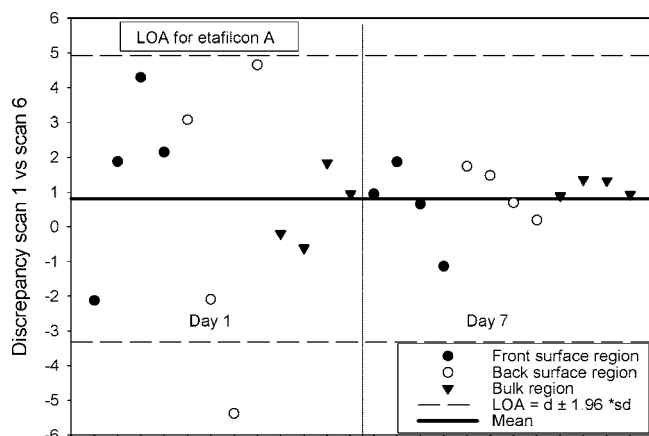

FIGURE 8.

Etafilcon A: Limits of Agreement. Discrepancies of the fluorescence intensity between the measurements at location 1 and 6 are plotted for all regions (front surface, back surface and "bulk") at day 1 and day 7. The average intensity loss for all measurements is plotted as the "mean."
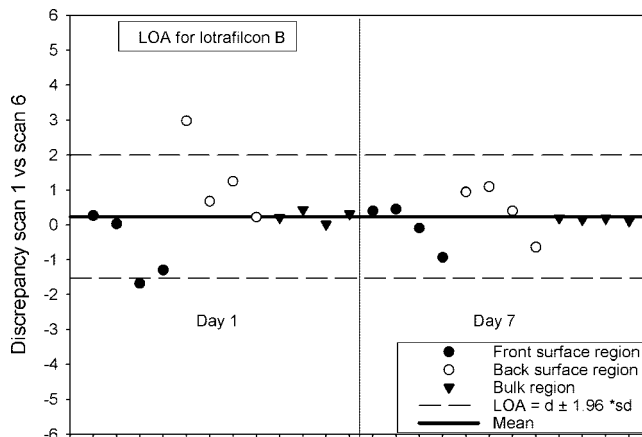

FIGURE 9.

Lotrafilcon B: Limits of Agreement. Discrepancies of the fluorescence intensity between the measurements at location 1 and 6 are plotted for all regions (front surface, back surface and "bulk") at day 1 and day 7. The average intensity loss for all measurements is plotted as the "mean."

bulk. After 7 days of incubation this trend is maintained for lotrafilcon B. However, for etafilcon the amount of absorbed and adsorbed BSA becomes equilibrated after this 7 days incubation period. Looking at the two different contact lens materials incubated in the PBS control solution, a significant fluorescent signal was already detectable on the surface of lotrafilcon B, without the addition of the dye (Figs. 3B and 6). This finding is of major importance, because the fluorescence intensity from the labeled BSA was only slightly stronger than the control sample, indicating that only a small amount of BSA adsorbed on the surface of lotrafilcon B. In contrast etafilcon showed no such surface peak (Fig. 3A), regardless of the incubation solution. To investigate the potential loss of fluorescence intensity due to light exposure (fluorescence loss in photobleaching; FLIP), two scans of the same location were taken on each lens and the discrepancies between these two measurements (from scans 1 and 6) were calculated and the Limits of Agreement plotted in Figs. 8 and 9. Exposure to the laser beam was between 60 and 80 s for each location. The average intensity loss for the lenses incubated with labeled BSA was 0.8 ± 2.1 units

for etafilcon and 0.2 ± 0.8 units for lotrafilcon B. Generally, a slightly lower intensity for the second scan was also found for both control groups (etafilcon 0.01 ± 0.01 units; lotrafilcon B 0.39 ± 0.42 units). CCC results were 0.98 for etafilcon and 0.99 for lotrafilcon B both calculated for the incubation in labeled BSA, confirming high concordance for repeated measurements and therefore consistent results for the different lenses. These results confirm that photobleaching effects were negligible.

The data from the lenses incubated in PBS with a very low concentration of DTAF, but without the addition of BSA, showed a fluorescent signal intensity on and inside both contact lens materials which was in the same range as found in our main study. The only difference to our labeled BSA solution was that we were able to detect significant fluorescent signal in the matrix of etafilcon A and, more importantly, also in lotrafilcon B, confirming that the pure dye does penetrate into both materials. This confirms that the amount of free dye in our solution had no impact on the results reported.

DISCUSSION

Hydrogels have been shown to be highly biocompatible, and as a result, they find application in various biomedical and pharmaceutical areas and are frequently used for implanted materials, including artificial blood vessels, catheters, or as drug delivery devices.[30,31] Albumin is the most abundant protein in human serum, and its adsorption on biomaterials is of major importance, because it is the initial event happening before cell attachments occur. The protein layer works as an interface between the biomaterial and the cellular tissue. However, this biochemical adsorption process can induce a higher risk of thrombogenicity due to conformational changes and irreversible adsorption of the protein on the surface.[32,33]

Contact lens complications due to protein deposition have been reported by many researchers.[1–6] The impact of albumin adhesion alone to contact lenses was studied by Taylor et al.[34] They demonstrated that increased albumin deposition to etafilcon A lenses resulted in increased adherence of *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*, with the opposite result occurring for polymacon lenses.[34]

Other studies confirmed that tear-coated contact lens materials are more likely to adsorb *Pseudomonas aeruginosa* compared to unworn lenses, but high individual variation was always reported.[35,36]

To determine protein adsorption on and/or absorption into hydrogel contact lenses, a variety of different imaging, immunological and microscopic techniques have been successfully used,[8,22–25] but none of these methods adequately describes the locations of proteins within the lens matrix or on the lens surface. A number of researchers have previously attempted to investigate protein penetration into hydrogel polymers, using both microscopic techniques and, more recently, CLSM. Refojo and Leong[37] used light microscopy and FITC-labeled lysozyme, BSA, and dextrans to look at the penetration of these substances into hydrogel polymer films of varying water contents and charge. The authors found that BSA penetrated into high water content gels but not into lower water content poly-HEMA gels and that lysozyme, with its lower molecular weight, penetrated further than BSA. Subsequently, Bohnert et al. used an "ultraviolet lamp" to investigate protein penetration,[16] but they could not detect any significant penetration of fluorescently labeled lysozyme or BSA into the bulk of a variety of hydrogel membranes. The most recent microscopy study investigating protein penetration into hydrogels used a staining technique (Coomassie brilliant blue) to investigate lysozyme and BSA penetration into all four FDA groups.[38] It is unclear whether the lenses investigated included silicone hydrogels, but their data showed that BSA was only located on the surfaces of the lens materials, with no visible penetration being observed, when compared with lysozyme, which showed penetration into FDA group IV materials.[38]

One of the most recent advances in microscopy relates to the development of confocal microscopy, which was patented by Minsky in 1961 and became even more popular with the addition of a laser in the late 1980s. Since then, various confocal microscopy techniques have been used extensively in ocular research to image cells and tissue, both in vivo and in vitro.[39–44] This form of microscopy has the significant advantage of being able to obtain images through thick samples using small step sizes. It has been previously used to provide information about depositions on both the contact lens surface and within the matrix, without the need to remove the protein of interest. Meadows and Paugh[45] used CLSM to study protein penetration in worn lenses and showed that protein penetrates through both etafilcon and poly-HEMA lens materials. To date, they are the only researchers to have used CLSM to study ex vivo lenses, and they were able to show that protein deposition increased in both materials over time. The most recent reports on protein penetration using CLSM are the studies by Garrett et al.[17,18,20] Their study examined both lysozyme and human serum albumin (HSA) penetration, using both commercially available conventional hydrogel materials and fabricated polymeric films of varying water content and charge. The result of this study showed that lysozyme penetrates in significantly greater quantities than HSA and that porosity and surface charge has a significant effect on lysozyme penetration, with ionic materials exhibiting greater penetration than neutral materials. Surface charge had no influence on HSA penetration, with very little penetration being seen after 1 day of exposure.

This study is unique in that we are the first to report on the use of CLSM to study the penetration of BSA with a molecular mass of 66 kDa into silicone hydrogel lens materials and one of the first to report that BSA can penetrate into conventional hydrogel materials. We found higher BSA uptake on an FDA group IV poly-HEMA-based conventional contact lens material (etafilcon A) compared to an FDA group I silicone hydrogel material (lotrafilcon B). Our results confirm previous studies, reporting that silicone hydrogel lenses adsorb very low levels of proteins compared to conventional poly-HEMA-based materials,[9–12] however, the advantage of this technique is that it does not only indicate differences in the amount of deposited protein, but can also locate the protein in terms of whether it is predominantly found on the surface or within the bulk providing the spatial and temporal distribution profile.

Fig. 3A and 3B show clearly that the location and degree of BSA deposited differ markedly between lotrafilcon B and etafilcon A. Figs. 4A to 7 demonstrate that a significant amount of labeled BSA penetrated into the matrix of etafilcon A after only 1 day of exposure, with no detectable BSA being found inside lotrafilcon B even after 7 days. Over time, the amount of BSA on and within the matrix of etafilcon A increased and the amount of absorbed BSA became similar to that adsorbed on the surface. No increasing uptake of labeled BSA was found for lotrafilcon B between day 1 and day 7.

Albumin absorption is influenced by many factors, including pH and ionic strength of the solution, water content and charge of the material, and, importantly, pore size.[46] Garrett et al. estimated two different models to calculate the actual pore size based on the water content of the hydrogel material.[17] They added different concentrations of methacrylic acid (MA) to poly-HEMA to increase the water content in the material and calculated the changing pore sizes. For a maximum concentration of 5% MA they calculated an average diameter of 34.7 and 29.3 Å for their two models, and therefore predicted that HSA, which has a diameter of approximately 55 Å, should not penetrate into their material, which they confirmed experimentally. However other researchers estimated bigger average pore sizes for various HEMA compositions: Gachon et al. reported pore sizes between 56 and 70.6 Å for poly(MMA-VP) lenses[47] and even bigger pores were found by Gatin et al. who investigated polyHEMA-based lenses and measured pore sizes of 428 Å.[48] Based on these studies it would be possible for BSA to penetrate into HEMA-based materials and our data support the conclusion that BSA with a molecular weight of 66 kDa can indeed penetrate into etafilcon A.

One final point to discuss is the surprising finding that the apparent degree of BSA deposition reduced on the lotrafilcon B material between days 1 and 7. This could be due to photobleaching or due to the dye intensity reducing over time. A previous study[49] showed that DTAF has comparably high fluorescence intensity to other dyes, but does tend to bleach faster. In our study we adjusted the argon laser to a very low intensity of 5% to prevent extensive light exposure, which could lead to bleaching effects. Figs. 8 and 9 demonstrate that for both materials only minor intensity losses were seen in the second scan at the same location, ruling out the possibility of photobleaching being significantly involved. This result confirms that the DTAF had good short time stability for the confocal laser, but it was not stable enough under long incubation conditions at 37°. Fading in the intensity of the dye was confirmed in a separate free-dye study (data not pre-

sented), confirming that the reason for the small, but statistically significant, reduction in fluorescence intensity after 7 days for the lotrafilcon B material was due to weakening of the DTAF and not BSA desorption. The increased amount of BSA adsorbed onto the etafilcon material prevented this small reduction in intensity being detectable. Further work is underway to locate a dye that remains stable over long periods of incubation.

CONCLUSIONS

CLSM is a useful technique to examine the penetration profile of the tear film protein albumin into different contact lens materials. After incubating etafilcon A in 0.5 mg/ml fluorescently labeled BSA, significant uptake on the surface and within the matrix was seen, which increased over time. The lotrafilcon B material adsorbed very little BSA on the surface, and no significant BSA was found in the matrix after 7 days of exposure. This confocal technique is applicable to any study in which biomaterials come into contact with any body fluid containing proteins.

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