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BIOMACROMOLECULE ADSORPTION AT THE CELLULOSE–LIQUID INTERFACE

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ABSTRACT

A novel methodology is developed to visualize and quantify biomolecules adsorption at the cellulose film-liquid interface. Hydrogenated cellulose (HC) films were made from cellulose acetate and deuterated cellulose (DC) films produced using deuterated bacterial cellulose. Deuterated bacterial cellulose was obtained by growing the *Gluconacetobacter xylinus* strain ATCC 53524 in D_2O media. Horse Radish Peroxidase (HRP), a robust and well know enzyme, was selected as model functional biomacromolecule to adsorb at the cellulose interface. The film thickness and quantification of adsorbed HRP molecules were characterized by X-ray and neutron reflectivity (NR) measurements. Reflectivity data analysis reveals the cellulose films to be smooth (low roughness) and uniform. The HC and DC films are 206 Å and 92 Å thick, respectively, and both films swell in the aqueous buffer solution. In NR measurements, it is difficult to trace the adsorbed HRP layer on HC film due to the small scattering length

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density (SLD) difference between HC and HRP providing no contrast. However, using deuterated cellulose (DC) film provides sufficient SLD difference (contrast) with respect to the SLD of HRP. The adsorbed HRP layer is 110 Å thick and occupies a volume fraction of 20%. Using deuterated cellulose films enabled the quantification of thin and partial layers of proteins at the liquid interface. Quantifying and controlling the morphology and functionality of biomolecules at the cellulose interface enables to efficiently develop and optimize low cost cellulose based diagnostics devices with superior functionalization.

Key words: Horse Radish Peroxidase (HRP), Deuterated cellulose, Thin films, Neutron reflectometry, Hydrogel, Adsorption, Liquid–solid interface, Regenerated cellulose

INTRODUCTION

Cellulose is a natural polymer which can easily be engineered into novel food packaging and biomedical applications [1, 2]. Cellulose is widely available, low cost and biodegradable. It has a unique glucosidic structure with three liable –OH groups per monomer which can easily be functionalized [3, 4]. The adsorption of functional biomacromolecules, such as enzymes, antibodies or even cells, onto the cellulose surface plays an important role in the development of numerous biomedical applications, especially diagnostics [5, 6]. The efficiency of paper biodiagnostics often depends on the conformation, stability, longevity and quantity of the biomolecules adsorbed onto the cellulose surface.

Sustaining antibody stability on the cellulose thin film surface is a difficult task and a limiting factor for the shelf life of point-of-care devices. The partially released antibodies in the lateral flow devices has caused concerns in commercialisation [7].

Visualizing functional biomolecules such as enzymes and antibodies on paper is difficult for many reasons. A first is that most functional biomolecules are protein, glycoproteins and polysaccharides which have a polymeric chemical composition fairly similar to cellulose. A second issue is that the critical dimension of these functional proteins typically ranges from 2 to 30 nm, which is much smaller than the dimension of fibers, paper pore size and even the pulp fiber roughness. Biomolecules are thus indifferent from the paper or pulp fibers onto which they are adsorbed. Using smooth model cellulose films is a way to alleviate the effect of paper morphology from the study. However, it is still a challenging task to quantify and visualize biomolecules on cellulose thin films. Many

characterization methodologies can be employed to quantify the adsorption process on cellulose such as Quartz Crystal Microbalance (QCM), Ellipsometry, Grazing Incidence X-ray Diffraction (GI-XRD), Atomic Force Microscopy (AFM) and X-ray (XR) and neutron reflectivity (NR) [8]. AFM probes the surface morphology while QCM quantifies at the nano-gram the mass adsorbed onto the thin film surface. These methods have their advantages and limitations such as resolution and radiation damage. Reflectivity using neutrons is a low energy non-destructive characterization method suitable to visualize and quantify biomolecules at the solid–liquid and solid–air interface; it however requires thin (5–100 nm) and smooth film. Biomolecules mainly consists of C, H, O and N atoms. Neutrons are sensitive to the scattering cross section of Hydrogen (H) and Deuterium (D), allowing selective contrast of the interphase. Exchanging H with D at different ratios allows to make significant changes in the contrast between the biomolecules and the background matrix, or in our case, the thin film surface. This contrast helps to visualize and quantify the adsorbed entity onto the thin film surface.

This study aims to visualize and quantify bio-macromolecules adsorbed directly onto the cellulose surface without using markers, dyes or chemical modification. Hydrogenated cellulose (HC) and deuterated cellulose (DC) films were prepared by spin coating. XR and NR measurements at the film–air interface were made to probe the quality of the films. Horse Radish Peroxidase (HRP), a widely used and well characterized enzyme, was selected as model bio-macromolecule to be adsorbed onto the cellulose thin film surface. NR measurements were made at the solid–liquid interface to quantify cellulose swelling and HRP adsorption. Quantification of the adsorbed HRP molecules was evaluated by model fitting the NR curves. The effect of cellulose deuteration on improving the visualization of adsorbed biomolecule is investigated.

EXPERIMENTS

Materials

1-Butyl-3-methylimidazolium chloride (BMIM chloride, ≥98.0%, HPLC), acetic anhydride (99.5%), pyridine (anhydrous, 99.8%), hexamethyldisilazane (HMDS, ReagentPlus, 99.9%), chloroform (PCR Reagent, containing amylenes as stabilizer, ≥99%), toluene (anhydrous, 99.8%), ethanol and acetone (AR, ≥99.5%) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Polished silicon wafers (50.8 mm diameter and 12 mm height, n-type Si:P, [100]) were obtained from SIL'TRONIX (Archamps Technopole, Archamps, France). As supplied wafers were cleaned by soaking in a mixture of ammonium hydroxide, hydrogen

peroxide and water (volume ratio = 1:1:5) at 70 °C for 15 min, allowed to air dry in a dust-free environment and used as substrates for spin coating. Spin coating was performed with a WS-650-23B spin coater (Laurell Technologies Co. North Wales, PA, USA).

Hydrogenated cellulose from cellulose acetate

Hydrogenated Cellulose (HC) was obtained by dissolving the cellulose acetate in acetone and then followed by spin coating onto the Si blocks for 30 sec at 4,000 rpm. Later, the film-coated Si blocks were kept in the sodium-methoxide solution for 12 h to obtain hydrogenated cellulose film [9].

Deuterated cellulose from deuterated bacterial cellulose

Deuterated Cellulose (DC) films were made from deuterated bacterial cellulose (DBC). Deuterated bacterial cellulose was obtained by growing the Gluconacetobacter xylinus strain ATCC 53524 in deuterated glycerol carbon sources dissolved in growth media containing D_2O. Deuterated cellulose soluble derivatives were obtained by cellulose trimethylsilylation in ionic liquid followed by solution in toluene and spin coated on polished silicon blocks to produced thin films [10, 11]. Final cellulose film was obtained by regeneration of cellulose derivative films with HCL acid vapours (Figure 1).

X-ray and neutron reflectivity

X-ray reflectivity (XR) measurements were performed at the solid–air interface on a Panalytical X'Pert Pro instrument with Cu K_α X-ray source (λ = 1.54 Å). Neutron reflectometry (NR) measurements were performed at the PLATYPUS beamline of the Australian Nuclear Science and Technology Organization (ANSTO, Sydney, Australia) nuclear facility. Measurements were performed at three different angles (0.5, 0.85 and 3.8 degree). The theory of the NR is given elsewhere [12]. Measurements were made both at the air–film and liquid–film interface. Data reduction and analysis was performed by using the Igor Based macro MOTOFit [13].

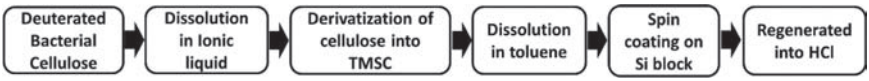

Figure 1. Synthesis process of deuterated cellulose film spin coated from deuterated cellulose bacteria.

RESULTS

X-ray reflectivity (XR) measurements were made on the hydrogenated cellulose (HC) film at the air–film interface to monitor the quality of the cellulose film with respect to film thickness, smoothness and roughness. Figure 2 shows the measured XR profile from the HC film spin coated onto the Si-block. The multiple fringes observed in the profile reveal that the film thickness is uniform. Further characterization is evaluated by fitting the XR profile using the software MOTOFit [13]. A single layer model was used to fit the cellulose layer followed by a layer of pre-formed SiO_2 onto the Si block. The thickness, X-ray SLD and roughness were the fitting variables. The solid line in Figure 2 reveals the quality of the fit and the respective X-ray SLD profile is plotted in the inset. The evaluated parameters are given in Table 1. The thickness of the SiO_2 layer is 10 ± 3 Å and the HC layer is 206 ± 10 Å thick with a roughness of 18 Å.

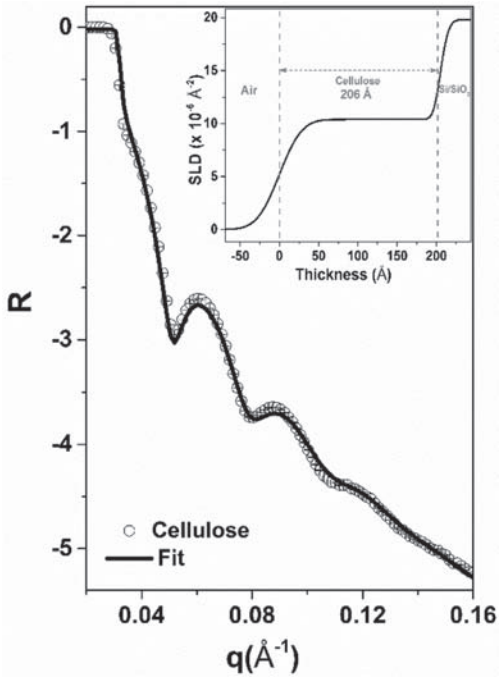

Figure 2. X-ray reflectivity profile of the hydrogenated cellulose film (from cellulose acetate) measured at the air–film interface. The solid line shows the single layer model fit. Inset: SLD profile of HC film with respect to the thickness calculated from curve fitting.

A solution of horseradish peroxidase (HRP) molecules dispersed in a 0.9%NaCl–D_2O saline buffer at a concentration of 1mg/mL was passed over the cellulose surface. The HRP molecules adsorbed onto the HC film surface via physio sorption to form a thin layer. Non-destructive NR measurements were made at the film–liquid interface on the HC film with and without adsorbed HRP layer. NR measurement allows to quantify the HC film behaviour in the buffer, HRP layer thickness, roughness and the volume fraction of HRP molecules adsorbed. Measurements were also performed in the saline buffer solution of ϕ.9%NaCl–70%D_2O–30%H_2O for additional contrast. The measured NR profiles of the HC and HC/HRP films are shown in Figure 3(a). No significant differences are observed in the NR profile of the HC and the HC/HRP film; cellulose and HRP have similar SLDs. It is therefore difficult to segregate and characterize the adsorbed HRP layer. Both reflectivity profiles can be fitted with the single layer model, as shown in Figure 3(b). The solid line represents the fit and the respective

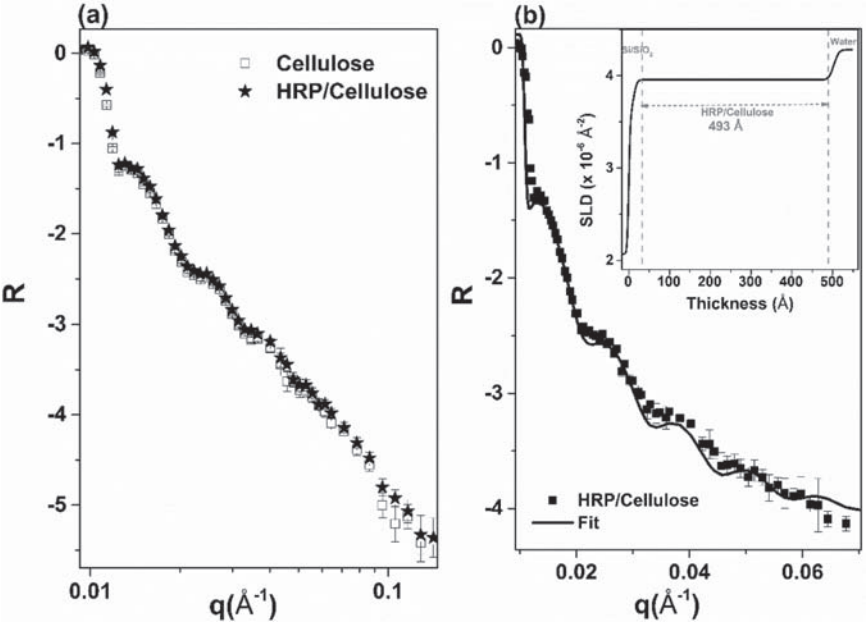

Figure 3. (a) Neutron reflectivity profile of the Hydrogenated cellulose (HC) film and HC/HRP film measured at film–liquid interface. Measurement performed in 0.9%NaCl in 70%D_2O–30%H_2O. (b) The solid line shows the single layer model fit for the HC/HRP curve. Inset: SLD profile of HC/HRP film with respect to the thickness calculated from fitting.

SLD profile obtained from fitting is given the inset of Figure 3(b). The single layer model fitting reveals the HC film at the liquid interface to be 493 ± 10 Å thick; this is 2.4 times the thickness of the dry film measured by XR at the air–film interface.

To enhance the contrast between cellulose surface and HRP molecules, deuterated cellulose (DC) films were made from deuterated bacterial cellulose (Figure 1). The NR profile of the DC film measured at air–film interface is shown in Figure 4. A single layer model fits well the experimental data; the solid line represents the fit and the evaluated SLD variation profile with respect to thickness is shown in inset. The DC film is 92 ± 5 Å thick with a roughness of 18 Å and a SLD of the DC film in air is 5.45×10^{-6} Å^{-2}.

Figure 5 shows the neutron reflectivity curves of the DC film without and with the HRP adsorbed layer measured at the liquid interface. The measurements were performed in the ϕ.9%NaCl–70%D_2O–30%H_2O saline buffered solution. The

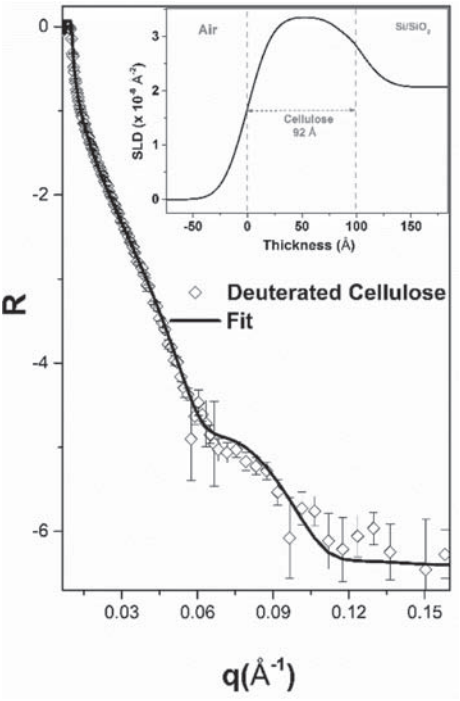

Figure 4. Neutron reflectivity profile of the DC film measured at the air–film interface. The solid line shows the single layer model fit. Inset: SLD variation profile of DC film with respect to film thickness.

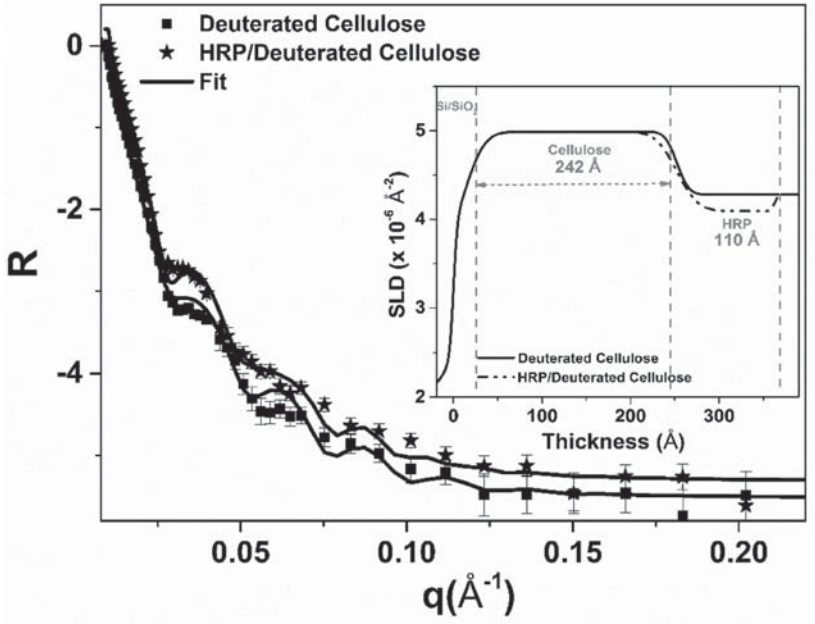

Figure 5. Neutron reflectivity curves for deuterated cellulose (DC) film and with HRP adsorbed at the film–liquid interface. NR measurement performed in 0.9%NaCl in 70%D_2O–30%H_2O. The solid line shows the single layer model fit for cellulose and an addition layer model for HRP. Inset: SLD profile of DC film and HRP layer with respect to film thickness (from curve fitting).

multiple fringes observed in both curves reveal uniform and smooth films. A noticeable difference emerges between the curve for HRP/DC (filled stars) and pure DC (filled box). Quantification by modelling the NR curves reveals good fit with a single layer model for the DC film and with an additional layer model for the DC film with the HRP layer. The solid line in Figure 5 shows the fits; the respective SLD profiles with respect to the thickness are presented in the inset. The structural parameters are presented in Table 1. The thickness of DC film in buffer is 242 ± 10 Å with a roughness 19 Å; the thickness of the adsorbed HRP layer on cellulose is 110 ± 8 Å with a roughness of 18 Å. The volume fraction of deuterated cellulose ranges between 30 and 40% and that for the HRP layer is between 15 and 20%. The SLD value for the DC is 6.3×10^{-6} Å^{-2} and 3.2×10^{-6} Å^{-2} for the HRP layer. The difference in SLD value between DC and HRP is much larger than that between HC and HRP, clearly showing the benefits of deuterating the cellulose substrate layer.

Table 1. Parameters obtained after fitting of the NR and XR curves at air–film and liquid–film interface. Film thickness, SLD, solvent fraction and film roughness were the fitting variables. Saline in the sample name represents the ϕ.9%NaCl–70%D_2O–30%H_2O saline buffered solution.

Sample	*Thickness* (Å)	*SLD* (10^{-6} Å^{-2})	*Roughness* (Å)	*Fraction of solvent* (%)
HC_Air_XR	206	—	20	—
HC_Saline_NR	493	2.9	12	76
DC_Air_NR	92	5.45	18	62
DC_Saline_NR	242	6.27	19	64
HRP_Saline_NR	110	3.2	19	80

DISCUSSION

Uniform and smooth hydrogenated and deuterated cellulose films (HC and DC) were prepared to visualize and quantify the adsorption of Horse Radish Peroxidase (HRP) at the cellulose–liquid interface. Both cellulose films swell in saline buffer solution. The thickness of the HC film is 493 Å in 0.9% NaCl–7ϕ% D_2O–30%H_2O, which is about 2.4 times the original dry film thickness (206 Å). However, in the same solution, the thickness of the deuterated cellulose (DC) is 242 Å, swelling by a factor of 2.6 (92 Å). Water molecules diffuse into the cellulose chains of the thin film which relax to ease the thermodynamically unstable structure locked in during the spin coating/fast drying process; this gain in mobility results in the important cellulose film swelling [14, 15]. Deuteration of the cellulose backbone did not affect swelling. This swelling means that the regenerated cellulose films essentially forms hydrogel layers in water.

XR and NR measurements show both HC and DC films to be uniform, smooth and suitable to characterize biomolecules adsorption layers. There was basically no difference between the NR curves of HC and HC/HRP due to the near identical SLD of cellulose and HRP providing no contrast. The cellulose SLD of composition $C_6H_{10}O_5$ is 1.7×10^{-6} Å^{-2}; cellulose has three liable hydrogens identified by the dotted circle in Figure 6. In 70%D_2O–30%H_2O, these three liable H's are replaced with D's which increase the HC SLD to 2.9×10^{-6} Å^{-2}. The SLD of the HRP evaluated from fitting the NR curve (Figure 5) in D_2O is 3.2×10^{-6} Å^{-2}, close to the SLD of cellulose. This small SLD difference between cellulose and HRP enzyme provides a very low contrast between the phases/layers, which renders differentiation difficult.

Two methodologies can be employed to increase the SLD difference between adsorbed HRP and cellulose surface: deuterate either the HRP molecules or

cellulose. To deuterated an enzyme biochemically is difficult, and so is the determination of the liable protons exchangeable into deuterium (D). However, deuteration of cellulose is easier and now much better understood [10, 11]. Cellulose contains only 10 H per monomer with three liable H's (Figure 6). It is easy to trace the exchangeable Hs or Ds in neutrons experiments thanks to the large scattering cross section difference between H and D characterized by neutrons.

Here, DC thin films are produced from deuterated bacterial cellulose grown in D_2O media. Earlier reported work on ATR-FTIR measurements on the deuterated cellulose in D_2O gave evidence that bacteria grown in D_2O medium contains C–D and O–D bonds but no C–H or O–H bonds proving high deuteration level [10]. AFM characterization revealed that after regeneration in acid vapours, the deuterated cellulose films are smooth with a decreased roughness (around 10 Å) [10]. The SLD value of DC ($C_6D_{10}O_5$) in D_2O buffer is 7.2×10^{-6} Å^{-2} while in the 70%D_2O–30%H_2O it decreases to 6.3×10^{-6} Å^{-2}. The reason behind this difference is due to the three liable Ds of the fully deuterated cellulose which can exchange with the Hs of the buffer (Figure 6). The SLD of the adsorbed HRP layer (3.2×10^{-6} Å^{-2}) shows a large SLD difference compared to the DC film (6.3×10^{-6} Å^{-2}). This large SLD difference allows to differentiate and quantify the HRP molecules adsorbed onto the DC film (Figure 5).

HRP is a robust enzyme widely used for catalysis, bio sensors, biotechnology, diagnostics and biomedical applications [16–20]. The average size of HRP is about $52 \times 34 \times 30$ Å^3 with a molecular weight of 44 kDa. Good fitting of the experimental NR results was achieved during modelling of the layer formed by adsorbed HRP molecules onto cellulose (Figure 5). A film thickness of 110 Å with a HRP volume fraction of 20% were measured, corresponding to an average surface coverage of 3.8 mg/m^2. This HRP layer thickness twice as high as the longest dimension of the molecule combined with the high surface coverage suggests the aggregation of HRP onto cellulose (Figure 7). The SLD profile

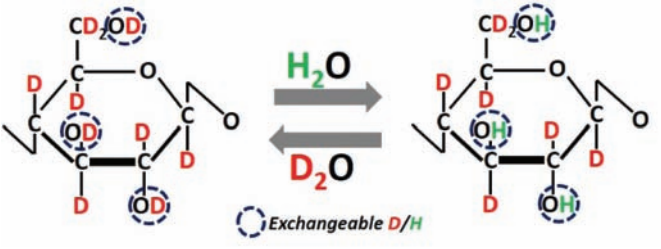

Figure 6. Exchangeable Ds in deuterated cellulose films in Hs in H_2O and reverse in Ds in D_2O

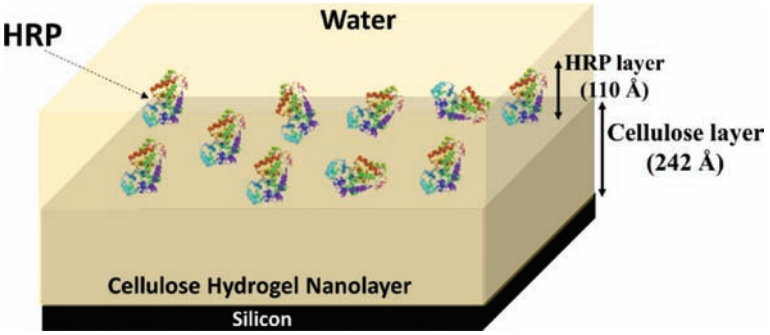

Figure 7. Schematic of the adsorption of Horse Radish Peroxidase (HRP) onto the cellulose surface.

between the interface of DC and HRP is not sharp which reveals diffusion of HRP into the cellulose layer. This is plausible as the cellulose film forms an hydrogel layer once swollen in water.

CONCLUSION

Smooth and uniform films of hydrogenated cellulose (HC) and deuterated cellulose (DC) were prepared from cellulose acetate and deuterated bacterial cellulose by spin coating. Both films swells by factor of about 2.5 in the saline buffer solution (0.9% NaCl–70%D_2O–30%H_2O), forming in essence a thin layer of hydrogel. A widely used and well characterized enzyme, Horse Radish Peroxidase (HRP), was selected as model biomolecule. The adsorption of HRP at the solid–liquid interface was measured by neutron reflectometry onto both the HC and DC films. HRP adsorption onto HC surfaces shows no contrast, and therefore no detection, by neutron reflectivity; this is due to the minute difference in scattering length density (SLD) between cellulose (2.9×10^{-6} Å^{-2}) and HRP enzyme (3.2×10^{-6} Å^{-2}), providing no contrast. However, for sensitive and accurate measurements the cellulose layer be deuterated. With a SLD of 6.3×10^{-6} Å^{-2}, DC films provide the contrast to visualize HRP molecules adsorbed at the solid–liquid interface. A HRP enzyme layer 110 Å thick with a volume fraction of 20% and corresponding to a surface coverage of 3.8 mg/m^2 are calculated. These results reveal that the HRP preferentially adsorbed as aggregates, and not-individual molecules, forming a partial layer onto cellulose. Further, the measured SLD profile suggests some diffusion of the HRP enzyme within the cellulose hydrogel layer. Deuteration of cellulose combined with neutron reflectometry of

the liquid–solid interface provides a new methodology to study the structure of cellulose layers and to better understand the effect of crystallinity and interaction with water. The ability to precisely measure the conformation of biomolecules at the cellulose interface, especially functional proteins such as enzymes and antibodies, empowers the engineering of efficient and low-cost paper/ cellulosic biodiagnostics.

ACKNOWLEDGEMENTS

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Transcription of Discussion

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Roger Gaudreault University of Montreal

Beautiful work Gil, congratulations. I also want to congratulate you on bringing your blood test to the commercial stage, which is an outstanding achievement. I have one question. Can we look at the QCM slides? So, QCM is a great method and a negative frequency shift of about –80 Hz is significant, meaning that molecules adsorb on the thin film surface. Did you use the *Sauerbrey* equation to calculate the adsorption of 4 mg/m^2?

Gill Garnier Monash University

I believe so, I said I believe because I should really turn this question to Vikram. Am I correct, Vikram?

Vikram Singh Raghuwanshi Monash University

Yes, we used the Sauerbrey equation $\Delta m = C\frac{\Delta f_n}{n}$ (where C is approximately –0.177 mg/m^2.Hz for the 4.95 MHz gold-coated quartz sensors used. Usually the

change in frequency for a specific overtone, Δfn, is divided by the overtone number, n, to determine the normalised frequency shift), and equation $\Gamma = \frac{10^{20} M_w \phi T}{N_a V}$ (where M_w is the molecular weight, ϕ is the volume fraction, T is the thickness, N_a is the Avogadro number and V in the molecular volume of an IgG molecule). By taking parameters from the neutron reflectivity data analysis and putting them in above equations we obtained these coverage values.

Roger Gaudreault

Okay, so on one occasion you adsorbed 2.5 mg/m^2 and another one you adsorbed 4 mg/m^2 on surfaces. My point is regarding the challenge with QCM where you need a rigid molecular system in justifying the use of the Sauerbrey equation to calculate the adsorbed mass.

Gill Garnier

Yes, this is.

Roger Gaudreault

And my concern is that you mentioned having a soft gel-like surface. Although you may have a rigid molecule, it is adsorbing on a gel-like surface. So can you comment on the accuracy of the calculated adsorbed mass?

Gill Garnier

Well, there are two considerations in our analysis. The first is accuracy; I was stunned to see, that we found 2.5 mg IgG /m^2 coverage by neutron reflectivity experiments, and 4 mg IgG/m^2 with this QCM technique. These techniques rely on completely different principles and to get similar results is truly amazing. The second consideration analyses the system. We basically have a big ball (IgG molecules) suspended on a spring (Cellulose hydrogel). We are well aware of that and we used the conventional technique and I think we now need to refine it a bit to understand more about how much of a spring the cellulose is. Is it really a hydrogel? What are its visco-elastic properties? It is very different working on cellulose hydrogels, and we saw yesterday with our Japanese friend from Oji, that these gels can be very viscous at only 2%; here we have 40%. Can I assume that the cellulose hydrogel behaves like a solid surface? This is our initial hypothesis used in the work present. However, as you commented, this has to be verified.

Vikram Singh Raghuwanshi

I would like to make one more comment, which is that the cellulose film we used for neutron reflectivity is regenerated from bacterial cellulose and the cellulose film coated sensor we used for QCM-D was from supplier which was regenerated from microfibrillated cellulose. Both are cellulose material but have a different morphology.

Gill Garnier

And if I may comment on that. What we didn't show is that we spent 2 years just to learn how to properly dissolve deuterated bacterial cellulose in organic solvents for proper film preparation by spin coating. We investigated many deuterated cellulose derivatives and discovered the mechanisms are very poorly understood. We were hoping to simply rely on a well-established recipe and that is what we initially tried. However, we found a lot of surprises with partial and no solubility at all. For example, if you select cellulose acetate, you must achieve a DS of 2 for solubility in acetone, because if you reach a DS higher than 2.5 it is no longer soluble in acetone. But how do you interrupt a reaction mid-step to reach a DS of 2? And what does an average DS of 2 really means? These are interesting issues. There are a few possibilities to reach cellulose acetate of DS 2. You can either have all cellulose chains having 2 hydroxy groups derivatized for each glucose, or you can have a mixture with half of the cellulose chains with the 3 hydroxyl groups fully substituted (DS 3) and half with only 1 Hydroxyl substituted (DS 1). The way these cellulose derivatives would dissolve in solvent is completely different, and therefore the films you obtain are also completely different. So what we found is that it is much easier to reach a full DS of 3. I give credit to Bank and team for the TMSC in toluene method. With complete substitution of all hydroxyl on all chains to DS of 3 you are sure all will be soluble. However, there is a lot of subtlety possible to achieve cellulose films of different morphology. This is an unexplored and promising field of research for our community.