In situ quantitative visualization and characterization of chitosan electrodeposition with paired sidewall electrodes†

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We report the first in situ quantitative visualization and characterization of electro-induced chitosan hydrogel growth in an aqueous environment. This was enabled with a pair of sidewall electrodes within a transparent fluidic system, which allowed us to resolve the electrogelling mechanism and interpret the dominant causes responsible for the formation and density distribution of the deposited hydrogel. The pH and the time-dependent growth profiles of the chitosan hydrogel were directly visualized, analyzed, and characterized. The results indicate that the gelation and immobilization of chitosan onto the cathode at a pH above its pK_a value (∼6.3) are due to the electrochemically generated concentration gradient of reactant OH^- ions, and their subsequent neutralization of the NH_4^+ groups of chitosan chains in solution near the cathode. The increased gel density around the fringes of the electrodes was demonstrated and correlated with the electrophoretic migration of chitosan cations during deposition. Simulation of the electric potential/field distribution, together with the corresponding dry film topography confirmed the non-uniform, electric field-dependent density distribution of deposited hydrogel. This report provides fundamental understanding towards the mechanism and the kinetics of the electro-induced chitosan gel formation. It also provides important guidelines for pursuing its application in bio-components integrated microsystems. The method in use exemplifies a simple, effective and non-destructive approach for in situ characterization of electro-responsive biopolymers in an aqueous environment.

Introduction

Rapid development of miniaturized bio-electronics and biomedical devices such as bio-microelectromechanical systems (bioMEMS), biosensors, biochips, and lab-on-chip has promoted the integration between soft, organic biological substances and hard, inorganic, micro-fabricated devices. Researchers are constantly seeking candidates as bio-device interfaces which are capable of successfully bridging soft organic biological systems and inorganic solid state materials at the device-level, and which could be assembled simultaneously on demand in response to localized signals.

Chitosan, a unique type of bio-reactive and biocompatible polysaccharide polymer which is responsive to localized electrical signals, serves as an excellent candidate for this purpose. The pH-responsive solubility of chitosan has proven a useful consequence of its pK_a value (∼6.3), arising from the primary amino groups of chitosan. This property allows the chitosan hydrogel to be electro-assembled from an aqueous bulk solution onto a conductive surface in response to cathodic signals. The soluble-insoluble transition point of chitosan gelation is close to the narrow physiological pH range in which most biological substances and physiological systems function optimally. This feature, combined with the nucleophilic property of its primary amine groups and the highly water adsorbent hydrogel format, makes chitosan an ideal carrier for assembling biological molecules and systems onto any conductive surface with three-dimensional spatial control. In fact, chitosan has served as a robust and reproducible scaffold for the assembly of versatile biological components including proteins, nucleic acids, catalytically active enzymes by electrodepositing chitosan onto electrode surfaces. Although there are increasing numbers of reports on fabrication and applications of functional devices or hybrid composites in which chitosan is electro-deposited, the origin of its electro-stimulated deposition and the structure of as-deposited hydrogel in the aqueous format are not well understood. It is generally believed that the electro-induced deposition of chitosan on the cathode falls within the category of electrophoretic deposition and the deposited film is assumed to be uniform, mostly based on the...
electrophoretic study of the chitosan cation and the post-characterization of the dehydrated chitosan film deposited at the cathode surface.

In this report, we investigate the deposition mechanism by direct, quantitative, \textit{in situ} visualization of the deposition process using paired sidewall electrodes in a transparent fluidic channel. This configuration enables a non-destructive way of characterizing the internal conformation of the chitosan hydrogel which is relevant to the physical crosslink density and amount of water adsorbed within the hydrogel structure. The pH profile of the chitosan polyelectrolyte during the deposition was demonstrated by using a universal pH indicator solution. A sharp pH gradient was clearly observed defining the interface between the insoluble hydrogel and soluble chitosan polyelectrolyte. Electrophoretic migration of positively charged, fluorescently tagged chitosan was monitored and the migration paths showed consistency with the simulated electric potential profiles. Time-dependent growth profiles of the electrodeposited hydrogel at different current densities were characterized. Gelation and immobilization of chitosan onto the cathode were observed at a pH above its pK\textsubscript{a} value ($\sim$6.3). Further analysis indicates a non-uniform, electric-field-dependent density distribution of the deposited hydrogel; both the electrophoretic migration of fully protonated chitosan in the bulk solution and partially neutralized chitosan within the gel contribute to the inhomogeneity. From these results we are able to draw some firm conclusions about the role of pH and electrical field during the deposition as well as the density distribution of the wet hydrogels. By revealing the impacts of processing parameters, rational synthesis of complex three-dimensional hydrogel structures with controlled configuration and conformation would be possible. Such technique and method are of significant interest and will be potentially useful in biofabrication and biomedically related applications where the stimuli-responsive gel-forming properties provide unprecedented opportunities to integrate biology into devices for point of care diagnosis, high throughput screening and potentially even implantable devices.

Results and discussion

\textbf{Sidewall electrodes in transparent fluidic channel}

For direct visualization of the electrodeposition process of chitosan, we employ an all-transparent microfluidic channel system with integrated sidewall electrodes. Fig. 1a shows the schematic three-dimensional view of the device structure. It consists of two thin layers of polydimethylsiloxane (PDMS) as the bottom and ceiling of the fluidic channel and two glass slides as the sidewalls. Gold stripes on the channel sidewalls were patterned as the sidewall electrodes by angled thermal evaporation with a shadow mask. The channel is 1 mm high, 1 mm wide and 1 inch long with an active sidewall electrode area in the fluidic channel of 1 mm $\times$ 1 mm. This paired sidewall electrode system enables unprecedented \textit{in situ} visualization and further characterization of chitosan electrodeposition.

To record the time-dependent evolution of chitosan electrodeposition, a CCD camera attached to an optical microscope with transmitted light from the bottom was used. The microscope lens located above the device is focused on the electrodes and electrolyte area within the channel. Such a setup allows us to observe transmitted light intensity change due to sol–gel transition during electrodeposition.

To simultaneously visualize the pH profile of the electrolyte during electrodeposition, we used a pH indicator solution whose color varies with different pH levels. The color–pH relationship is obtained by analyzing the color of buffers at different pH values within the fluidic system under experimental conditions. Prior to using the system for chitosan electrodeposition, we verify its viability by testing the hydrolysis of water. In order to clearly demonstrate the pH distribution, we mix pH indicator with 100 mM PBS buffer at pH 7 and then apply an electrical signal to it. Fig. 1b is an optical image taken after 85 seconds of constant current density of 4 A m$^{-2}$ applied between the electrodes, indicating a pH gradient spreading from acidic (pH 4) at the anode electrode (right) to basic (pH 10) at the cathode electrode (left). Different pH contours (from 10 to 4) are indicated by white solid lines and corresponding pH values are labeled by white numbers. We can clearly see the influence of steady proton and hydroxide ions fluxes and resulting concentration changes primarily due to the electrochemical reduction and oxidation that take place at the cathode and anode because of their electrochemistry:

\begin{align*}
\text{cathode:} & \quad 2\text{H}_2\text{O} + 2e^- \rightarrow \text{H}_2 + 2\text{OH}^- \quad (1) \\
\text{anode:} & \quad 2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{H}^+ + 4e^- \quad (2)
\end{align*}

\textbf{In situ visualization, pH mapping and mechanism of chitosan electrodeposition}

With the capability of \textit{in situ} visualization using our microfluidic system, we then characterize the electrodeposition of chitosan hydrogels. Acidic chitosan polyelectrolyte solution is prepared by dissolving chitosan powder with HCl and DI water (details in
The fluidic system was separately filled with static acidic chitosan solution and a mixture of chitosan solution with pH indicator for characterizing the hydrogel and pH profile of the polyelectrolyte during electrodeposition. Optical micrographs of chitosan solution during electrodeposition were recorded at time frames of 85, 130, and 175 seconds at a current density of 4 A m\(^{-2}\). Images of chitosan hydrogel while the chitosan solution was transparent. For the growth profiles without pH indicator, we observed a light brown color of neutralized chitosan hydrogel while the chitosan solution was transparent. 

Because these microscopic images were taken with transmitted light from the bottom, the decreased transparency of the chitosan hydrogel is most likely due to light scattering through the physically cross-linked chitosan hydrogel or chitosan crystal domains that have been observed in hydrated chitosan films. This scattering randomizes the direction of the incoming light and therefore fewer photons are detected by the CCD image sensor.

With pH indicator, acidic chitosan polyelectrolyte appears pink (pH 5), and the deposited hydrogel exhibits a purple color (pH 10) indicating a basic environment. A sharp pH gradient (less than 40 \(\mu\)m wide, as shown in the inset of Fig. 2f), where pH changes dramatically (Fig. 2g), separates the hydrogel from the bulk solution. A similar sharp, localized pH gradient has also been observed at the interface between flowing chitosan and basic buffer solutions in a recent report on the fabrication of a chitosan hydrogel membrane in microfluidic devices.

Based on the fact that the location of the sharp pH gradient matches the front of chitosan hydrogel and they both propagate away from the cathode surface, we conclude that the rapid growth of the hydrogel is due to the electrochemically generated OH\(^-\) ions at the cathode. Our conclusion that the electrochemically generated pH gradient is responsible for chitosan electrodeposition is consistent with previous electrochemical studies that showed a correlation between chitosan deposition and water electrolysis. The generation of OH\(^-\) at the cathode is believed to neutralize chitosan and induce its localized sol–gel transition as indicated by the reaction:

\[
\text{Chit-NH}_2^+ + \text{OH}^- \rightarrow \text{Chit-NH}_3^+ + \text{H}_2\text{O}
\]

Therefore, the cathodic gelation and immobilization of chitosan under an electric stimulus can be explained as follows. Chitosan molecules in the bulk solution are neutralized by charge transfer from the electrochemically generated OH\(^-\) anions. As the OH\(^-\) ions generated at the cathode surface continue diffusing to the neutralization front to compensate for the dramatic decrease in OH\(^-\) concentration there, they cause further neutralization and immobilization of chitosan molecules from solution. Consequently, the gel front propagates into the bulk chitosan electrolyte solution. Expansion of the pH gradient at the gel–solution interface is suppressed due to the buffering effect of the chitosan neutralization. A schematic figure and detailed discussion can be found in the ESI (Fig. S1†). Electrophoretic migration also contributes to chitosan’s electrodeposition (see below) but it is not the determining factor responsible for massive gelation. Moreover, the higher mobility of the hydroxyl ion as compared to that of the chitosan cation makes an explanation of gelation on the basis of electrophoretic deposition even more improbable. In summary, the electro-induced continuous gelation of chitosan is attributed to the elevated pH which results from the electrochemical generation of OH\(^-\) at the cathode. 

Chitosan’s conformation and its interactions are pH dependent. At low pH, chitosan’s amines are protonated, the polymer has a high charge density and electrostatic repulsions between monomeric units tend to stiffen the chain leading to a rigid rod conformation. At higher pH, the amines become de-protonated and ionic repulsions are reduced thus allowing the individual chains to collapse and also allowing associations between
chains through attractive interactions (e.g., hydrogen bonding and hydrophobic interactions). These inter-chain associations form the physical crosslinks responsible for hydrogel formation. Interestingly, de-protonation does not need to be complete for gels to form and it has been reported that gels can form when nearly 40% NH$_3^+$ are present. Our observed pH gradient indicates that a dramatic change in charge density occurs over a very short distance (less than 40 microns). This change in charge density also indicates that chains in the narrow interfacial region experience considerable differences in electrostatic force exerted by the applied electric field. Finally, this narrow interfacial region also separates the solution from the gel and thus there are dramatic differences in chain mobility across this interface.

**Dimensional characterization of electrodeposited hydrogel**

With the advantage of direct, in situ optical visualization of the chitosan hydrogel distinguished from the solution state, we then characterize the impacts of electric current density on the dimensions of deposited hydrogel. Fig. 3a–c show the time dependence of the volume (Fig. 3a), the middle thickness (Fig. 3b) and the lateral expanding thickness exceeding the electrode edge (Fig. 3c) of the as-deposited chitosan hydrogel. The applied current density ranges from 1 A m$^{-2}$ (top) to 10 A m$^{-2}$ (bottom) with a step of 1 A m$^{-2}$. Fig. 3a shows a roughly linear dependence of hydrogel volume on the deposition time as well as on the current density. The current density dictates the rate of OH$^-$ ions generated at the cathode assuming the conversion rate of OH$^-$ at cathode is uniform, while the deposition time and the current density together determine the overall number of electrons passing through the cathode. Therefore, for a given amount of time, a higher current density leads to more neutralized chitosan molecules as more OH$^-$ ions are generated at the cathode. Accordingly, we observe proportional growth rate increases of the chitosan hydrogel volume, middle thickness, and lateral thickness with increased current density. The slight decrease in growth rate of the hydrogel middle thickness (Fig. 3b) after 100 s is caused by the depletion of positively charged chitosan molecules in the channel between the cathode and anode, since most of the protonated chitosan molecules migrated towards the cathode and were neutralized. On the other hand, the hydrogel lateral thickness (Fig. 3c) maintains a linear time dependence, as the fluidic channel is relatively long and protonated chitosan molecules are abundant.

To test the feasibility of our assumption, we compare the number of electrons passing through the cathode ($\Delta N_e = 2.5 \times 10^{13}$ s$^{-1}$) and the number of NH$_3^+$ sites being neutralized ($\Delta N_{NH_3} = 2.68 \times 10^{13}$ s$^{-1}$) per unit time in our system (details in ESI†). The fact that $\Delta N_e$ and $\Delta N_{NH_3}$ agree within 10% indicates a plausible hypothesis for the electro-induced chitosan hydrogel growth mechanism. Moreover, the case that the latter is slightly larger (7%) than the former suggests that the chitosan molecules are not fully neutralized within the hydrogel—about 7% of amine sites remain positively charged. This supports our picture of the pH-dependent ionization level of chitosan (protonation level of the amine groups) depicted in Fig. 2h. Furthermore, such a result is consistent with the observed limited migration of chitosan molecules within the gel after initial neutralization (Video S1†), which is critical for the formation of the electric field dependent density distribution of the electrodeposited hydrogel.

**Electrophoretic migration**

Historically, the cathodic electrodeposition of chitosan has been attributed to electrophoretic deposition since any charged colloidal particles which are capable of forming stable suspension can be used in electrophoretic deposition. However, the results which follow demonstrate that the electrophoretic migration of the chitosan cations is responsible for the density distribution rather than the gelation of deposited hydrogel.

To visualize the electrophoretic migration of chitosan in bulk polyelectrolyte solution, we fluorescently tagged the chitosan molecules and observed their movement using fluorescence microscopy. Fig. 4a is a fluorescence micrograph illustrating the distribution of NHS–rhodamine tagged chitosan molecules after 115 seconds of electrodeposition at 4 A m$^{-2}$. NHS–rhodamine was used as the fluorescent label for its excellent photostability in a wide range of pH levels, even in acidic environments. From the fluorescence image (Fig. 4a) and corresponding fluorescence intensity surface plot (Fig. 4b) it is apparent that positively charged chitosan molecules are repelled away from the anode which results in a half-oval dark area centered at the anode, while at the cathode, chitosan molecules are gathered, neutralized, physically crosslinked, and reorganized, leading to a highlighted half-oval shaped hydrogel (Videos S2 and S2†). The migration of positively charged chitosan macromolecules here is governed by Nernst–Planck equation: the flux of any charged molecule is driven simultaneously by the influence of both an ionic concentration gradient and an electric field (details in ESI†). Although the presence of chitosan over most of the channel width demonstrates the role of a diffusion gradient impeding electrophoretic migration, the motion of the molecules is primarily governed by electrostatic forces which enable and drive chitosan deposition on the electrode.
A volumetric shrinkage induced by chitosan chain collapse, from solution to hydrogel, leads to a temporary, narrow depletion region (interphase layer) of chitosan molecules at the interface between the neutralization front and the bulk solution. Such a transition interphase layer is observed in Fig. 4a as a very thin, half-oval, dark zone between the fluorescently highlighted chitosan hydrogel and the polyelectrolyte solution.

To evaluate the dominant mechanism behind the migration, we simulate the electric potential mapping with experimental conditions (voltage reading between cathode and anode is about 2 V under 4 A m$^{-2}$ current density) using COMSOL Multiphysics (Fig. 4c). The simulation is carried out by setting the potential 0 V on the cathode and 2 V on the anode (or by setting the current density to 4 A m$^{-2}$ and the electric conductivity of the electrolyte to 1.52 $\times$ 10$^{-4}$ S m$^{-1}$). It is obvious that the experimental migration pattern is geometrically similar to the contour of simulated equipotential lines, supporting our conclusion that the movement of the chitosan is dominated by their electrophoretic migration.

**Electric field (electrostatic force) induced density distribution**

To further address the impact of electric field on as-deposited hydrogel properties, we examined both optical and fluorescence images captured during electrodeposition. Fig. 5a shows a captured bright field optical image with transmitted light from the bottom after 120 seconds of electrodeposition at a 6 A m$^{-2}$ current density. A highly non-uniform transmitted light intensity (TLI) distribution of the hydrogel indicates an inhomogeneous density allocation (Video S1†). The reduction of the TLI reflects increase in chitosan mass per unit volume. Therefore lowest TLI is observed at dimmest area where adsorption of transmitted light by chitosan reaches maximum. Fig. 5b and c are the 2D and 3D TLI surface plots of the area marked with a yellow dotted line in Fig. 5a, with colors representing TLI level (red represents lowest TLI, indicating a maximum light adsorption at highest gel density while purple represents the opposite). The Z axis direction is inverted here for easy comparison with the fluorescence intensity plots. We can clearly see a mountain-like shape of the TLI distribution within the gel with two dimmest spots at the edge of the cathode where the maximum electric field occurs (Video S1†). This morphology can be explained by the high electric field values near the surface of the cathode, enhanced at the edge of the cathode (fringing effect) where charged chitosan molecules should experience greater electrostatic forces. Second, during initial gelation, partially charged chitosan molecules are not fully de-protonated and are still subject to electrostatic forces from the electric field, resulting in chain drifting and reorganization towards the highest electric field point, where chitosan chains are further neutralized and the condensation starts to
reach maximum. This leads to hydrogels with a higher physical cross-linking density due to pH dependent interactions between deposited chitosan layers.\textsuperscript{36}

To examine and confirm the inhomogeneity of the deposited hydrogel, we also employed NHS–fluorescein labeled chitosan. Fig. 5d represents a captured fluorescence micrograph at 120 seconds of electrodeposition at a 6 A m\(^{-2}\) current density (same as in Fig. 5a) with a white dashed rectangle indicating the position of the cathode. Fig. 5e and f are the 2D and 3D fluorescence intensity surface plots of the marked area in Fig. 5d (yellow dotted rectangle). The analysis shows similar inhomogeneous density distribution as in Fig. 5a, indicating a genuine field dependent density distribution of the electrodeposited chitosan hydrogel. Fig. 5g and h exhibit a simulated electrical field profile between two electrodes using COMSOL Multiphysics software. The similarity among the electric field distribution, wet hydrogel density distribution, and the dry film topography (details in ESI†) demonstrates a causal relationship between electric field and the hydrogel density.

Profilometry studies of the corresponding dry chitosan film (4 A m\(^{-2}\) deposition for 85 s) allowed us to estimate the volume ratio of the chitosan hydrogel before and after dehydration. Together with the analysis of transmitted light intensity and fluorescence intensity of the hydrogel compared with bulk solution, we confirmed the hydrogel contains about 0.7% (w/v) chitosan and over 99% water after 85 s deposition at a 4 A m\(^{-2}\) current density (details in ESI†).

**Experimental**

The sidewall electrodes and leads were defined by angled thermal evaporation of Cr (10 nm) and Au (120 nm) onto a tilted glass slide using a bent stainless steel shadow mask with 1 mm wide slits. Oxygen plasma was used to bond a pair of patterned glass slides onto PDMS (Dow Corning). 0.5% (w/v) chitosan solutions were prepared by dissolving chitosan flakes (Sigma-Aldrich, 85% deacetylated) in HCl and distilled water till pH 3 and titrated dropwise to pH 5.3 by adding NaOH.\textsuperscript{4} NHS–fluorescein and NHS–rhodamine (Thermo Scientific) were separately mixed with acetic acid (pH 4–5), monobasic sodium phosphate (pH 6–7), tris-hydroxymethylaminomethane (pH 8–9), and bicarbonate (pH 10) buffers to prepare chitosan solutions. The pH of chitosan solution was obtained by comparing the RGB values of chitosan and over 99% water after 85 s deposition at a 4 A m\(^{-2}\) current density (details in ESI†).

**Conclusion**

To conclude, we have directly visualized and systematically characterized the electrodeposition of chitosan hydrogels using paired sidewall electrodes in a transparent fluidic channel. The pH, geometric and density profiles of the hydrogel during deposition were studied, resulting in our conclusion that the boundary of the hydrogel is controlled by the migration of electrochemically generated OH\(^-\) ions, while the density distribution of the hydrogel is determined by the electrophoretic migration of chitosan (i.e., the pH-dependent charge density together with the local electric field). These experimental results contribute fundamental understanding towards the unique ionization, conformation, and solubility characteristics of chitosan and provide essential guidelines for creating, controlling, and optimizing the complex polymeric structures for biofabrication, biofunctionalization, and biocomponent assembly with electro-addressing ability in miniature devices.

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**References**

26 Here the light blue backgrounds of Fig. 2a–c are due to the white balance settings (1.67 for cyan/red, 1.09 for magenta/green, and 0.23 for yellow/blue) of AxioVision imaging software during images capturing. The settings remain the same for all the optical images reported in this paper.