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ORIGINAL PAPER

The Effect of Fluorescent Labels on Protein Sorption in Polymer Hydrogels

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Abstract Hydrogels are an increasingly important class of medical device materials that enable diverse and unique function, but can also be subject to significant biofouling and contamination. Although it is challenging to accurately quantify protein biofouling in hydrogels, spectroscopic detection of fluorescently labeled proteins is one method with the potential to provide direct, sensitive quantitation in transparent materials. Therefore, it is important to understand how fluorophores can affect protein-material interactions in hydrogels. This work uses an independent method, native ultraviolet fluorescence (native UV) of proteins, in conjunction with labeled protein fluorescence and the bicinchoninic acid assay (BCA), to assess the effect of fluorescent labels on protein sorption in polymer hydrogels. Bovine serum albumin (BSA) and lysozyme (LY) were labeled with two common but structurally different fluorophores and used as model biofouling proteins in three contact lens hydrogel materials. Native UV was used to directly measure both labeled and unlabeled protein sorption, while orthogonal measurements were performed with extrinsic fluorescence and BCA assay to compare with the native UV results. Sorption of labeled proteins was found to be <2-fold higher than unlabeled proteins on most protein-material combinations, while differences of up to 10-fold were observed for labeled BSA in more hydrophobic hydrogels. Fluorescence recovery after photobleaching (FRAP) also showed that the fluorescent label chemistry can significantly affect surface adsorption of sorbed proteins on the internal surfaces of hydrogels. This study reveals the complex nature of fluorophore-protein-material interactions and shows the potential of native UV for investigating unlabeled protein biofouling in hydrogels.

Keywords Hydrogel · Fluorophore · Fluorescent label · Sorption · Biofouling · Protein · Labeling · Interference · Contact lens · Medical device · Adsorption · Absorption · Fluorescence · Fluorescence recovery after photobleaching (FRAP) · Photobleaching · Intrinsic fluorescence · UV fluorescence · Residues · Microplate · Structure · Lysozyme · Ionic · Hydrohobic · BCA assay · Calibration · Response · Transparent · (Hydroxyethyl) methacrylate (HEMA) · Silicone · Bicinchoninic acid · Polymer

Introduction

The porous water-filled structure of polymer hydrogels enables their use in diverse applications for medical devices such as scaffolds, delivery vehicles, barriers,contact lenses, fillers, and bulking agents [1–3]. Although hydrogels are hydrophilic by design, proteins and other biomolecules with hydrophobic functionalities can bind to internal domains by coulombic (charge-charge) interactions [4]. Similar to non-porous materials, hydrogels can undergo protein adsorption and biofouling, resulting in tissue inflammation [5,6], microbial colonization [5], spread of infectious or allergenic agents [7], and performance degradation [8]. Moreover, new hydrogel materials being used or currently being developed for medical devices often contain complex hydrophobic domains to

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enhance performance attributes such as oxygen transport. [9] These domains may result in increased lipid and hydrophobic protein motif adsorption. In contrast to non-porous materials where soil is confined to the surface, the vast internal surface area of some hydrogels can absorb soil, potentially interfering with device function and/or safe use. It is important to be able to quantify protein sorption in order to better understand if biofouling has a role in adverse events, such as, for example, keratitis in contact lens wearers [10].

Measurement of protein adsorption on non-porous surfaces has been studied extensively in the literature for new and existing materials [11–13], but absorption of proteins and subsequent adsorption on hydrogel internal domains is more challenging to study. It is difficult to re-create many sophisticated hydrogel materials in-situ directly on sensor surfaces, and label-free methods such as surface plasmon resonance [14] and ellipsometry [12] are poorly suited for analysis because of the thickness of hydrogels used in medical devices. Scanning electron microscopy and atomic force microscopy can only image proteins on the surface of device materials. While it is helpful to understand interactions at the surface, interactions in the bulk material are characterized by different surface tension, different domains formed during polymerization, and in some cases, different coatings or treatments applied to the surface of the hydrogel.

Methods employed to study protein biofouling of medical device hydrogels include extraction followed by separation and quantification [15,16], direct measurement by fluorescence [17], and mass spectrometry [18]. Extraction is subject to challenges with strongly adsorbed species and heterogeneous recovery on different material types. In addition, large samples are needed to provide sufficient sensitivity. Confocal fluorescence is low-throughput and is generally a semi-quantitative method. Other fluorescent methods such as staining and derivatization have been used to directly observe biofouling on device surfaces [19].

More recently, a microplate-based fluorescence quantification strategy for measuring biofouling and cleaning of contact lens hydrogel materials has also been demonstrated [20]. The high-throughput capability of this method has great potential for improving the study of hydrogel interactions. Small samples (D=3 mm) are obtained with a biopsy punch and placed in 384-well microplates. Sampling potentially changes the surface of materials at the edge of the punch-out (for example, with surface treated materials), but the edge surface area comprises only a small portion of the total. The punch-outs are then exposed to fluorescently labeled protein, and sorption and cleaning processes can be evaluated using a plate reader with fluorescence spectroscopy.

An important question about the use of fluorophore labeled proteins is how they might alter secondary and tertiary structures [21,22] and affect protein-hydrogel interactions, potentially changing surface adsorption behavior [23]. Recent work using confocal fluorescence combined with radiotracers has suggested that some probes can have a noticeable effect on adsorption in contact lens hydrogels [24]. Thus, it is important to understand the limitations of using fluorescent probes for measuring protein-hydrogel interactions.

The goal of this work is to understand how fluorophores might affect the accuracy of fluorescence-based quantification of protein sorption in hydrogels. We measured labeled and unlabeled protein sorption using the intrinsic fluorescence of aromatic amino acids (tyrosine, tryptophan, and phenylalanine) in proteins when exposed to ultraviolet radiation [25] (native UV) [26–29]. The results were compared with two orthogonal methods: fluorophore fluorescence (for labeled proteins) and colorimetric BCA assay [30] (for unlabeled proteins). Contact lens materials used as model hydrogel substrates were biofouled by tear proteins, which contain high concentrations of bovine serum albumin (BSA) and lysozyme (LY) [31]. The overall UV fluorescence quantum yield of BSA and LY is about 15.2 and 6.0 %, respectively [32]. This results in reduced sensitivity compared to fluorescent probes (e.g. fluorescein quantum yield = 93 % [33]), but sufficiently high protein concentrations of BSA and LY may provide valuable information to compare sorption of labeled and unlabeled proteins. BSA and LY are also good representatives of a range of properties found in body fluid proteins: BSA (66.5 kDa/isoelectric point (pI) of 4.8), has a slight negative charge at pH 7.4 and has hydrophobic (Sudlow I/II sites) [34] and hydrophilic (Trp-134) [35] tertiary structures; LY (14.3 kDa/pI 11.35) has a significant positive charge at pH 7.4 and a two-domain tertiary structure.

The proteins were labeled with two common, but structurally different fluorophores (Fig. 1) to help elucidate how fluorophore chemistry might impact sorption. For each of the three quantification methods, a calibration curve was created using direct protein deposition and used to determine the amount of protein adsorption from quasi-equilibrium sorption experiments. Several contact lens hydrogels representing diverse properties of water content, ionic charge, and hydrophobicity were tested for each unlabeled/labeled protein combination. The results of fluorophore fluorescence and native UV were then compared for each labeled protein/material pair, while the results of BCA and native UV were compared for each unlabeled protein/material pair. Finally, the ratio of labeled/unlabeled protein sorption as quantified by native UV was evaluated. To better understand the mechanism of the observed differences, the mobility and diffusion rate of the proteins was studied using fluorescence recovery after photobleaching (FRAP). The combination of these techniques enabled a more complete and quantitative assessment of how fluorophores can affect protein sorption in medical device hydrogels.

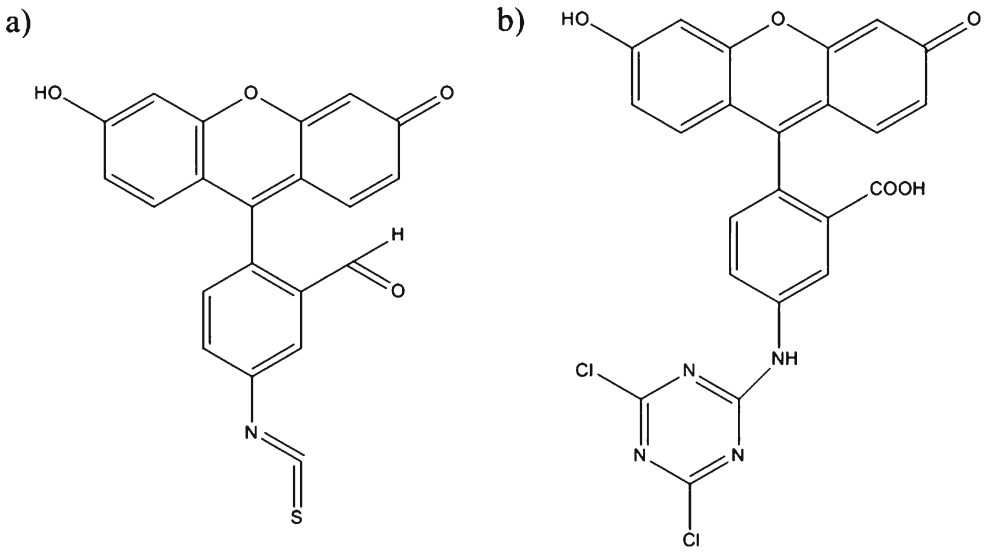


Fig. 1 Chemical structures: **a** fluorescein (FITC), which is similar to HiLyte, **b** 5-([4,6-Dichlorotriazin-2-yl] amino) fluorescein hydrochloride (DTAF)

Experimental Methods

Materials

Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM Potassium Chloride, 10 mM phosphate) was prepared from 10x stock solution purchased from VWR (Philadelphia, PA). Sodium bicarbonate buffer (0.1 M, pH 9), bovine serum albumin (BSA), lysozyme (LY), and 5-([4,6-Dichlorotriazin-2-yl]amino)fluorescein hydrochloride (DTAF) were purchased from Sigma-Aldrich (St. Louis, MO). DTAF is a highly reactive fluorescein derivative that reacts with not only amino groups and N-terminal amino acids of proteins but also with polysaccharides. It has been reported to have greater purity and stability than fluorescein isothiocyanate (FITC) [36] and an average extinction coefficient of 62,500 (M^{-1} cm^{-1}) [37,38]. HiLyte Fluor 488 SE Protein Labeling Kit including desalting columns (MWCO: 6k) was obtained from AnaSpec (Fremont, CA). HiLyte 488 SE (HiLyte) has similar spectra to that of fluorescein and forms stable carboxamide bonds in proteins via a succinimidyl ester with an extinction coefficient of 68,000 M^{-1} cm^{-1}. It is reportedly similar to fluorescein isothiocyanate (FITC) but more resistant to photobleaching [39]. Spin desalting columns (MWCO: 7 k) and Slide-A-Lyzer Dialysis Cassettes (MWCO: 20 k) were purchased from Thermo Scientific (Waltham, MA). Dialysis membrane (MWCO: 3.5 k) was obtained from SpectrumLabs (Rancho Dominguez, CA). White, 384- and 96-well plates and clear, 384-well plates were purchased from Fisher Scientific (Pittsburgh, PA). Three contact lens materials (HyA, HyB, HyC) were used to cover a range of material properties. HyA is a negatively charged silicone-containing hydrogel with low water content, HyB is a neutral silicone-containing hydrogel with low water content, and HyC is a negatively charged non-silicone hydrogel with high water content. The silicone material in HyA and HyB increases the overall hydrophobic character of the hydrogels.

Preparation of Protein

Preparation of Unlabeled Protein

Stock concentrations of unlabeled BSA and lysozyme were prepared in PBS and bicarbonate buffer from lyophilized powder and diluted serially.

Preparation of Labeled Protein

HiLyte was prepared by dissolving in DMSO and then adding to PBS protein solutions. The fluorophore DTAF was dissolved in DMF and added to sodium bicarbonate buffered-protein solutions. The mixtures were incubated in darkness for 1 h with gentle shaking. Following conjugation, free fluorophore was removed with desalting columns for HiLyte-labeled proteins and DTAF-labeled BSA. A spin desalting column was used to purify DTAF-labeled lysozyme for better recovery. The labeled dye solutions were dialyzed in PBS to remove any remaining free fluorophore and exchange buffer. The degree of labeling (DOL) determined spectrophotometrically for HiLyte-BSA, HiLyte-LY, DTAF-BSA, and DTAF-LY were 1.3, 0.5, 1.5, and 0.3, respectively. The difference in DOL between BSA and LY is appropriate because of the difference in the molecular weight of the proteins. BSA, at 66.5 kDa is ~5x the molecular weight of LY at 14.3 kDa, and similar DOL for BSA [40] and LY [41] have been reported elsewhere in the literature.

Calibration Procedure

Calibration curves for each contact lens materials were obtained by depositing known amounts of protein onto each sample. Protein solution (5 μL) at several concentrations was placed directly onto the surfaces of the punch-outs and then allowed to absorb into the samples until no visible droplet could be seen (approximately 15 min) (Fig. 2a). The calibration volume (5 μL) was optimized by dropping various volumes of PBS onto a punch-out until one was found that covered the surface without spilling over. PBS (20 μL) was deposited over each sample and intrinsic fluorescence (excitation: 290±5 nm, emission: 335±10 nm) and extrinsic fluorescence measurements (HiLyte – excitation: 490±5 nm, emission: 520±10 nm; DTAF – excitation: 495±5 nm, emission: 516±10 nm) were obtained using a recently developed method for contact lens hydrogels incorporating a Tecan m1000 microplate reader [20]. The concentration-response curves were aligned with either a quadratic or linear fit of the corresponding calibration data where appropriate.

Quasi-Equilibrium Mass Sorption Assay Procedure

Fluorescence/Native UV Coupons of each contact lens hydrogel were obtained with a 3 mm biopsy punch and equilibrated in PBS overnight. The samples were placed into a 384-well microplate and deposited with 20 μL unlabeled or labeled protein solution (0.01–3 mg/mL). The plate was covered and incubated for 20 h in a humidity chamber at 32 °C (Fig. 2b). The following day, samples were rinsed inside their wells 4x with PBS (50 μl). PBS (20 μL) was deposited over each sample and fluorescence measurements were obtained as described above. *BCA*: The bicinchoninic acid (BCA) assay is a nonspecific protein quantification assay commonly used due to its compatibility with surfactants, reagent stability, protocol flexibility, and high sensitivity [42]. After fluorescence/native UV measurement, an additional 20 μL of PBS was then deposited over the previous 20 μL and freshly prepared BCA reagent (40 μL) was added to each well. The microplate was then covered and placed back into a preheated 60 °C humidity chamber for 15 min [43]. The reacted solution in each sample well was transferred into a 384-well transparent microplate and absorbance (562 nm) was measured with the microplate reader.

FRAP Procedure

Coupons of HyC were placed into a 96-well plate and soaked overnight in 10 μL of 0.5 mg/mL HiLyte-BSA, HiLyte-LY, DTAF-BSA, or DTAF-LY and then rinsed with 200 μL of PBS. Each sample was mounted onto a coverslip and immersed in 20 μL of PBS. The coverslip was placed onto a Leica SP8 confocal laser scanning microscope and imaged using an Argon ion laser (488 nm). The use of a 20x objective with a small (0.7) N.A. and an open pinhole minimized the effect of diffusion from the non-planar (Z) axis on recovery. Three pre-bleach images were acquired, followed by bleaching, and three sets of post-bleach images (every 5, 10, and 30 s). Fluorescence recovery curves fit well to a single exponential and calculation of the immobile fractions and time constants was performed by the Leica LAS AF software.

Results and Discussion

Calibration

Calibration curves for both BSA and lysozyme were constructed to characterize the relationship between total protein

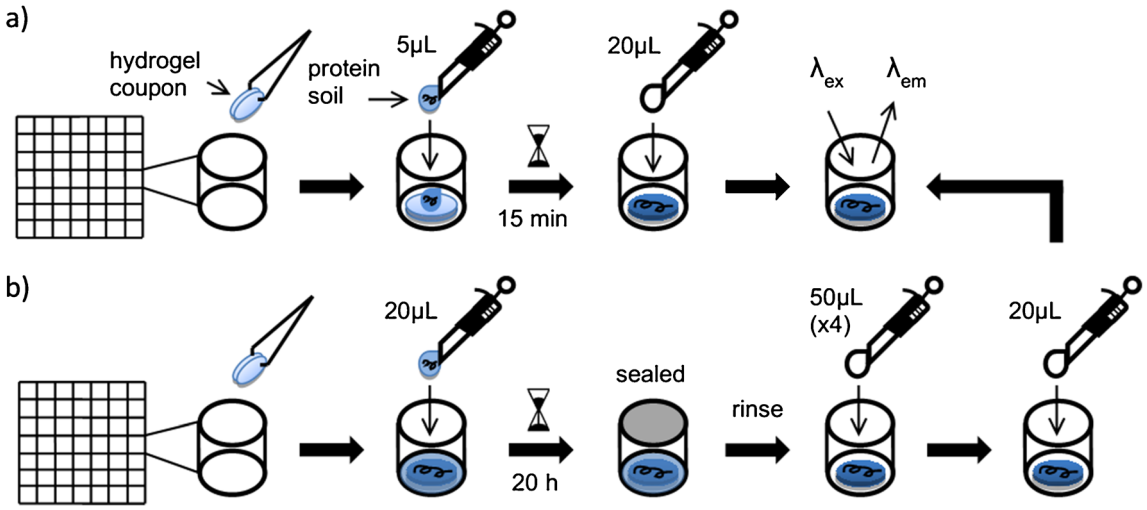


Fig. 2 Schematic showing **a** calibration protocol and **b** experimental assay protocol used for fluorescence and native UV quantification

soil mass and assay response (i.e. fluorescence intensity or absorbance) for each fluorophore-protein-hydrogel material combination. Here, for the sake of demonstrating the calibration procedure, we discuss the results for HyB (Supplementary Information (SI) Figure 1) with unlabeled BSA and labeled BSA-HiLyte. The data for HyA/HyC is found in SI (Figures 2, 3). It is important to use calibrations to obtain quantitative results because of method- and material-related variations in response. A similar strategy is routinely employed in medical device cleanliness testing on planar surfaces to minimize the effects of sample measurement variation on different materials and provide quantitative evaluations of cleaning efficacy [44,45]. Here we show that calibration is especially important for hydrogels. Due to autofluorescence and material thickness, the effects of material differences are amplified.

For fluorescence (SI Figure 1, left column), both BSA (top row) and LY (bottom row) with HyB show a linear response range followed by a non-linear range for higher amounts of deposited protein. The BSA response is linear from 0 to 1 μg deposited (6325RU), where the slope (4881RU/μg) starts to decrease slowly until reaching a plateau between 2.5 and 4 μg at 13,897RU. Between 4 and 16 μg, the response decreases to 9703RU. Although non-linear saturation of signal and plateau behavior can be caused by the inner filter effect in these ~300 μm thick hydrogels, the noticeable drop in signal seen at higher concentrations may also indicate fluorescence quenching. Labeled species may be concentrated in close proximity within the hydrogel pores. A similar response behavior has been seen in previous work by our group [20] and underscores the importance of using appropriate dilutions of labeled species to measure protein adsorption in hydrogels. The interaction of labeled lysozyme with HyB was also strong, with a linear response from 0 to 0.5 μg (15,288RU), where the slope (29,449RU/μg) starts to decrease slowly before reaching a plateau at 2.5 μg (22,040RU). The difference in the fluorescence level at which the two proteins reached a plateau may be attributed to the differences in the molecular weight of the two proteins (for a given concentration, there are 4-fold more LY than BSA molecules) and their DOL. Differences in protein conformation may also effect the fluorescence response [46].

For native UV calibration (SI Figure 1, center column), the relationship between BSA mass and fluorescence intensity is linear. However, greater sensitivity is exhibited towards unlabeled (slope = 2153RU/μg) than labeled (slope = 438RU/μg) protein. This may be due to steric changes in molecular structure, or possibly overlapping emission/absorbance transitions between the intrinsic and extrinsic fluorophores, resulting in intra- or intermolecular resonance energy transfer that reduces the emission yield at 335 nm. For BSA, a linear relationship is observed for both unlabeled and labeled protein from 0 to 5 μg. Similar to the results for BSA, the unlabeled LY response (slope = 3690RU/μg) is higher than the labeled response (slope = 2269RU/μg). These differences show the importance of correcting the native UV results using calibration data in order to make an accurate comparison between adsorption of labeled and unlabeled species.

For the BCA assay (SI Figure 1, right column), there is a linear calibration response at all masses tested for both LY and

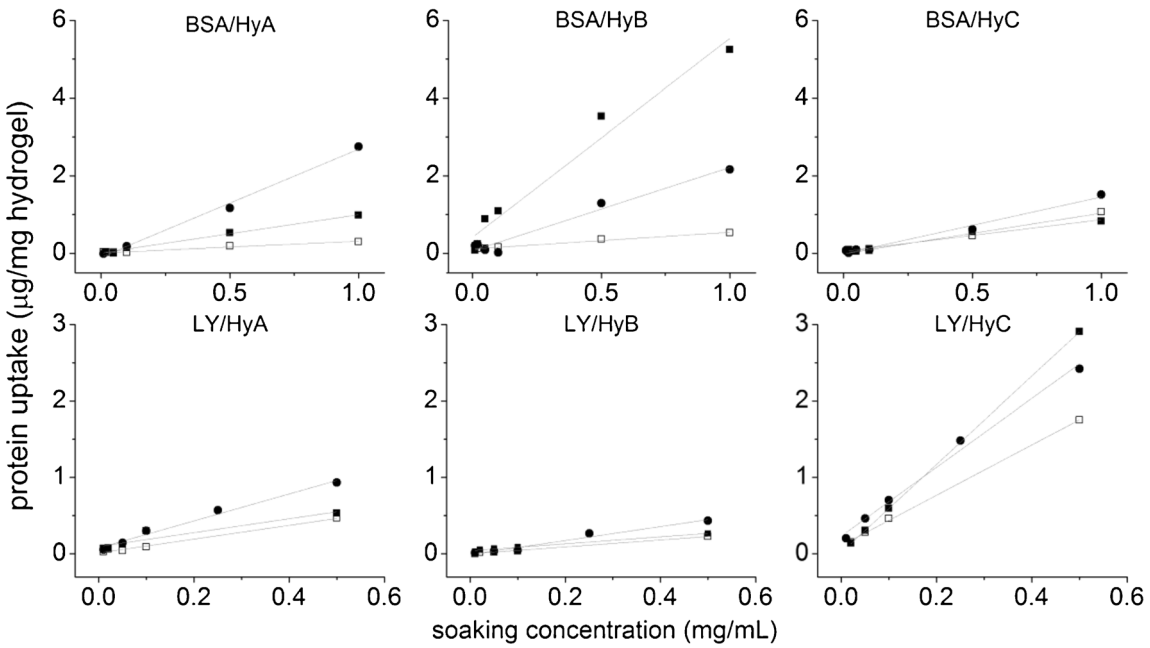


Fig. 3 Calibrated native UV data for HiLyte-BSA (*top row*), HiLyte-LY (*bottom row*), DTAF-BSA (*top row*), DTAF-LY (*bottom row*) on HyA, HyB, and HyC (HiLyte-labeled protein-solid *circles*, DTAF-labeled protein-solid *squares*, unlabeled protein-open *squares*)

BSA. However, there is greater sensitivity for LY (slope = 2.22RAU/μg) than for BSA (slope = 0.31RAU/μg). This is likely due to the greater number of LY molecules than BSA molecules per unit mass. Another factor may be the relative difference in the number and accessibility of BCA assay-reactive amino residues between the two proteins.

For all three contact lens materials, data in the monotonic range were fit to either a linear or quadratic function and used to calculate real masses in the concentration-adsorption studies carried out below.

Quasi-Equilibrium Sorption of Unlabeled and Labeled Protein

To observe concentration-dependent sorption of unlabeled and labeled protein in hydrogels, the three contact lens materials were soaked 20 h at corneal temperatures (32 °C) in an excess of several concentrations of protein solutions. After rinsing, the amount of protein in the hydrogel was detected using native UV (for both labeled and unlabeled proteins), fluorescence (for labeled proteins), and BCA assay (for unlabeled proteins).

Sorption of Labeled and Unlabeled Protein by Native UV

The use of native UV protein quantification allows for a direct comparison (once calibrated) between Hilyte-labeled, DTAF-labeled, and unlabeled protein sorption in hydrogel materials.

Sorption of HiLyte-Labeled Protein by Native UV

The HiLyte-labeled BSA (Fig. 3, top row) had the greatest overall sorption in HyA (0.97 μg) and less sorption in HyB (0.80 μg) and HyC (0.42 μg) for the 0.5 mg/mL soaking concentration. The trend (HyA > HyB > HyC) shows preference for a material that has low water content but also ionic and hydrophobic silicone components. HiLyte-labeled BSA had greater sorption than the unlabeled protein in all three materials for 0.5 mg/mL soaking concentration, with a difference of 0.81 μg/143 % for HyA, 0.58 μg/114 % for HyB, and 0.11 μg for HyC/30 %. The trend (ΔHyA > ΔHyB > ΔHyC) suggests an increasing effect of the label on BSA sorption with silicone-containing materials HyA and HyB.

The HiLyte-labeled LY (Fig. 3, bottom row) had the greatest overall sorption by HyC (1.67 μg), less sorption by HyA (0.78 μg) and even smaller sorption by HyB (0.27 μg) for the 0.5 mg/mL soaking concentration. The trend (HyC > HyA > HyB) follows a pattern of ionic interaction between the cationic LY and ionic materials HyC and HyA. HiLyte-labeled LY also showed greater sorption than unlabeled LY in all three materials with a difference of 0.39 μg/67 % for HyA, 0.13 μg/63 % for HyB, and 0.46 μg/32 % for HyC for the 0.5 mg/mL soaking concentration. This trend (ΔHyA > ΔHyB > ΔHyC) suggests a similar mechanism of interaction as for HiLyte-labeled BSA. The difference in HiLyte-labeled and unlabeled LY sorption was less than for BSA on all three substrates.

Sorption of DTAF-Labeled Protein by Native UV

Native fluorescence measurements for sorption of DTAF-labeled protein were similar to those for sorption of HiLyte-labeled protein in some but not all fluorophore/material combinations.

The DTAF-labeled BSA (Fig. 3, top row) had the largest sorption by HyB (2.20 μg), less sorption by HyA (0.44 μg), and even smaller sorption by HyC (0.38 μg) for the 0.5 mg/mL soaking concentration. The trend (HyB > HyA > HyC) shows increasing sorption in more uncharged, hydrophobic material. DTAF-labeled proteins also had greater sorption than the unlabeled protein in all three materials with a difference of 0.28 μg/93 % for HyA, 1.98 μg/164 % for HyB, and 0.07 μg/20 % for HyC for the 0.5 mg/mL soaking concentration. The trend order (ΔHyB> > ΔHyA> > ΔHyC) showed a strong, increasing effect of the label on BSA sorption with increasingly neutral, silicone-containing material.

The DTAF-labeled LY (Fig. 3, bottom row) had the greatest overall sorption in HyC (2.00 μg), less sorption by HyA (0.44 μg), and even smaller sorption by HyB (0.17 μg) for the 0.5 mg/mL soaking concentration. The trend (HyC > HyA > HyB) follows a pattern of ionic interaction between the cationic LY and ionic materials HyC and HyA. DTAF-labeled LY also showed greater sorption than unlabeled LY in all three materials with a difference of 0.05 μg/12 % for HyA, 0.03 μg/19 % for HyB, and 0.79 μg/49 % for HyC for the 0.5 mg/mL soaking concentration. Similar to HiLyte-labeled proteins, there was a significant increase in sorption of labeled proteins over unlabeled protein, and the differences in DTAF-labeled LY sorption were smaller on all materials when compared with DTAF-labeled BSA. The order of difference in labeled and unlabeled protein (ΔHyC > ΔHyB > ΔHyA) is the opposite as that for HiLyte-labeled LY, showing a larger effect on hydrophilic materials than hydrophobic materials.

Based on both the HiLyte and DTAF native UV data, it became clear that there were differences in the adsorption of labeled and unlabeled proteins for all materials tested, and significant differences in sorption between the two labeled proteins.

Quasi-Equilibrium Sorption of Labeled Protein by Fluorescence

To validate the results obtained by native UV and better understand the trends observed, the mass of labeled protein sorption was confirmed by comparing quasi-equilibrium sorption values obtained using extrinsic (labeled) fluorescence.

Quasi-Equilibrium Sorption of HiLyte-Labeled Protein by Fluorescence

HiLyte-labeled BSA (Fig. 4, top row, circles) had the greatest overall sorption in HyC, where the mass at 0.5 mg/mL soaking concentration was beyond the monotonic calibration range. For a much lower concentration (0.05 mg/mL), HyC took up 0.28 μg of HiLyte-BSA. This mass is significant considering that only ~1 μg of HiLyte-BSA was taken up by HyA and HyB at the 10-fold higher 0.5 mg/mL soaking concentration. The sorption pattern (HyC > HyB ≅ HyA) diverged from that of native UV (HyA > HyB > HyC), with the largest difference between the HyC sorption values for fluorescence and native UV. A good explanation for this divergence may be related to poor UV transmission in HyC (confirmed by spectrophotometry). Although the calibration was designed to eliminate material effects, differences in the depth of protein penetration for the calibration (~15 min before drying) vs. the sorption experiments (20 h soak) may have led to greater attenuation of the UV excitation/emission energy for more deeply embedded proteins. Additional experiments performed with confocal microscopy (SI Figure 4) confirmed that the penetration depth of BSA in HyC increased in this time frame. Because BSA is ~5x the mass of LY, such an effect would likely be more pronounced for BSA than for LY.

HiLyte-labeled LY (Fig. 4, bottom row, circles) also had the greatest overall sorption (0.87 μg at 0.05 mg/mL) in the ionic, high-water HyC, followed by greatly decreased sorption in HyA (0.91 μg at 0.5 mg/mL) and even less sorption in HyB (0.43 μg at 0.5 mg/mL). The sorption trend (HyC > HyA > HyB) was similar to that seen using native UV.

Quasi-Equilibrium Sorption of DTAF-Labeled Protein by Fluorescence

DTAF-labeled BSA (Fig. 4, top row, squares) had the greatest overall sorption at the 0.5 mg/mL soaking concentration in HyA (0.16 μg), and similarly less sorption in HyB (0.14 μg) and HyC (0.10 μg). This sorption pattern (HyA > HyB > HyC) diverged from that obtained using native UV (HyB > HyA > HyC). Because BSA has both α-helix and β-sheet secondary structures, the hydrophobic nature of the DTAF label may drive preferential adsorption in hydrophobic domains of the silicone materials, resulting in different response behavior than HiLyte for the two detection methods.

DTAF-labeled LY (Fig. 4, bottom row, squares) sorption in HyC, similar to that of HiLyte-labeled BSA and LY, was beyond the monotonic response range. For the lower concentration reported above (0.05 mg/mL), the sorption was 0.95 μg. At the 10-fold higher 0.5 mg/mL soaking concentration, DTAF-LY sorption on HyA (0.88 μg) was greater than sorption on HyB (0.33 μg). The sorption pattern (HyC > HyA > HyB) was the same as that observed using native UV detection and was identical to the pattern seen for HiLyte-LY as well.

Quasi-Equilibrium Sorption of Unlabeled Protein by BCA Assay

Because extrinsic fluorescence could not be used to validate the native UV unlabeled protein sorption results, the BCA assay was used for this purpose.

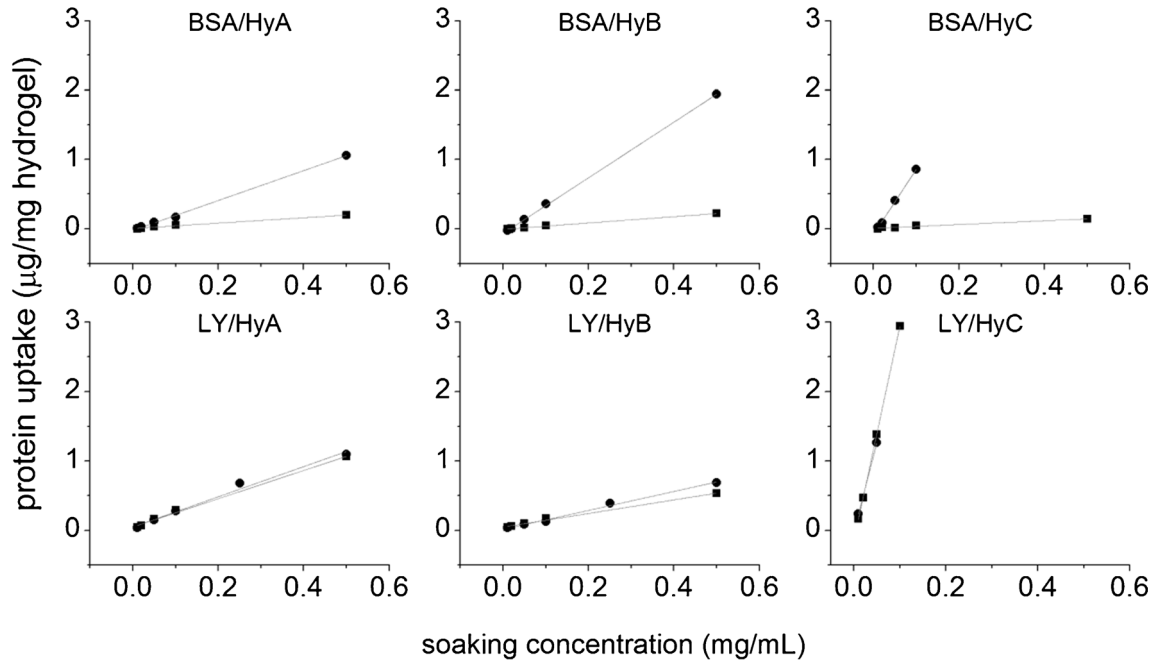


Fig. 4 Calibrated fluorescence data for Hilyte (*circles*) and DTAF (*squares*) labeled BSA (*top row*) and LY (*bottom row*) on HyA, HyB, and HyC

Sorption of Unlabeled Protein by BCA Assay

Unlabeled BSA sorption at the 0.5 mg/mL soaking concentration (Fig. 5, top row) was similar in HyA (0.12 μg) and HyC (0.10 μg) and slightly lower in HyB (0.07 μg). This trend (HyA ≅ HyC > HyB) diverged from that of native UV, where the order was HyC > HyB > HyA. The reason for this difference is not clear, but may be due to the much smaller effective pore size of HyA than the other two materials [47]. Since the BCA assay depends on diffusion of reagents to and from protein reaction sites, the small effective pore size of HyA may have limited the penetration of protein into the hydrogel matrix, causing a falsely low response for the other two materials where more deeply embedded proteins are more difficult to access.

Unlabeled LY sorption at the 0.5 mg/mL soaking concentration (Fig. 5, bottom row) was greatest for HyC (1.15 μg), followed by HyA (0.16 μg) and then HyB (0.07 μg). This trend (HyC > HyA > HyB) is identical to that seen for unlabeled protein sorption quantified by native UV.

Overall, quantitation by BCA assay produced lower total masses of protein compared to native UV. This difference is likely due to the difference in penetration depth for the calibration assay vs. the sorption assay. Because the BCA assay relies on diffusion of the reagents through the hydrogel matrix to react with embedded proteins, and diffusion of colored reagents back into the bulk for measurement, the shallow penetration of proteins in the 15 min calibration assay resulted in greater response than the deeper penetration observed after 20 h. This may explain why the smallest difference with native UV was seen for the largest protein (BSA) in the material with the smallest effective pore size (HyA).

Combining the Results of Native UV, Fluorescence, and BCA Assay

Hydrogel materials are more complex than planar surfaces and the use of different measurement techniques can result in unexpected variation.

Agreement Between Native UV and Fluorescence (Labeled Proteins)

The agreement between native UV and fluorescence results was expressed as the mass of protein determined by fluorescence divided by the mass of protein determined by native UV (Table 1, Column A (HiLyte) and B (DTAF)).

For HiLyte-labeled proteins, in all cases except for HyA/BSA, the protein mass obtained using fluorescence was greater than that obtained using native UV. The results (HyA/BSA-0.9, HyA/LY-1.2, HyB/BSA-1.5, HyB/LY-1.6, HyC/BSA-4.0, HyC/LY-2.7) show an average difference within 20 % for BSA and 35 % for LY with HyA and HyB. For HyC, where uptake mass is higher, the agreement between fluorescence and native UV was not as good (up to 4-fold difference in the results). This was likely due to the poor UV transmission of the HyC material, as discussed in Section 3.3.1 above.

For DTAF-labeled proteins, only for LY was the protein mass obtained using fluorescence greater than that obtained using native UV. The results (HyA/BSA-0.4, HyA/LY-2.0, HyB/BSA-0.1, HyB/LY-1.9, HyC/BSA-0.3, HyC/LY-4.5)

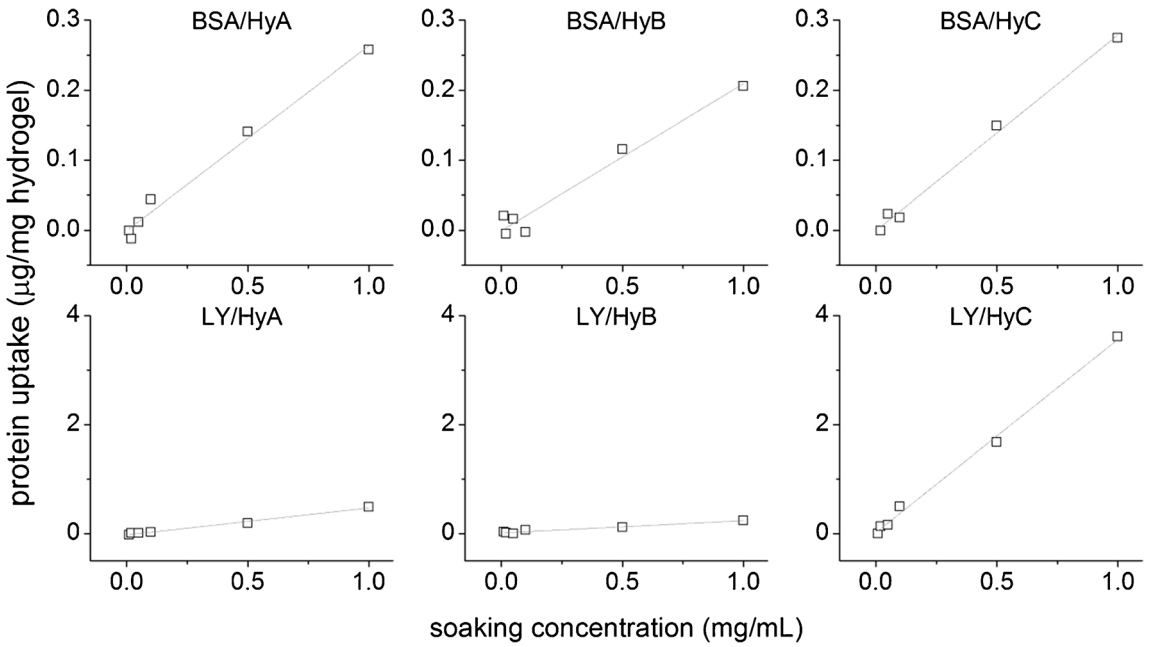


Fig. 5 Calibrated BCA assay data for unlabeled BSA (*top row*) and lysozyme (*bottom row*) on HyA, HyB, and HyC

Table 1 Tabulation of mass ratios between different quantitation methods across all fluorophore-protein-hydrogel combinations at 0.5 mg/mL soaking concentration unless otherwise indicated

		A	B	C	D	E
Hydrogel	Protein	HiLyte / $UV_{HiLyte\text{-}labeled}$	DTAF / $UV_{DTAF\text{-}labeled}$	$BCA_{unlabeled}$ / $UV_{unlabeled}$	$UV_{HiLyte\text{-}labeled}$ / $UV_{unlabeled}$	$UV_{DTAF\text{-}labeled}$ / $UV_{unlabeled}$
HyA	BSA	0.9	0.4	0.8	6.1	2.8
	LY	1.2	2.0	0.4	2.0	1.1
HyB	BSA	1.5	0.1	0.3	3.6	10.0
	LY	1.6	1.9	0.5	1.9	1.2
HyC	BSA	4.0 (at 0.05mg/mL)	0.3 (at 0.05mg/mL)	0.3	1.4	1.2
	LY	2.7 (at 0.05mg/mL)	4.5 (at 0.05mg/mL)	0.9	1.4	1.7

show an average difference within 120 % for BSA and 65 % for LY with HyA and HyB. Similar to the results for HiLyte-labeled proteins, the agreement for the HyC/LY combination (4.5-fold difference at 0.05 mg/mL) was not as good. This difference can be explained by the poor UV transmission of the HyC material. Although it is not clear why the agreement for HyC sorption was so much better for DTAF-labeled BSA than for HiLyte-labeled BSA, it may be due to the fact that the HyC sorption of DTAF-BSA was much lower than for other combinations. It is possible that the difference in measurement results, in addition to being affected by the material and protein, also scaled with the absolute amount of protein taken up by HyC.

Agreement Between Native UV and BCA (Unlabeled Proteins)

The agreement between native UV and BCA assay results was expressed as the mass of protein determined by BCA divided by the mass of protein determined by native UV (Column C).

The results (HyA/BSA-0.8, HyA/LY-0.4, HyB/BSA-0.3, HyB/LY-0.5, HyC/BSA-0.3, HyC/LY-0.9) show an average agreement within 75 % for BSA and 50 % for LY with all materials. All masses obtained by BCA assay were lower than those obtained by native UV measurement. Overall, the agreement between native UV and BCA for unlabeled proteins was better than the agreement between fluorescence and native UV for labeled proteins.

Effect of Labeling on Overall Protein Sorption

The protein sorption by HyA and HyB indicated by native UV was consistently within 100 % of the mass obtained by fluorescence (labeled proteins) and BCA assay (unlabeled proteins). As such, we deduce that native UV provides a reasonably good quantification of the effect of labeling on the protein sorption. Although the differences between native UV and fluorescence for HyC (4-5x) were greater than 100 %, the evidence points to poor UV transmission, a factor which would likely cancel out to some extent when comparing labeled and unlabeled response factors for a given protein.

Overall, the mass ratios for labeled/unlabeled sorption (Columns D (HiLyte), E (DTAF)) demonstrate that the effect of the fluorophore on sorption depends on the specific protein-material interaction.

The mass ratios for sorption of HiLyte-labeled/unlabeled protein were close to unity for both BSA and LY (BSA-1.4, LY-1.4) on the most ionic and hydrophilic material, HyC. For the uncharged and silicone containing material, HyB, the ratios (BSA-3.6, LY-1.9) indicate that the label had a greater effect on the sorption of the hydrophobic BSA than the charged LY. For HyA, which contains both ionic functionality and silicone, the ratios (BSA-6.1, LY-2.0) show an even stronger effect by the label on BSA sorption than on LY sorption. While the effect of HiLyte on LY was similar for all materials, its effect on BSA was more complex. In general, HiLyte caused increased sorption in the silicone-containing materials.

For DTAF-labeled proteins, a similar effect is observed, but with even greater perturbations induced by the fluorophore. The labeled/unlabeled mass ratios for HyC were again close to unity for both BSA (1.2) and LY (1.7). The mass ratio for DTAF-labeled LY sorption was also very similar in HyA (1.1) and HyB (1.2). However, for DTAF-labeled BSA, the ratio of 2.8 for HyA and 10 for HyB showed an increasing effect of the label on sorption with increasing neutrality and hydrophobicity of the hydrogel. The effect of DTAF on sorption had a similar pattern to HiLyte, with a small impact on LY and a larger impact on BSA in all materials.

In both cases, it appears that when electrostatic interactions are present between proteins and hydrogel materials, they dominate over other forces that drive labeled/unlabeled protein sorption. In the absence of strong ionic interactions, hydrophobic silicone components of the hydrogels were associated with significant increased sorption of the proteins.

Although some general patterns are observed here, the behavior is not predictable by simple rules alone, as there may be other effects besides fluorophore interactions with the hydrogel, such as effects of the fluorophore on protein conformation.

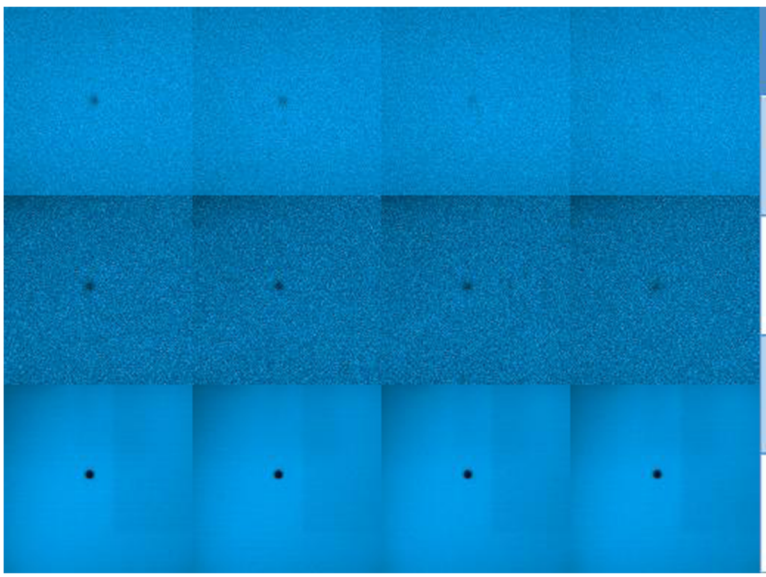
Fluorescence Recovery After Photobleaching (FRAP)

While the microplate method quantifies overall sorption of labeled proteins, it does not distinguish between absorbed and adsorbed protein. To better understand why there were such large differences in sorption between different fluorophore-protein-material combinations, fluorescence recovery after photobleaching (FRAP) was employed to quantify protein diffusion in protein soaked contact lens hydrogels (Fig. 6). The results of FRAP fitting to a model diffusion recovery equation revealed that HiLyte-labeled BSA had a higher mobile fraction (71 %) and a much shorter half-life for recovery (22 s) than DTAF-labeled BSA (44 %/76 s). This indicates less interaction between HiLyte-labeled protein and the HyC material. Both labeled LY proteins had much lower mobility than BSA, presumably due to stronger electrostatic interactions with the HyC material. Similar to the pattern with BSA, HiLyte-labeled LY had a larger mobile fraction (11 %) and shorter recovery half-life (4 s) than DTAF-labeled LY (7 %/104 s). LY, at about 23 % of the molecular weight of BSA, is expected to have a much faster diffusion rate and shorter recovery half-life than BSA. While this held true for HiLyte-LY, the much longer $T_{1/2}$ observed for DTAF-LY in conjunction with only 7 % mobile fraction may suggest that there may be a third state of partial interaction (neither completely free nor strongly bound) with the HyC material associated with the DTAF label only. This is further supported by the longer $T_{1/2}$ seen for DTAF-BSA (76 s) when compared with HyLyte-BSA (22 s).

The FRAP results confirm that the differences in sorption found above are due to surface interactions. Additionally, because they were done on HyC material, they show that the small differences in sorption between labeled and unlabeled proteins observed with HyC for both HiLyte and DTAF may not completely capture the nature of protein-material interaction. Because HyC is a high water material, a large fraction of the protein sorption measured may not represent surface adsorbed protein. This may hide smaller but significant differences in surface adsorption of labeled proteins.

Conclusions

Hydrogels, with their complex structures, raise unique complications in investigations of their interaction with biological molecules. Additional complications inherent to analytical methods may confound results further. Recent reports have suggested that fluorophore protein labels can greatly skew data on protein adsorption by materials. This work provides new insight on the many variables that can affect quantitative detection methods for protein sorption in hydrogels, and provides an assessment of fluorophore effects on protein sorption. First, we developed a framework necessary to ensure reliable, quantitative methods for comparing labeled and unlabeled proteins with a recently developed high-throughput microplate platform. The results were validated using orthogonal methods, and the extent of agreement was within 2-fold in most cases, except for the UV absorbing HyC material. Second, the method was employed to investigate how factors



Protein	Mobile Fraction	T ½ (s)
HiLyte-BSA	0.71	22
DTAF-BSA	0.44	76
HiLyte-LY	0.11	4
DTAF-LY	0.07	104

Fig. 6 FRAP montage (*left*) of HiLyte-BSA (*top row*: 0s,6s,26s,58s), DTAF-BSA (*middle row*: 0s,3s,23s,84s), and DTAF-LY (*bottom row*: 0s,6s,48s,108s) and mobility data (*right*) for labeled BSA and LY on HyC

such as material charge, material hydrophobicity, protein charge, protein hydrophobicity, and fluorophore chemistry affect fluorophore-protein-material interactions.

The most dominant effect associated with protein sorption in hydrogels was electrostatic attraction. Interactions between positively charged LY and anionic HyA/HyC resulted in the largest sorption and were not greatly affected by the presence of fluorophores. Water content was also important, with the higher water, ionic HyC material consistently taking up more charged LY protein than the lower water, ionic HyA material.

The effect of different fluorophores was less predictable. For the neutral, silicone-containing HyB, the DTAF had a greater effect on BSA sorption. But for the ionic, silicone-containing HyA, HiLyte had a greater effect on BSA sorption. FRAP studies showed that the differences observed using the high-throughput methods were due to surface interactions between proteins and material. Proteins labeled with the DTAF label also had greatly reduced mobile fractions, indicating that they were strongly adsorbed to the surfaces of the hydrogels. FRAP also revealed that despite overall similarities in sorption for the two fluorophores with HyC material, the nature of surface interaction with the labeled proteins is nevertheless greatly affected by the label chemistry.

Based on these results, it is prudent to use caution when employing any of the techniques demonstrated here for quantifying protein sorption in hydrogels. Linearity cannot be assumed without calibration, and non-monotonic regions of response could potentially lead to more pronounced errors. The use of fluorophores HiLyte and DTAF resulted in higher sorption for all labeled proteins. In most cases, the difference was less than 2-fold. The combination of BSA and silicone containing materials was especially problematic, resulting in up to 1 order of magnitude difference in sorption. Whether or not this effect is acceptable will depend on the reason for testing protein sorption. For example, in comparative tests used for high-throughput material interaction screening, the differences in sorption between labeled and unlabeled proteins may be of less concern. Where an absolute uptake mass is needed, as in biocompatibility testing, investigators should be aware of the real variations introduced by fluorescent labels. In particular, the DTAF label seems to have very strong surface interactions, even with a hydrophilic, high-water material. These interactions may be missed, especially with bulk detection methods in cases where a large amount of protein is absorbed by the hydrogel.

Native UV detection may be a good method for measuring unlabeled protein sorption in many applications. Because of the lower sensitivity when compared with labeled fluorophores, the method still needs to be improved in order to be used with more challenging applications such as cleaning studies, where trace amounts of protein in a hydrogel are measured. Efforts are currently underway to further improve the native UV method using UV laser-induced fluorescence (UV-LIF) combined with time-resolved spectroscopy to separate protein fluorescence from autofluorescence of the hydrogel materials.

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