Biopolymer-Induced Calcium Phosphate Scaling in Membrane-Based Water Treatment Systems: Langmuir Model Films Studies

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Abstract

Biofouling and scaling on reverse osmosis (RO) or nanofiltration (NF) membranes during desalination of secondary and tertiary effluents pose an obstacle that limits the reuse of wastewater. In this study we explored the mineral scaling induced by biopolymers originated from bacterial biofilms: bovine serum albumin (BSA), fibrinogen, lysozyme and alginic acid, as well as an extracts of extracellular polymeric substances (EPS) from bio-fouled RO membranes from wastewater treatment facility. Mineralization studies were performed on Langmuir films of the biopolymers deposited at the interface of a solution simulating RO desalination of secondary-treated wastewater effluents. All studied biopolymers and EPS induced heterogeneous mineralization of mainly calcium phosphate. Using IR spectroscopy coupled with systematic quantitative analysis of the surface pressure versus molecular-area isotherms, we determined the mineralization tendencies of the biopolymers to be in the order of: fibrinogen > lysozyme > BSA > alginic acid. The biopolymers and EPS studied here were found to be accelerators of calcium-phosphate mineralization. This study demonstrates the utilization of Langmuir surface-pressure area isotherms and a model solution in quantitatively assessing the mineralization tendencies of various molecular components of EPS in context of membrane-based water treatment systems.

Keywords: Calcium phosphate mineralization; biofilm; surface pressure-molecular area isotherms; biofouling; reverse osmosis; desalination.
1. Introduction

The reuse of treated municipal wastewater effluents is no longer a matter of choice in many parts of the world, where membrane-based water technologies play a key role. While enormous advances in membrane-based technologies have been achieved, fouling and scaling of the membranes are still unavoidable and pose obstacles for further advancement and application. Fouling is the accumulation by adsorption, adhesion or precipitation of solids, or build-up of gel-like deposits on the membrane surface. There are four major types of fouling: scaling of sparingly soluble salts; organic fouling by natural organic matter such as humic acid; particulate or colloidal fouling [1-3]; and biofouling - where microorganisms attach to the membrane and excrete extra-cellular polymeric substances (EPS), forming biofilms. The EPS, which includes polysaccharides, proteins, lipids, humic acids, as well as RNA and DNA, provides a nourishing and protective environment for microorganisms that are embedded in the biofilm [4].

Scaling by calcium phosphate minerals during desalination of secondary and tertiary effluents by reverse osmosis (RO) is considered a limiting factor for improving the yield of the process. This is due to the relatively high concentration of phosphate in domestic wastewater effluents, which can reach up to 6 mg/L [5]. Another reason is lack of appropriate anti-scalants, as existing commercial anti-scalants have thus far failed to prevent calcium phosphate scaling [5]. In the wastewater desalination site in Orange County, CA, microfiltration (MF) / ultrafiltration (UF) technologies were used as pretreatment instead of the traditional pretreatment procedure by acidification. However, these technologies did not prove successful in preventing scaling and fouling of the RO membrane. Calcium phosphate together with organic matter accumulated on the RO membrane; suggesting that nanoparticles or smaller colloids pass through microfiltration and ultrafiltration membranes and end up on RO membranes as cake-layer foulants [6].

The influence of organic fouling on scaling was also reported for calcium sulfate; scaling of CaSO₄ was enhanced in the presence of biofilm on RO/NF membranes as was evident by micron size crystals covering the surface [7]. In addition, a rapid flux decline was observed in nanofiltration (NF) membranes from natural waters in the presence of divalent cations due to binding of ions to humic carboxylic functional groups. This binding led to a reduction of the humic macro-molecules’ charge, eventually resulting in a decrease in the repulsion between the macro-molecules, which led to a denser fouling layer [8-9].
Scaling in biofilms resembles biomineralization in nature, as both processes involve macromolecules that induce the adsorption and precipitation of mineralizing ions, in addition to influencing the kinetics, size and phases of crystals obtained in these complex systems. Langmuir films studied by surface-pressure versus molecular-area ($\pi$-A) isotherms have been widely employed for monitoring early stages of mineralization induced by specific molecular systems [10].

A Langmuir ($\pi$-A) isotherm system was previously used by our group to study the influence of organic chemical groups on calcium phosphate mineralization in a model solution with an ionic profile simulating secondary-treated wastewater effluents (denoted SSE solution). Mineralization was induced by organic functional groups to varying extents, according to the following order: -PO$_4$H$_2$ > -COOH ~ -NH$_2$ > -COOH : -NH$_2$ (1:1) > -OH > -ethylene glycol [11]. Another study demonstrated through small-angle neutron scattering that proteins (BSA and lysozyme) in bulk SSE solution led to the buildup of protein-mineral particles [12]. Small angle neutron scattering was also used for studying the influence of BSA-grafted gold nanoparticles (BSA-GNPs) on calcium phosphate mineralization from SSE solution. The BSA-GNPs exposed to SSE solution induced immediate mineralization which led to stable composite particles (organic/inorganic materials) of about 0.2 $\mu$m diameter, and a mineral volume fraction between 50 and 80% [13].

Here we studied the influence of several biomolecules: BSA, fibrinogen, lysozyme, and alginic acid [14-16] - representing biofilm components, as well as EPS extract, on calcium phosphate mineralization in Langmuir films at the interface of a SSE solution. The effects of the mineralization induced by these films were monitored by surface pressure-area isotherms, Brewster angle microscopy (BAM) and polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS).

2. Material and Methods

2.1 Chemicals

Bovine serum albumin (BSA) 96% purified by electrophoresis (MW = 66.6 kDa), fibrinogen (fraction I, type I-S, 4% carbohydrate content according to manufacturer information) from bovine plasma (MW = 340 kDa), alginic acid from brown algae (MW of 16.6 kDa as determined
by micro-viscosity measurements and 24 kDa by SANS measurements; see description of molecular weight determination in Appendix A), lysozyme from chicken egg white (MW=14.3 kDa), 1-pentanol ≥ 99%, 1,4-piperazinediethanesulfonic acid (PIPES), potassium dihydrogen phosphate, phenol, and sulphuric acid were purchased from Sigma Aldrich (St. Louis, MO). Sodium chloride, sodium sulfate, and magnesium chloride hexahydrate were purchased from Frutarom (Haifa, Israel). Sodium bicarbonate and hydrochloric acid were purchased from Bio-Lab (Jerusalem, Israel). Calcium chloride dehydrate was purchased from Carlo Erba Reagents (Rodano, Italy). Ultrapure water (resistivity 18.1 MΩ·cm) was used for all purposes. Bradford reagent was purchased from Bio-Rad (Jerusalem, Israel).

2.2 Preparation of SSE solution
SSE solution simulating effluents from Shafdan plant in Tel-Aviv region wastewater reclamation plant [11,17] at a stage of 80% desalination was prepared as previously described [12] by dissolving 17.1 mg of KH$_2$PO$_4$, 460 mg NaCl, 2.5 mL of 1M HCl, 1370 mg of MgCl$_2$·6H$_2$O, 1645 mg of CaCl$_2$·H$_2$O, and 663.5 mg of Na$_2$SO$_4$ in 500 mL of water. Then, 835 mg of NaHCO$_3$ were dissolved in 50 mL of water and added slowly to the mixture. Finally 20 mL of 0.5 M PIPES buffer was added. The final volume of the solution was completed with water to 1 liter and the pH of the solution was 6.9. The solution was filtered through a 0.22µm PVDF filter and stored up to 10 days at room temperature. A phosphate deprived SSE solution was prepared similarly but with sodium chloride (25.6 mg) instead of the 17.1 mg of KH$_2$PO$_4$, compensating for the ionic strength of the phosphate salt; the pH was measured and if needed adjusted to 6.9 during preparation of the solution after dissolving all salts, just before final volume completion.

2.3 EPS extraction and composition
EPS was extracted from a microbial biofilm developed on a fouled RO membrane after desalination of ultrafiltration permeate effluents of a membrane bioreactor (MBR) [18-19]. EPS is usually characterized by the weight ratios of its polysaccharides, proteins, nucleic acids, lipids, and other polymeric substances [20-21]. EPS composition was determined by a liquid chromatography–organic carbon detection (LC-OCD) system (model 8, DOC Labor manufactured by Dr. Stefan Huber, Karlsruhe, Germany)
with a gel-permeation chromatographic column, multidetection with UV absorbance at 254 nm [22]. Organic carbon and organic nitrogen detection were used to determine the amounts of dissolved organic carbon (DOC) of EPS fractions extracted from the fouled membrane (see more details in Appendix B). According to LC-OCD the EPS comprised 5.8% biopolymers (polysaccharides, proteins, amino-sugars), 56.6% humic substances, 17.3% building blocks (breakdown products of humics) and 17.3% low molecular weight neutrals (mono- and oligosaccharides, alcohols, aldehydes, ketones). The detailed analysis of EPS by LC-OCD is given in Table B.1, Appendix B. The EPS was also analyzed by colorimetric methods to determine proteins and polysaccharides content, which is described in Appendix B.

2.4 Langmuir- (π-A) isotherms

Surface pressure versus molecular-area isotherms were conducted in a mini-trough (KVS Instruments, Helsinki, Finland). Biomolecules were first dissolved in a spreading solvent and then dispersed using a syringe on the interface in a fully open trough, area = 250 cm². The deposited film was then left for 30 minutes to equilibrate at the interface, allowing the solvent to evaporate and molecules to equilibrate on the interface. Changes in surface pressure were measured by a Wilhelmy plate, as a function of nominal area per molecule, determined by the amount of material residing at the interface, and the available area that is controlled by two barriers. Films were compressed at a constant rate of 5 mm/min [23]. To study mineralization induced by the film at the interface, isotherms were recorded for monolayers formed over deionized water, SSE, and for a film first incubated for 18 hours over SSE and then compressed. Visible mineralized films on the surface were collected with a spatula. The accumulated material was acidified with 70% analytical grade nitric acid, and analyzed for elemental composition with Varian 720-ES-ICP optical emission spectrometer (Varian, Inc., Walnut Creek, CA) measuring with standard deviation of 2.6 % at confidence level of 99%.

BSA solution (pI 4.7, 583 amino acids) was prepared by dissolving 1 mg/mL of the protein in water containing 0.05% (v/v) 1-pentanol to improve the spreading process, following the procedure by Xue et al. [24] The BSA monolayer at the air-liquid interface was formed by spreading 13 µl of this solution over water or SSE. Fibrinogen (pI = 5.8, 225 amino acids) monolayers were prepared following the procedure of Sankaranarayanan et al. [25] Briefly,
fibrinogen was dissolved in phosphate buffer (pH = 7.5) at a concentration of 1 mg/mL, and then 9 µl were spread over the subphase. Lysozyme monolayers were formed following the procedure of Thakur et al. [26]. Lysozyme (pI = 11.3, 163 amino acids) was dissolved in water at concentration of 1 mg/mL and then 110 µl were spread on the subphase. Alginic acid (0.1 mg/mL) was dissolved in water at pH = 8 then 1 mL was spread over the subphase. As all these compounds could have become dissolved in the subphase during spreading, the amount of material residing at the interface after deposition was determined (described below). The extracted EPS was dissolved in water (380 µg/mL) and 200 µl of this solution were spread on the subphase. Since EPS is composed of different substances, the exact composition of which are not known, the isotherms of this film were plotted as a function of the trough area rather than the mean molecular area.

The Langmuir films were compressed after 30 minutes of equilibration at the interface, taken as time zero, or following 18 hours of incubation at the most expanded state of the film. All experiments were conducted at room temperature. The surface pressure versus area per molecule measurements were conducted so to start at low or close to zero surface pressure at the "gaseous-phase" region of the isotherm. Compression of the film leads to an increase in surface pressure and to the appearance of condensed phases along the isotherm that are characterized by different slopes, hence compressibility values (see equation 1). The isotherms were analyzed with respect to the following characteristics: the area per molecule corresponding to the onset in surface pressure, that is the area per molecule at which transition from zero surface pressure to the first increase of 0.5 mN/m. The next region denoted was the compressed state, characterized by a steep increase in surface pressure in which the molecules become closely packed. This limiting area was calculated from the intersection of a tangent line to the compressed state of the isotherm at its steepest slope, with the axis of abscissa as will be illustrated in Figure 3A. The steep increase in surface pressure is described by the minimum compressibility value (k) along this region, i.e. the normalized change of mean molecular area A divided by the change in surface pressure, π:

\[ k = - \frac{1}{A} \frac{\Delta A}{\Delta \pi} \]  

(1)

Where A is the mean molecular area occupied by the biopolymer, ΔA is the change in mean molecular area, Δπ is the change in surface pressure, (ΔA/Δπ) is the temperature dependent
inverse of the isotherm's slope at the region around the limiting area per molecule, \( T \) is the
temperature. Following the compressed state the surface pressure may level off whereby the
monolayer collapses into a multilayered thick film.

The mineralization tendencies of the various biopolymers were compared by a value denoted as
\( \Delta \pi_5 \), which was calculated for each mineralized film. This value stands for the increase in surface
pressure at the end of 18 hours of incubation, at the area per molecule where the film on SSE at
time zero shows 5 mN/m (a value selected arbitrarily), as illustrated in Figure 3A. Furthermore,
we defined \( \overline{\Delta \pi}_5 \) as normalized \( \Delta \pi_5 \) (Table 2), that is \( \Delta \pi_5 \) divided by the surface density of the
biopolymers at the interface. The surface density was calculated by dividing the amount of
biopolymer residing at the interface by the surface area of the trough. Noteworthy, comparison
between \( \Delta \pi_5 \) and \( \overline{\Delta \pi}_5 \) of the different biopolymers is relying on the consideration that the
compressibility of the films in this region is similar.

2.5 Polarization Modulated Infrared Reflection Adsorption Spectroscopy (PM-IRRAS)

IR measurements of the various biopolymers and EPS over SSE solution were performed at time
zero and after 18 hours of mineralization using a Nicolet 6700 spectrophotometer (Thermo,
Madison, WI) coupled with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector.
The detector angle at which the measurements were done was set to 72°, while the incident
infrared beam was polarized by a ZnSe polarizer and modulated by a ZnSe photoelastic
modulator between parallel (p) and perpendicular (s) polarizations to the plane of incidence. For
each spectrum, 1000 scans were collected at a modulation frequency of 1600 cm\(^{-1}\) and at a
resolution of 8 cm\(^{-1}\). The resultant signal is the differential reflectivity spectrum \( \Delta R/R = (R_p-R_s)/(R_p+R_s) \),
where \( R_p \) and \( R_s \) are the polarized reflectivities of the plane of incidence. The
contribution of the SSE solution was taken into account by dividing each spectrum by the
spectrum of SSE solution alone.

2.6 Estimation of the amount of bio-polymers at the solution-air interface

An estimation of the amount of bio-polymers remaining at the interface of SSE solution
following film deposition was conducted in Teflon® mini-troughs (cylindrical shape of 6.8 cm
diameter and 1 cm height, volume 36 mL). Each trough was placed in a petri-dish and filled with
the SSE solution. Then specific volumes of each of the bio-polymers were spread to form a
monolayer film. Following an equilibration period of 30 minutes, 2.5 mL of chloroform were
injected directly to the bottom of the mini-troughs forming a separate phase, without mixing with
the aqueous solution. The chloroform layer pushed ~2.5 mL of the upper layer out, presumably
with the interfacial film, into the petri-dish.
Quantification of BSA, fibrinogen, and lysozyme in the overflown samples were analyzed using
the Bradford assay [27] according to the following procedure: 200 µl of the overflown protein
solution were added to 800 µl of Bradford reagent (100 ppm of coomassie brilliant blue G-250 in
1 M PBS), the mixture was vortexed, and after 5 minutes absorbance was measured with a
spectrophotometer at λ= 595 nm. The concentration was then deduced using a calibration curve.
In the case of alginic acid, the whole subphase excluding the overflown sample from the
interface part was collected and alginic acid was quantified as follows: the subphase solution was
concentrated to 2.5 mL by evaporating the water at 90°C. Alginic acid was determined in the
concentrated solution by the phenol-sulphuric-acid method: 200 µl of 5% (w/v) phenol were
added to 200 µl of sample, the mixture was vortexed, then 1 mL of concentrated sulphuric acid
was added, after one hour absorbance of the samples was measured at λ= 490 nm [28] and
compared to a calibration curve.

2.7 Brewster Angle Microscopy (BAM)
A Brewster angle microscope (EP3SW-BAM, NFT, Göttingen, Germany) mounted on Langmuir
trough was used for in situ visualization at the interface. The light source of the BAM was a
frequency-doubled Nd:YAG laser with a wavelength of 532 nm and 50 mW primary output
power in a collimated beam. The BAM images were recorded with a CCD camera. The samples
were scanned with a Nikon super long working distance objective with nominal 10×
magnification and a diffraction-limited lateral resolution of 1 μm. The images were corrected to
eliminate side ratio distortion originating from the microscope’s non-perpendicular line of vision.
All images of the Langmuir films were taken without compressing the films either at time zero or
after 18 hours of incubation. The gray levels correlate with film thickness, with brighter
reflections indicative of a thicker film.
3. Results

3.1 Mineralization studies monitored by BAM on Langmuir trough

Mineralization experiments were performed in a Langmuir trough and visualized by BAM. Films of the four biopolymer foulant-model molecules BSA, fibrinogen, lysozyme, alginic acid and the EPS over water, showed no visible features (data not shown), whereas over SSE - adsorbed material was observed at the interface at time zero (Figure 1). Images of the same films taken after 18 hours of incubation over the SSE, showed a more condensed substance, pointing to continuous accumulation of ions at the interface. In particular, the ion accumulation in the presence of lysozyme and alginic acid which was hardly detectable by BAM at time zero over the SSE, appeared to be similar in density to the other studied bio-polymers and the EPS, after 18 hours incubation.

3.2 IR spectra of the mineralized film at solution-air interface

PM-IRRAS spectra were measured after 18 hours incubation on SSE solution (Figure 2A). The most intense peaks in the region 900-1200 cm\(^{-1}\) may be attributed to phosphate absorption, in both octacalcium phosphate and hydroxyapatite [29-30] phases. The peak at around 1460 cm\(^{-1}\) is associated with the asymmetric stretch of the carbonate \(-\text{CO}_3^{2-}\) group which indicates the concurrent formation of calcium carbonate [31-32] or the replacement of phosphate and/or hydroxide groups in hydroxyapatite by carbonate [33-34]. In a control experiment BSA film over phosphate-deprived-SSE solution (Figure 2A) showed no peaks at all, hence lack of mineralization, pointing to the importance of phosphate ions in the process. The PM-IRRAS measurements could not be performed at longer incubation durations due to evaporation of the subphase.

The PM-IRRAS spectrum of the EPS film incubated over SSE for 18 hours was somewhat different from those of the other biopolymers, with a broad and relatively weak peak between 750 and 1200 cm\(^{-1}\) (Figure 2B). This peak can be attributed to a combination of hydrogen phosphates in addition to octacalcium phosphate and hydroxyapatite [35]. This finding implies
that the EPS induces mineralization of the calcium-phosphate minerals at a lower extent and of less stable phases compared to the pure biopolymer films. Nonetheless, it was previously shown that calcium-phosphate minerals tend to convert to the more thermodynamically stable octacalcium and hydroxyapatite phases with time [36].

3.3 Surface pressure-area isotherms of selected biopolymers on SSE solution

The surface pressure-area isotherm of BSA over water (Figure 3A) exhibited a similar pattern to that previously reported [24] with an onset in surface pressure at area per molecule of ~17,000 Å². In an attempt to estimate the limiting area for BSA we considered a hypothetical scenario in which the protein with its 583 amino acids is fully unfolded at the interface. In this scenario, the projected area per BSA is $583 \times 16 \text{ Å}^2 = 9328 \text{ Å}^2$ (16 Å² is the projected surface of one amino acid, based on values obtained by diffraction measurements for amphiphilic peptides at interfaces [37]). The difference between the detected onset and the estimated limiting area value suggested that the proteins are far from being closely packed. Indeed, Brewster angle microscope images (Figure 1) demonstrated a net of spacious filament-like domains. A limiting area per molecule of ~15,000 Å² was determined by a tangent line drawn to the steepest part of the compressed state (Figure 3A; see Table 1). Compressing the BSA film beyond the limiting area led to a steep increase in the surface pressure (up to area per molecule of ~10,000 Å²), characterized by the compressibility value $k = 21 \text{ m/N}$. The compressed state is then followed by a plateau characterizing the collapsed film region.

The isotherm of BSA monolayer over SSE at time zero was similar in shape to that observed on water with an increase of only ~ 7% in the limiting area per molecule (of note, in a separate experiment aiming to determine the amount of biomolecule residing at the interface following the film deposition over SSE, BSA was found not to dissolve in this subphase). The increase in limiting area per molecule may be explained by the proteins' conformational changes and/or immediate adsorption of ions from the SSE solution to the BSA film. The BSA monolayer incubated for 18 hours on SSE prior to compression showed a further increase in the apparent limiting area per molecule, ~23000 Å², which is 44% larger than the limiting area detected at time zero. This increase corroborates with the accumulation of minerals detected by the BAM and the PM-IRRAS measurements. The overall shape of the isotherm on SSE solution (after 18
hours of incubation) was appreciably different than the corresponding isotherm obtained at time
zero. The isotherm exhibits two compressed phases separated by a plateau region, the first one
with $k = 30 \text{ m/N}$ and a second (at $\sim 8000 \text{ Å}^2$) with $k = 8 \text{ m/N}$ characterizing "solid-like"
monolayers. The isotherm of the BSA monolayer incubated over water for 18 hours (control
experiment, see Figure 3A and Table 1) exhibited essentially the same pattern as that obtained at
time zero over water, indicating that the increase in BSA surface pressure on SSE solution was
due to the mineral accumulation at the interface.
The limiting area per molecule of fibrinogen film was found to be at $\sim 8000 \text{ Å}^2$ (Figure 3B).
Fibrinogen which is composed of 225 amino acids, may hypothetically show a limiting area per
molecule of $225 \times 16 \text{ Å}^2 = 3600 \text{ Å}^2$. Hence, similarly to consideration discussed above for the
BSA film, the fibrinogen molecules according to the isotherm do not form a closely packed film
at this state. The fibrinogen isotherm on SSE at time zero showed a limiting area per molecule
even larger, $\sim 15000 \text{ Å}^2$, almost double the corresponding one detected on water. BAM images of
fibrinogen on SSE (Figure 1) showed a hollow network of the protein most probably enhanced
by adsorbed ions definitely not closely packed. Analyses of the fibrinogen film at the interface
indicated that it does not dissolve in the SSE subphase (Table 1). Hence, the increase in the
limiting area per molecule on SSE points to possible conformational changes of the protein as
well as to strong and immediate binding interactions between the protein and ions originating
from the SSE solution. The immediate mineralization did not have a strong effect on the
compressibility of the fibrinogen film, with values of $k = 30 \text{ m/N}$ and $47 \text{ m/N}$ on water and SSE
solution, respectively.
After 18 hours of film incubation over SSE, the isotherm was of the same shape obtained on SSE
at time zero, however, with an offset of $\sim 10 \text{ mN/m}$ in surface pressure for compression ($k = 44$
$m/N$) up to $\sim 7000 \text{ Å}^2$/molecule, an area per molecule which is quite close to that starting the
compressed state of the film on water. The curve then showed a second steep increase in surface
pressure, indicative of a "solid-like" resistant film, $k = 16 \text{ m/N}$, pointing to extensive
mineralization process and film reorganization that took place along the incubation period
(Figure 3B and Table 1). The curve obtained for the control experiment with fibrinogen film
incubated on phosphate-deprived-SSE solution was similar to that obtained over SSE for time
zero, yet with a small offset in surface pressure. Hence, the main interactions and precipitation
obtained on the interface were formed by calcium phosphate minerals (discussed further for the alginate system below).

Alginic acid spread over the interface of water appeared to be fully dissolved in the subphase (Figure 3C). Alginic acid film was generated on SSE solution by spreading 1 mL of 0.1 mg/mL alginate solution. This amount of spread material showed a low surface pressure region extending down to ~550 Å²/molecule (Figure 3C), followed by a compressed phase (k = 22 m/N). The area per molecule is based on the fraction of the polysaccharide remaining at the SSE interface, which was found in a separate set of experiments to be 21% (Table 2). The compression curve obtained for the alginate film that was incubated for 18 hours over SSE solution resembled in shape that on SSE at time zero, however, with a surface pressure higher by ~ 10 mN/m throughout the compression range, indicative of the more extensive mineralization that occurred over this period of time.

The reference isotherm, of alginate incubated over phosphate derived SSE solution, exhibited a constant surface pressure of ~ 5 mN/m throughout the whole range, with a transition to a somewhat less compressible film in an area lower than 250 Å²/molecule. These isotherms highlight phosphate-calcium interactions that contribute to the mineralization process. It is possible that calcium ions that adsorb to the alginate at the interface may further interact with phosphate ions in the subphase and these together can create crystallization nuclei. It may also be that calcium-phosphate clusters or nuclei would form first within the SSE solution and then become adsorbed to the interface.

Lysozyme spread over water could not be stabilized as a film at the interface (Figure 3D) yet, over SSE solution a Langmuir (π-A) isotherm could be obtained with a limiting area per molecule at ~200 Å²/molecule (Figure 3D), and a compressed state characterized by k = 31 m/N (Table 1). Calculating the hypothetical area per molecule in a similar manner to that described for BSA and fibrinogen showed that the fully unfolded state of lysozyme occupies an area of 163 ×16 = 2608 Å²/molecule. Unlike the BSA and fibrinogen films, in this case the isotherm as well as the measurement of protein residing at the interface, 13.5% (Table 2), indicate that most of the lysozyme film dissolved in the SSE subphase. Incubation of the lysozyme film over SSE solution for 18 hours prior to compression resulted in an isotherm which already at 400 Å²/molecule
appeared in the compressed state, \( k = 39 \) m/N, indicative of the continuous accumulating minerals at the interface (Figure 3D and Table 1).

3.4 Surface pressure-area isotherms of extracellular polymeric substances

Figure 4 shows Langmuir (\( \pi-A \)) isotherms of EPS over water and over SSE solution. Since EPS comprises a mixture of different biomolecule components and concentrations, the isotherms were presented as a function of the actual Langmuir trough area rather than an area per molecule as in the individual biomolecules. As a reference isotherm we used EPS material spread on deionized water which induced a \( \sim 5 \) mN/m increase in surface pressure at the fully compressed state of the film. The same EPS amount spread over SSE solution induced a continuous increase in surface pressure up to \( 20 \) mN/m at the fully closed position of the trough (Figure 4), with minimum compressibility of \( k = 43 \) m/N (Table 1). The differences between the isotherms on water and on SSE point to immediate interactions of the EPS biomolecules with the ionic substances of the SSE solution.

An isotherm of EPS film incubated for 18 hours over SSE prior to compression showed, similarly to isotherms of the single bio-polymers, an appreciable increase in surface pressure, reaching \( 54 \) mN/m at the fully compressed state, with a minimum compressibility value of \( k = 14 \) m/N (Table 1) suggesting the formation of film highly resistant to compression forces. Hence, continuous mineralization processes, at the interface of the SSE solution, reinforced the EPS. The pressure-area isotherm of EPS spread over the SSE solution with no phosphate exhibited a pattern very similar to that obtained on SSE at time zero, only with a slightly higher surface pressure (<5 mN/m; Figure 4). Similar to the studies of single biomolecules, this signifies that phosphate ions play a major role in interactions with calcium leading to the mineralization at the film-EPS interface.

3.5 Comparison of biopolymer tendencies to induce mineralization

In order to compare the tendencies to induce mineralization by the studied biopolymers, for each of the biopolymers’ isotherms we extracted a value representing the increase in surface pressure over the 18 hour incubation time, denoted \( \Delta \pi_5 \). This increase was measured at the area per molecule where the biopolymer film on SSE at time zero was \( \pi = 5 \) mN/m. \( \Delta \pi_5 \) for BSA
isotherms is indicated by the arrow in Figure 3A. This increase in surface pressure was also normalized to the actual amount of material per unit area remaining at the interface denoted $\Delta \pi_5$. The results are summarized in Table 2.

The $\Delta \pi_5$ value showed that all studied biopolymers have the tendency of inducing mineralization; whereas, $\Delta \pi_5$ revealed that the increase in the mineralization tendencies follows the order of: fibrinogen > lysozyme > BSA > alginic acid. The normalized $\Delta \pi_5$ value is meaningful, as it provides valuable information in respect to the induction of mineralization at interfaces by partially soluble biomolecules, since even a soluble biopolymer may be held by physical or covalent interactions in a biofilm on RO membrane and, as such, exert its intrinsic influence on the mineralization. Moreover, the tendency of the biopolymer to induce mineralization cannot be simply correlated only with the pI or the charge of the molecule and specific chemical groups constituting the biomolecule. The molecular and supramolecular structures of the biopolymers may also play an important role in this process as demonstrated here.

3.6 Biopolymer comparison by chemical composition analysis of mineralized films

The mineralized films formed during incubation of the biopolymers over SSE solution were collected, acidified, and analyzed by ICP for the elemental ratio between calcium and phosphorus (Ca/P). It should be noted that the Ca/P ratio for the SSE solution is 89. The Ca/P ratio of hydroxyapatite (HAP), which is considered the most stable form of calcium phosphate minerals, is 1.67. The ratios found for the mineralized films (Table 3) are much lower than that of SSE, yet slightly higher than the expected ratio in HAP. Ca/P values of BSA, fibrinogen, lysozyme and EPS were between 4 and 8, indicating a large extent of mineralization; the exceptional ratio of ~ 14 found for alginic acid may be explained by high concentration of calcium ions due to sorption interactions with the negatively charged carboxylic groups of the polysaccharide. Lysozyme which has a pI value of 11.3 and is positively charged in the SSE solution, might adsorb phosphate ions and increase phosphate local concentration at the interface, as indeed indicated by the smallest observed Ca/P ratio (i.e. high phosphate content). Mineralization occurred to a larger extent at prolonged incubation times; the Ca/P ratio of BSA that was incubated for 48 hours with SSE was lower than that of the BSA incubated for 18 hours.
Mineralization experiments longer than 48 hours could not be carried out with BSA since the mineralized films appeared to sink to the bottom of the trough, as a result of their increased weight.

4. Discussion

The biopolymer films and the EPS were found to induce calcium-phosphate mineralization. Using surface pressure - area isotherms the extent of mineralization could be characterized qualitatively and also quantified by several parameters. The mineralization started immediately probably through attractive electrostatic interactions, as could be deduced from the higher surface pressure, or larger limiting area per molecule, on the SSE solution at time zero compared to the water system. The electrostatic charge of the biopolymer (i.e. its pI value) can be referred to in order to estimate the tendency of the biopolymer to interact with mineralizing ions. BSA and fibrinogen have pI values of 4.7 and 5.8 respectively and in the SSE mineralization solution (pH 7.0) both are negatively charged and hence may form electrostatic interactions with calcium ions and other cations from the subphase. BSA and fibrinogen contain a high percentage (16.5% and 13.1%, respectively) of anionic amino-acids (Asp and Glu) which are reported as the most active residues for inducing mineralization of calcium salts [38]. We found that the mineralizing tendencies of the BSA and fibrinogen as deduced by the normalized increase in surface pressure, \( \Delta \pi \), were different, with fibrinogen inducing mineralization to a larger extent than BSA. Further studies, beyond the scope of this manuscript, are needed to elucidate more details on the factors that lead to such differences. This study revealed that there are factors other than pI that could influence the mineralization process, such as the structure of the biomolecules, their conformation and interactions with other biomolecules, within the film at the interface. Interestingly, these insights, in particular of conformational changes and mineralization processes at interfaces may potentially be related to recent studies aiming at unraveling the links between protein composition and conformations and their role in controlling biomineralization [39].

Alginic acid was used in this study as a model polysaccharide foulant because acidic polysaccharides were identified as major components of organic matter in wastewater effluents.
Alginic acid showed the least extent of mineralization, as compared with the other biopolymers. All biopolymers exhibited increased compressibility when spread over SSE or after the incubation, as a result from the accumulated calcium-phosphate minerals at the interface. However, a pronounced decrease in compressibility with increasing incubation time was noted for EPS, indicating increased resistance to compression in comparison to the other biopolymers tested. Noteworthy, this finding may be related to the composition of the EPS film, which is mainly of fatty acid-like molecules (56.6% humic substances) whereas the other films were polymeric (biopolymer) materials.

As mineralization evolves over time the biopolymers may exhibit different tendencies, as for example: Alginic acid and lysozyme may have an inhibitory role in the nucleation stage of mineralization by sequestering calcium ion species [39] but at later stages induce mineralization similar to the other biopolymers. Indeed in the experiments carried out here both alginic acid and lysozyme showed no signs of mineralization in BAM measurements at time zero, but after 18 hours showed extensive mineralization as the other biopolymers, according to BAM and to Ca/P ratios measured by ICP. The Ca/P ratios of films collected over the SSE solution after 18 hours of incubation were much lower than the ratio in the SSE solution. The Ca/P ratio of the BSA film which was incubated for 48 hours was substantially lower in comparison to that of the 18 hour incubation (4.2 and 7.0, respectively), hence more minerals are accumulating at the interface with time, resulting in much higher P concentrations relative to the SSE value (Ca/P = 89). Another possible cause for lower Ca/P values in 48 hour is that the calcium-phosphate minerals accumulated at the interface may be transformed into the more stable hydroxyapatite phase over time, as the value obtained is closer to the value of hydroxyapatite (Ca/P = 1.67). Other experiments with such long incubation times were not successful as the minerals accumulating on the interface became heavy and films sunk into the subphase. Another reason for the deviation of the detected Ca/P ratio from the theoretical value of hydroxyapatite can be the simultaneous mineralization of calcium carbonate, as can be seen in the PM-IRRAS spectra of the different model biopolymers, where a peak around 1460 cm\(^{-1}\) (representing carbonates) was detected. It is worth mentioning that both calcium phosphate and calcium carbonate have positive saturation indices in SSE solution: 6.68 and 0.79 respectively [12].
Although amorphous calcium phosphate (ACP) is below saturation in SSE solution (saturation index -3.21) ACP prenucleation centers of 180 Å diameter and 8×10^{-6} volume fraction were observed in four days old SSE solution using small-angle neutron scattering (SANS) as published previously [12]. Stabilization of these particles by magnesium and other ions of the SSE solution as proposed in [42] cannot be excluded. This study showed for the first time that various biopolymers representing foulants in wastewater treatment systems, as well as EPS, induce heterogeneous mineralization, mainly of calcium-phosphate, at the interface of SSE solution. We showed that once both calcium and phosphate became adsorbed and created nucleation sites, the mineralization continued to evolve. The methodology presented here could be further utilized to characterize and quantify the extent, phases and rates of mineralization by different biofoulants and different types of EPS. The importance of this work lies in understanding the influence of EPS and biofouling-born substances on calcium phosphate scaling in membrane based wastewater desalination. The results support the previously reported notion [12-13] in desalination processes that pre-treatment by removal of biopolymers as well as phosphate from the feed effluents before reverse osmosis desalination, may be highly efficient in minimizing membrane fouling and scaling.

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Appendices: Supplementary data
Describing the determination of molecular weight of alginic acid by viscosity measurements and SANS measurements, and extraction procedure of EPS from fouled RO membranes and chemical composition of EPS as measured by LC-OCD.

References


