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The role of the protein-water interface in dictating proton conduction across protein-based biopolymers

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Abstract

Proton conducting polymers became central in recent years, and especially for energy related applications. As such, unraveling their proton transport mechanism is of prime importance, and specifically the different contribution of proton transport across bulk water inside the polymer vs. the transport at the interface between the polymer and water. In recent years, proton conducting biopolymers are proving to be a green and sustainable alternative for traditional polymers. Unlike traditional synthetic polymers, the protein-based biopolymer that we use here can uptake significant amount of water reaching 150 wt.% water, which might suggest a large contribution of through bulk water proton transport vs. the protein-water interface one. We directly tackle the latter question and deciphering the contribution of the protein-water interface in mediating proton conduction across our electrospun biopolymer by introducing two alternative experimental approaches. The first is to follow proton conduction across highly aligned mat in a parallel vs. perpendicular to the fiber direction, while the second is to measure proton conduction across a ‘completely dry’ network of the protein fibers. We conclude that although the protein-based mat is containing substantial amount of water, proton transport is mediated along the protein-water interface.

Keywords: bovine serum albumin, impedance spectroscopy, electrospinning, proton transport, Grotthuss mechanism, proton exchange membranes
Introduction

Proton conducting materials are the subject of numerous studies in recent years toward understanding their proton transport mechanism and their utilization in a variety of applications and especially energy-related ones, such as fuel cells, batteries, and capacitors. Common studied materials including metal-organic frameworks, solid acids, ceramic oxides, and synthetic polymers.\textsuperscript{1–4} One of the challenges of this community, and specifically for proton conduction synthetic polymers, is to decipher the exact proton transport mechanism while distinguishing between the possible proton transport across bulk water to proton transport involving the polymeric backbone and its interface with water molecules. Far away from such materials, proton translocation across specific pathways is a fundamental process in nature, whereas proteins are nature’s choice as the proton mediating moiety, and mostly across a biological membrane. Inspired by nature, several recent works have utilized proteins for the formation of proton conducting material.\textsuperscript{5–9} Such bioinspired polymeric conductive materials are gaining momentum toward their integration in biomedical applications.\textsuperscript{10,11} In addition to their applicative route, much effort was targeted toward deciphering how protons are being transported along such protein-based materials. Gorodetsky and coworkers formed drop-casted thin films out of a cephalopod structural protein that exhibit relatively high proton conductivity of $0.1 \text{ mS cm}^{-1}$ at room temperature and 90% relative humidity.\textsuperscript{7} This performance is attributed to the large number of water molecules and the carboxylic acids in the protein structure. Ashkenasy and coworkers also demonstrated the importance of the carboxylic acid in promoting proton conduction across a self-assembled peptide fibrils.\textsuperscript{12,13} Based on this knowledge, they showed recently a highly proton conducting peptide fibrils network, which is merely 5-7 times lower than the conductivity of Nafion\textsuperscript{®} films.\textsuperscript{6} The role of charged amino acids has been also proven essential in supporting very high proton conductivity across protein-based polymers\textsuperscript{5,9}. Another example is the bovine serum albumin (BSA) protein, that has been manipulated (by denaturing the protein) through electrospinning process to receive a free-standing material with a fibrillar structure.\textsuperscript{14} The main difference between the use of BSA to all the previously reported protein-/peptide-based materials is the sustainable nature of the BSA protein. Being one of the waste products of the bovine industry, the use of this protein for making new functional materials is promoting a circular economy by using waste products. Furthermore, the protein can be isolated in bulk quantities, resulting in a very low price-tag of the protein raw materials for the formation of the electrospun mats, and there is no need for any genetic expression or synthesis. We previously showed that after immersing the BSA mats in water, the hydrated mats could support proton conduction on a millimeter range,\textsuperscript{8} which can be disrupted using chemical modifications of the protein surface making them more hydrophobic.\textsuperscript{15} In here, we wish to explore the role of water in
mediating long-range proton conduction across the BSA mats, in comparison to the protein contribution. To do so, we use two different approaches: 1) using the fully hydrated BSA mat while controlling the matrix alignment; and 2) measuring protonic conduction across a completely dry BSA thin fiber network as a function of relative humidity (RH). We show by several electrical measurements that the protein surface and its interface with water molecules are crucial for proton transport along the studied BSA electrospun system. We further compare our results to the ‘more traditional’ drop-casting technique of the same BSA protein used for electrospinning, thus emphasizing the role of the microstructure of the BSA electrospun fibers and its interface with water.

Results and Discussion

Before diving into the results, it is of prime importance to distinguish the BSA electrospun system from other bioderived polymers. In all the reported proton-conducting protein-based polymers, the hydrated form of the polymer contained 12-20% water\textsuperscript{5,16}, which corresponds also to the water uptake of proton-conducting polysaccharide-based biopolymers.\textsuperscript{17–19} Importantly, no proton conduction has been observed for <10% water uptake for such polymers. Following the electrospinning process, the dry BSA mat contains just 4 wt.% water\textsuperscript{8}, however, upon immersing it in water, the BSA fibers act as a sponge, absorbing a vast amount of water, resulting in a staggering water uptake of 150 wt.% for the hydrated BSA mat. Taking into consideration the substantial amount of water within the BSA mat, we wish to explore here the exact role of water in mediating long-range proton conduction across the BSA mats, in comparison to the protein contribution. This large amount of water molecules within the BSA mat might well result in proton conduction solely across the bulk water within the mat in a Grotthuss-type proton diffusion process (Figure 1, blue pathway). Another possibility is that proton transport takes place on the surface of the protein comprising the mat, in which both protonable side chains of amino acids (i.e., can participate in a hydrogen-bond network) as well as some water molecules at the surface of the protein are responsible for the proton diffusion (Figure 1, red pathway).
Figure 1 – Suggested pathways of proton diffusion via a Grotthuss-like mechanism. Blue pathway: proton diffusion solely via water molecules situated within the BSA mat. Red pathway: proton diffusion involving water molecules and functional groups on the surface of the protein fibers.

**Electrical measurements with fully hydrated and aligned BSA mats**

In our first approach to decipher the role of water in proton conduction, we investigated how the matrix alignment can influence the proton conduction across hydrated electrospun BSA mat by changing the protein matrix orientation from having a completely random network to having a well-aligned one. To create a randomly oriented free-standing BSA mat, we performed an electrospinning process using pre-processed BSA protein (details in experimental section) with an aluminum collector. Several studies reported the ability to control the matrix alignment during electrospinning of different polymers, in which one of the straightforward methodologies to achieve such alignment is by inducing a high insulating gap in the conductive collector. As a result, the applied electrostatic field induces the fibers to bridge between the conductive strips. Here, we created fiber alignment by placing a strap of Teflon tape in the center of the aluminum foil collector (**Figure 2a**). To date, few studies have addressed the effect of matrix alignment on the conduction of electrospun materials, all of them using synthetic polymers with moderate water content (<50%). As discussed, the water uptake of the BSA-based electrospun mat (150%) is exceptionally high. We hypothesize that if proton transport solely across the bulk water within the BSA electrospun mat is the predominant mechanism, we expect to observe no differences in the protonic conductivity between the parallel and perpendicular
orientation (or randomly distributed fibers) (Figure 2b). Since the water portion of the hydrated BSA mat is significantly higher than the protein-content itself, it is fair to exclude the formation of discrete water channels within the protein polymer that might influence any differences between the measured conductance of the parallel and perpendicular orientation. On the other hand, if proton transport is taking place along the protein interface with water (involving amino acid side chains), then we expect to see a higher protonic transport efficiency for the parallel orientation as the percolating pathway along the fibers will be shorter in this orientation compared to the perpendicular one (Figure 2b).

Figure 2 – (a) Schematic of the electrospinning setup. (b) Schematic of the BSA mat environment in the electrical measurements of this section, bringing into attention the large amount of water within the hydrated BSA mats, with suggested pathways of proton transport across bulk water vs. the protein-water interface. SEM images of the (c) aligned BSA mat, and (d) random oriented BSA mat. The scale in both images represents 100 μm. Fiber radial distribution calculated for the (e) aligned BSA mat, and (f) random oriented BSA mat.
To confirm fiber alignment and to explore their microstructure, we analyzed the scanning electron microscopy (SEM) images of the aligned mat (such as the one in Figure 2c) in comparison to the SEM images of the randomly oriented mat (Figure 2d). The images verify the generation of fiber alignment when using the modified collector. In terms of the microstructure of the aligned mat, it consists of fibers with a diameter of 1.58±0.29 µm, which is in line to the fiber’s diameter in the randomly oriented mat, albeit the fibers in the aligned mat are denser with distance between fibers of around 1-3 µm. We used ImageJ software to determine the fiber radial distribution in both cases (Figure 2e and 2f). As expected, the regular mat fibers do not show any preferred direction, while most of the aligned mat fibers order at the same angle. To explore the effect of matrix alignment on the mat’s ability to conduct protons we turned to electrochemical impedance spectroscopy (EIS) measurements. For that cause, we repeated the same measurement protocol used for the random BSA mat. First, we soaked the aligned mat in purified water until full hydration. Next, we placed the mat on gold electrodes and dried away the excess of unbound water with tissue paper before measurement (in this stage, the mat contained ~150 wt.% water). Each mat sample was measured in parallel and perpendicular to the fiber direction, simply by rotating the same sample of aligned mat in respect to the electrodes. Figure 3a displays the Nyquist plots (impedance imaginary part as a function of the impedance real part) of the aligned mat in both directions for different electrode separation distances. The curves show a semi-circle and a tail corresponding to the bulk proton conduction and the formation of a double-layer capacitance in the mat-electrode interface. This behavior is evidence of ionic conduction within blocking electrodes. We extracted the mat resistance ($R$) from the EIS data by an equivalent circuit model (Figure 3a, inset). The conductivity ($\sigma$) was calculated considering the mat geometry using: $\sigma = \frac{l}{R \cdot A}$, where $l$ is the electrode separation distance, and $A$ is the cross sectional area (mat width times the mats thickness). Figure 3b summarizes the mat conductivity in both directions for different electrode separation distances, showing that the mat conductivity in fiber direction (parallel) is around two-fold higher than the conductivity perpendicular to the fibers, whereas the latter is in the same order of magnitude to the conductivity measured with a randomly oriented native BSA mat. The higher conductivity of the mat, when measured in a specific direction, indicates the fundamental role of the protein interface in transporting protons through the material. The matrix alignment providing new favored percolating pathways for proton transport and thus enhance the conductivity in the fiber direction. However, this enhancement is slowly diminishing as the distance between the electrodes becomes larger, suggesting that the preferred percolating pathway of the aligned BSA mat becomes less sustainable for very long distances. Interestingly, a similar change in conductivity between the parallel to the fibers orientation and the perpendicular one has been observed also for synthetic ionic conducting polymers with...
moderate water uptake, hence suggesting similar proton transport mechanism albeit the very different water content of the materials.\textsuperscript{27,28}

**Figure 3** – (a) EIS Nyquist plots of the aligned BSA mat in a parallel and perpendicular to the fibers orientations at different electrode separation distances, along with (b) the extracted conductivity values. The inset shows the equivalent circuit used to extract the resistance of the mats.

**Electrical measurements with a dry electrospun network of BSA fibers**

While in the previous section we concluded that the protein-water interface has a fundamental role in supporting proton transport across our fully hydrated protein matrix (i.e., containing bulk water), in this part we intend to directly confront the ability of the protein surface to mediate protons in a ‘water-less’ environment. To resolve it, we performed the electrospinning of the BSA fibers directly onto our electrodes (Figure 4a), while making a layer of fibers network between the electrodes (the inset shows an optical microscope image of the formed fibers network), meaning not a free-standing mat as before. Unlike the previous section, in which we used the hydrated (150 wt.\%) form of the BSA mat, we performed the electrical measurements in this section with the as prepared dry state of the BSA fibers, containing merely 4 wt.\% of tightly bound water molecules to the protein structure,\textsuperscript{8} while slowly increasing the RH values.\textsuperscript{32} Importantly, this low water content is much lower than other reported protein-based polymers.\textsuperscript{5,16} Our hypothesis in this section is that if proton transport across bulk water is the predominant proton conduction mechanism of the BSA mat, we should not detect any conductance of the dry network regardless of the RH, however, if the protein-water interface is the main proton transport relay, we should observe proton conduction even at low RH values which will be RH-dependent (Figure 4b). Our EIS measurements of the formed layer at different RH levels (Figure 4c) exhibited a semi-circle that is attributed to the conduction of the protons through the fibers. We determined the resistance values by applying the same equivalent circuit used for the aligned mat
(Figure 3a, inset). We observed a decrease in the extracted resistance values as a function of increasing the relative humidity levels (Figure 4c, inset). This behavior of elevated conduction upon increasing the RH levels is one of the hallmarks of proton conduction. We further examined the direct current (DC) electrical characteristics of the BSA thin layer. One major drawback of using the Au blocking electrode for measuring protonic conduction is the proton accumulation near the surface of the electrode. Hence, we decided to use here palladium hydride (PdH) electrodes that can inject and accept protons, whereas for each proton injection, an electron will be transferred for the leads: 

$$\text{PdH} \rightarrow \text{Pd} + \text{H}^+ + e^-$$

In this way, protonic current transforms into a measurable electronic current,$^7,17,18,33–36$ resulting also in a significant reduction in the contact resistance between the PdH electrode and the (bio-)material.$^37$ Accordingly, we performed electrospinning of the BSA network layer directly onto palladium electrodes and measured the device I-V characteristics at room humidity (~75%) (Figure 4d). As can be clearly seen in the figure, upon the exposure to hydrogen gas, the current response increased by more than an order of magnitude, hence proving that protons are the charge carriers in the measured current. Overall, the different electrical setups and measurements in this section prove that a ‘completely dry’ protein-based material can mediate long-range proton conduction via the protein surface – water interface, meaning that water molecules are improving the uniformity of the H-bonds network on the surface of the protein (as displayed in Figure 1).
Figure 4 – (a) Schematic of the electrospinning setup. The inset shows an optical microscope image of the BSA fiber monolayer. (b) Schematic of the BSA mat ‘dry’ environment in the electrical measurements of this section, emphasizing the RH-induce formation of hydration shell around the BSA fibers, which facilitating the proton transport on the surface of the protein fiber. (c) Nyquist plot of the BSA network layer at different RH level. The inset shows the extracted resistance values as a function of RH. (d) Current-voltage characteristic of the BSA network layer on palladium-hydride electrodes. The inset shows a zoom-in of the curve before hydrogenating the Pd electrodes.

*Electrical measurements with a drop-casted BSA film*

Till now, we have established that the surface of the BSA fiber, formed in the electrospinning process, is important for mediating protons across our polymers. As mentioned in our introduction, several other proteins have shown proton mediating capabilities,\(^5,7,9\) however, all of these works have used a drop-casted version of the proteins, meaning lacking a microstructure such as in our electrospun fibers.
Accordingly, the last section of our study will serve as a control of comparing our results obtained with the BSA electrospun fibers to a drop-casted BSA film (Figure 5a) with similar geometrical parameters as our electrospun mat. Importantly, drop-casted BSA film are not free-standing as the BSA mat, they do not have a clear microstructure, and do not absorb the vast amount of water as the BSA electrospun mat. As shown in Figure 5b, the measured resistance using EIS is highly RH-dependent, whereas at 90% RH, the calculated conductivity reaches a value of around 0.1 mS/cm, which is about 3 times lower than the conductivity measured with the aligned mat at the parallel configuration for the same distance between electrodes (1 mm). Nevertheless, the most important conclusion from this control experiment is the fundamentally different RH-dependency of the drop-casted BSA film (Figure 5c) to the one measured with the network of the electrospun BSA fibers (Figure 4c). While the decrease in resistance upon going from 60% to 90% RH is 40 folds for the electrospun BSA fibers, for the drop-casted BSA film, it is more than 3 orders of magnitude (~2700 folds) for the same range of RH %. This fundamental difference can be attributed to the microstructure of the electrospun BSA fibers, and accordingly the state of water molecules around it and the mode of proton transport (as shown and discussed in Figure 1). The surface of the BSA fiber is exposed to water molecules and can form an H-bond network between the amino acids on the surface of the BSA fiber to nearby water molecules. We have previously shown that these water state and H-bond network can be interrupted by introducing hydrophobicity to the surface of the BSA fiber.\(^1\) For the electrospun BSA fibers network, the rate-limiting step is the formation of a percolating H-bonds pathway, consists of water molecules and some amino acids of the protein capable to participate in this H-bonds network. Hence, upon forming this network (as illustrated in the schematics of Figure 4b), adding more water to the system by increasing RH is resulting in a moderate decrease in resistance. On the other hand, for the drop-casted BSA film, there is no microstructure, and proton transport can be related to the formation of water channels, assisted by amino acids of the BSA protein. Accordingly, when more water molecules going into the film, they will significantly contribute to the proton conduction efficiency, resulting in the vast dependency of the measured resistance as a function of RH (Figure 5c).
Figure 5 – (a) Schematic of the drop-casting process together with an image of the drop-casted film on the metallic electrodes. (b) Nyquist plot of the drop-casted BSA film at different relative humidity level. The inset shows a zoom-in of the high RH values. (c) The extracted resistance values from the curves in (b) as a function of RH.

Concluding remarks

In this study, we investigated the proton conduction across electrospun BSA fibers, and specifically the role of the protein surface and its interface with water molecules in supporting such protonic conduction. In the first part of our work, we investigated the effect of fiber alignment on the fully hydrated (~150 wt.%) mat conductivity, where we found that the parallel configuration of the aligned mat (compared to the electric field between the electrodes) is around 2-folds more conductive than the perpendicular orientation. This finding suggests that even in the fully hydrated mat, the protein surface and its interface with bulk water are crucial for the proton transport mechanism. In the second part of our study we directly examined if the presence of bulk water is necessary for proton conduction by removing water. To do so, we have used a dry network layer of the BSA electrospun fibers, i.e., not a free-standing mat. We showed that this network can support some protonic conductivity, which is RH-dependent, hence bulk water is not mandatory for the protonic transport. We further proved that protons are indeed the main charge carrier by using proton-transparent hydrogenated Pd electrodes. We compared our results with the electrospun BSA fibers to a control sample of a drop-casted BSA layer. Unlike the BSA mat, a drop-casted BSA film is not free-standing and does not have any microstructure. We showed that the RH-dependency of the BSA drop-casted sample is fundamentally different than the one of the electrospun BSA fibers, and suggesting that while for the BSA electrospun
fibers, the fiber/water interface is the predominant proton transport pathway, for the drop-casted sample, it is water channels that support proton conduction. All in all, the various electrical measurements used here to investigate the role of the protein-water interface in supporting proton conduction indicate that proton transport is taking place on the protein interface with water and not in bulk water. As shown in our illustration in Figure 1, and as suggested by the results of using the dry BSA fiber network, to support the protonic transport across the BSA fibers there is a need of a hydrogen bond network including both amino acids residues as well as some water molecules to bridge between them. Importantly, the ‘dry’ environment of the BSA fiber network has some resemblance to the local environment within natural proton transporting proteins, such as the transmembrane proton shuttling protein transporters. It is well understood that proton transport within these proteins is involving both water molecules and amino acids that can participate in hydrogen bonding, and especially glutamic and aspartic acids. However, while in biological system, this mode of proton transport is taking place for very short distances of <5 nm, and usually across a membrane, we show that it can support very long proton transport on the millimeter scale. Outside of the biological world, the role of proton transport mechanism, and the involvement of bulk water in it, is a vivid line of research in the wide field of proton conducting polymers and ionomers. Accordingly, our new findings on the role of the interface between the proton conducting (bio-)polymer and water in supporting protonic conduction can shed some clues on the general proton transport mechanism of various proton-conducting polymers.

**Experimental Section**

**Electrospinning of BSA mats** - Electrospinning solution was prepared by dissolving BSA (MP Biomedicals) in 90% 2,2,2-trifluoroethanol (Apollo Scientific) for a final BSA concentration of 14% (w/v). After 12 hours, 5% (v/v) β-mercaptoethanol (Alfa Aesar) was added and kept on stirring for 3 hr. Electrospinning was performed in a custom-built system with grounded aluminum collector (for the random oriented mat). A 15 kV bias was applied on a 24-gauge blunt needle with injection rate of 1.5 ml/hour. The needle was fixed 12 cm above the collector. For the randomly oriented mat, the spinning was directly onto the aluminum collector, and the final mat thickness was around 60 μm. To create the aligned mat, a strap of Teflon tape was placed in the middle of the aluminum collector, and in this configuration, the aligned mat final thickness was thinner and around 30 μm. This latter thickness of the aligned mat is an upper limit as thicker mats in this configuration will lose the effect of the
Teflon tape induced orientation of the electric field, and will result in a randomized orientation above this thickness.

**Metal finger electrode fabrication** - Heavily p-doped silicon wafers with SiO<sub>2</sub> dielectric layer (110 nm) and glass microscope slides were used as substrates for the devices used in the DC and impedance measurements, respectively. The substrate was sonicated for 5 min. in the following series of solvents: acetone followed by methanol, isopropanol, and finally ethanol. After the last sonication, silicon substrates were washed with distilled water, and dried on a glass Petri dish heated to 120°C. Glass substrates were washed with distilled water and dried using hot air. Using an E-beam evaporator at a deposition rate of 2 Ås<sup>-1</sup> under 1 Torr at room temperature, 100 nm Au/Pd on 10 nm Cr were evaporated through shadow masks. Mats were placed on finger electrodes and gently dried with filter paper to remove excess water. The water weight percentage within the BSA mat in all electrical experiment was around 150 wt.%.

**Electrospinning of BSA thin layer network** - Similar solution has been used as described above. A 15 kV bias was applied on a 24-gauge blunt needle, 12 cm above the collector, with an injection rate of 1.5 ml/hour for 30 seconds. BSA network was formed on Au electrodes with inter-electrode distance of 1 mm and 2 cm width and Pd electrodes with inter-electrode distance of 100 µm and 2.7 cm width.

**Impedance measurements** - Impedance measurements were performed using an MTZ-35 impedance/gain-phase analyzer (Bio-Logic). The gold electrodes were contacted using a probe station micromanipulator (TP102SV-PS, INSTEC). A 50 mV AC bias was applied during the measurements without DC bias. A frequency range of 10MHz to 10Hz was used. Humidity dependence was studied inside the probe station using a custom-built gas system, where N<sub>2</sub> gas was passed through water to control the humidity.

**DC current-voltage measurements** - DC current-voltage measurements were performed using a source measurement unit (B2901A, Keysight). A BSA thin layer network was deposited on Pd finger electrodes with a distance of 100 µm between electrodes, which were placed inside a probe station (TP102SV-PS, INSTEC). The palladium electrodes were contacted using the probe station micromanipulator. The current was measured as a function of voltage in the range of −1 V to 1 V. To perform palladium hydride measurements, hydrogen gas was injected into the chamber for 30 minutes.

**Drop casted BSA film** – The film was prepared by drop-casting 10% (w/v) BSA aqueous solution onto a gold finger electrode with an electrode separation distance of 1 mm. The layer was then dried in the air for 12 hours to form a film. The final film thickness was around 30-40 µm.

**Conflicts of interest**

The authors declare no conflicts of interest.
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