

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

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23

24 **Abstract**

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

40

41

42 **Keywords:** Calcium phosphate mineralization; biofilm; surface pressure-molecular area
43 isotherms; biofouling; reverse osmosis; desalination.

44

45 **1. Introduction**

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

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

68 The influence of organic fouling on scaling was also reported for calcium sulfate; scaling of
69 CaSO_4 was enhanced in the presence of biofilm on RO/NF membranes as was evident by micron
70 size crystals covering the surface [7]. In addition, a rapid flux decline was observed in
71 nanofiltration (NF) membranes from natural waters in the presence of divalent cations due to
72 binding of ions to humic carboxylic functional groups. This binding led to a reduction of the
73 humic macro-molecules' charge, eventually resulting in a decrease in the repulsion between the
74 macro-molecules, which led to a denser fouling layer [8-9].

75 Scaling in biofilms resembles biomineralization in nature, as both processes involve
76 macromolecules that induce the adsorption and precipitation of mineralizing ions, in addition to
77 influencing the kinetics, size and phases of crystals obtained in these complex systems.
78 Langmuir films studied by surface-pressure versus molecular-area (π - A) isotherms have been
79 widely employed for monitoring early stages of mineralization induced by specific molecular
80 systems [10].

81 A Langmuir (π - A) isotherm system was previously used by our group to study the influence of
82 organic chemical groups on calcium phosphate mineralization in a model solution with an ionic
83 profile simulating secondary-treated wastewater effluents (denoted SSE solution). Mineralization
84 was induced by organic functional groups to varying extents, according to the following order: -
85 $\text{PO}_4\text{H}_2 > -\text{COOH} \sim -\text{NH}_2 > -\text{COOH} : -\text{NH}_2 (1:1) > -\text{OH} > -\text{ethylene glycol}$ [11]. Another study
86 demonstrated through small-angle neutron scattering that proteins (BSA and lysozyme) in bulk
87 SSE solution led to the buildup of protein-mineral particles [12]. Small angle neutron scattering
88 was also used for studying the influence of BSA-grafted gold nanoparticles (BSA-GNPs) on
89 calcium phosphate mineralization from SSE solution. The BSA-GNPs exposed to SSE solution
90 induced immediate mineralization which led to stable composite particles (organic/inorganic
91 materials) of about 0.2 μm diameter, and a mineral volume fraction between 50 and 80% [13].

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

98

99 **2. Material and Methods**

100 *2.1 Chemicals*

101 Bovine serum albumin (BSA) 96% purified by electrophoresis (MW = 66.6 kDa), fibrinogen
102 (fraction I, type I-S, 4% carbohydrate content according to manufacturer information) from
103 bovine plasma (MW = 340 kDa), alginic acid from brown algae (MW of 16.6 kDa as determined

104 by micro-viscosity measurements and 24 kDa by SANS measurements; see description of
105 molecular weight determination in Appendix A), lysozyme from chicken egg white (MW=14.3
106 kDa), 1-pentanol \geq 99%, 1,4-piperazinediethanesulfonic acid (PIPES), potassium dihydrogen
107 phosphate, phenol, and sulphuric acid were purchased from Sigma Aldrich (St. Louis, MO).
108 Sodium chloride, sodium sulfate, and magnesium chloride hexahydrate were purchased from
109 Frutarom (Haifa, Israel). Sodium bicarbonate and hydrochloric acid were purchased from Bio-
110 Lab (Jerusalem, Israel). Calcium chloride dehydrate was purchased from Carlo Erba Reagents
111 (Rodano, Italy). Ultrapure water (resistivity 18.1 M Ω ·cm) was used for all purposes. Bradford
112 reagent was purchased from Bio-Rad (Jerusalem, Israel).

113

114 *2.2 Preparation of SSE solution*

115 SSE solution simulating effluents from Shafdan plant in Tel-Aviv region wastewater reclamation
116 plant [11,17] at a stage of 80% desalination was prepared as previously described [12] by
117 dissolving 17.1 mg of KH₂PO₄, 460 mg NaCl, 2.5 mL of 1M HCl, 1370 mg of MgCl₂·6H₂O,
118 1645 mg of CaCl₂·H₂O, and 663.5 mg of Na₂SO₄ in 500 mL of water. Then, 835 mg of NaHCO₃
119 were dissolved in 50 mL of water and added slowly to the mixture. Finally 20 mL of 0.5 M
120 PIPES buffer was added. The final volume of the solution was completed with water to 1 liter
121 and the pH of the solution was 6.9. The solution was filtered through a 0.22 μ m PVDF filter and
122 stored up to 10 days at room temperature. A phosphate deprived SSE solution was prepared
123 similarly but with sodium chloride (25.6 mg) instead of the 17.1 mg of KH₂PO₄, compensating
124 for the ionic strength of the phosphate salt; the pH was measured and if needed adjusted to 6.9
125 during preparation of the solution after dissolving all salts, just before final volume completion.

126

127 *2.3 EPS extraction and composition*

128 EPS was extracted from a microbial biofilm developed on a fouled RO membrane after
129 desalination of ultrafiltration permeate effluents of a membrane bioreactor (MBR) [18-19]. EPS
130 is usually characterized by the weight ratios of its polysaccharides, proteins, nucleic acids, lipids,
131 and other polymeric substances [20-21].

132 EPS composition was determined by a liquid chromatography–organic carbon detection (LC-
133 OCD) system (model 8, DOC Labor manufactured by Dr. Stefan Huber, Karlsruhe, Germany)

134 with a gel-permeation chromatographic column, multidetection with UV absorbance at 254 nm
135 [22]. Organic carbon and organic nitrogen detection were used to determine the amounts of
136 dissolved organic carbon (DOC) of EPS fractions extracted from the fouled membrane (see more
137 details in Appendix B). According to LC-OCD the EPS comprised 5.8% biopolymers
138 (polysaccharides, proteins, amino-sugars), 56.6% humic substances, 17.3% building blocks
139 (breakdown products of humics) and 17.3% low molecular weight neutrals (mono- and
140 oligosaccharides, alcohols, aldehydes, ketones). The detailed analysis of EPS by LC-OCD is
141 given in Table B.1, Appendix B. The EPS was also analyzed by colorimetric methods to
142 determine proteins and polysaccharides content, which is described in Appendix B.

143

144 *2.4 Langmuir- (π -A) isotherms*

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

159 BSA solution (pI 4.7, 583 amino acids) was prepared by dissolving 1 mg/mL of the protein in
160 water containing 0.05% (v/v) 1-pentanol to improve the spreading process, following the
161 procedure by Xue et al. [24] The BSA monolayer at the air-liquid interface was formed by
162 spreading 13 μ l of this solution over water or SSE. Fibrinogen (pI = 5.8, 225 amino acids)
163 monolayers were prepared following the procedure of Sankaranarayanan et al. [25] Briefly,

164 fibrinogen was dissolved in phosphate buffer (pH= 7.5) at a concentration of 1 mg/mL, and then
165 9 µl were spread over the subphase. Lysozyme monolayers were formed following the procedure
166 of Thakur et al. [26]. Lysozyme (pI = 11.3, 163 amino acids) was dissolved in water at
167 concentration of 1 mg/mL and then 110 µl were spread on the subphase. Alginic acid (0.1
168 mg/mL) was dissolved in water at pH = 8 then 1 mL was spread over the subphase. As all these
169 compounds could have become dissolved in the subphase during spreading, the amount of
170 material residing at the interface after deposition was determined (described below).

171 The extracted EPS was dissolved in water (380 µg/mL) and 200 µl of this solution were spread
172 on the subphase. Since EPS is composed of different substances, the exact composition of which
173 are not known, the isotherms of this film were plotted as a function of the trough area rather than
174 the mean molecular area.

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

$$191 \quad k = - 1/A (\Delta A/\Delta\pi)_T \quad (1)$$

192 Where A is the mean molecular area occupied by the biopolymer, ΔA is the change in mean
193 molecular area, $\Delta\pi$ is the change in surface pressure, $(\Delta A/\Delta\pi)$ is the temperature dependent

194 inverse of the isotherm's slope at the region around the limiting area per molecule, T is the
195 temperature. Following the compressed state the surface pressure may level off whereby the
196 monolayer collapses into a multilayered thick film.

197 The mineralization tendencies of the various biopolymers were compared by a value denoted as
198 $\Delta\pi_5$, which was calculated for each mineralized film. This value stands for the increase in surface
199 pressure at the end of 18 hours of incubation, at the area per molecule where the film on SSE at
200 time zero shows 5 mN/m (a value selected arbitrarily), as illustrated in Figure 3A. Furthermore,
201 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
202 biopolymers at the interface. The surface density was calculated by dividing the amount of
203 biopolymer residing at the interface by the surface area of the trough. Noteworthy, comparison
204 between $\Delta\pi_5$ and $\overline{\Delta\pi_5}$ of the different biopolymers is relying on the consideration that the
205 compressibility of the films in this region is similar.

206

207 *2.5 Polarization Modulated Infrared Reflection Adsorption Spectroscopy (PM-IRRAS)*

208 IR measurements of the various biopolymers and EPS over SSE solution were performed at time
209 zero and after 18 hours of mineralization using a Nicolet 6700 spectrophotometer (Thermo,
210 Madison, WI) coupled with a liquid nitrogen-cooled mercury cadmium telluride (MCT) detector.
211 The detector angle at which the measurements were done was set to 72° , while the incident
212 infrared beam was polarized by a ZnSe polarizer and modulated by a ZnSe photoelastic
213 modulator between parallel (p) and perpendicular (s) polarizations to the plane of incidence. For
214 each spectrum, 1000 scans were collected at a modulation frequency of 1600 cm^{-1} and at a
215 resolution of 8 cm^{-1} . The resultant signal is the differential reflectivity spectrum $\Delta R/R = (R_p - R_s)/$
216 $(R_p + R_s)$, where R_p and R_s are the polarized reflectivities of the plane of incidence. The
217 contribution of the SSE solution was taken into account by dividing each spectrum by the
218 spectrum of SSE solution alone.

219

220 *2.6 Estimation of the amount of bio-polymers at the solution-air interface*

221 An estimation of the amount of bio-polymers remaining at the interface of SSE solution
222 following film deposition was conducted in Teflon® mini-troughs (cylindrical shape of 6.8 cm
223 diameter and 1 cm height, volume 36 mL). Each trough was placed in a petri-dish and filled with

224 the SSE solution. Then specific volumes of each of the bio-polymers were spread to form a
225 monolayer film. Following an equilibration period of 30 minutes, 2.5 mL of chloroform were
226 injected directly to the bottom of the mini-troughs forming a separate phase, without mixing with
227 the aqueous solution. The chloroform layer pushed ~2.5 mL of the upper layer out, presumably
228 with the interfacial film, into the petri-dish.

229 Quantification of BSA, fibrinogen, and lysozyme in the overflown samples were analyzed using
230 the Bradford assay [27] according to the following procedure: 200 μ l of the overflown protein
231 solution were added to 800 μ l of Bradford reagent (100 ppm of coomassie brilliant blue G-250 in
232 1 M PBS), the mixture was vortexed, and after 5 minutes absorbance was measured with a
233 spectrophotometer at $\lambda= 595$ nm. The concentration was then deduced using a calibration curve.
234 In the case of alginic acid, the whole subphase excluding the overflown sample from the
235 interface part was collected and alginic acid was quantified as follows: the subphase solution was
236 concentrated to 2.5 mL by evaporating the water at 90°C. Alginic acid was determined in the
237 concentrated solution by the phenol-sulphuric-acid method: 200 μ l of 5% (w/v) phenol were
238 added to 200 μ l of sample, the mixture was vortexed, then 1 mL of concentrated sulphuric acid
239 was added, after one hour absorbance of the samples was measured at $\lambda= 490$ nm [28] and
240 compared to a calibration curve.

241

242 *2.7 Brewster Angle Microscopy (BAM)*

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

253

254

255 **3. Results**

256 *3.1 Mineralization studies monitored by BAM on Langmuir trough*

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

266

267 *3.2 IR spectra of the mineralized film at solution-air interface*

268 PM-IRRAS spectra were measured after 18 hours incubation on SSE solution (Figure 2A). The
269 most intense peaks in the region $900\text{-}1200\text{ cm}^{-1}$ may be attributed to phosphate absorption, in
270 both octacalcium phosphate and hydroxyapatite [29-30] phases. The peak at around 1460 cm^{-1} is
271 associated with the asymmetric stretch of the carbonate -CO_3^{2-} group which indicates the
272 concurrent formation of calcium carbonate [31-32] or the replacement of phosphate and/or
273 hydroxide groups in hydroxyapatite by carbonate [33-34]. In a control experiment BSA film over
274 phosphate-deprived-SSE solution (Figure 2A) showed no peaks at all, hence lack of
275 mineralization, pointing to the importance of phosphate ions in the process. The PM-IRRAS
276 measurements could not be performed at longer incubation durations due to evaporation of the
277 subphase.

278 The PM-IRRAS spectrum of the EPS film incubated over SSE for 18 hours was somewhat
279 different from those of the other biopolymers, with a broad and relatively weak peak between
280 $750\text{ and }1200\text{ cm}^{-1}$ (Figure 2B). This peak can be attributed to a combination of hydrogen
281 phosphates in addition to octacalcium phosphate and hydroxyapatite [35]. This finding implies

282 that the EPS induces mineralization of the calcium-phosphate minerals at a lower extent and of
283 less stable phases compared to the pure biopolymer films. Nonetheless, it was previously shown
284 that calcium-phosphate minerals tend to convert to the more thermodynamically stable
285 octacalcium and hydroxyapatite phases with time [36].

286

287 *3.3 Surface pressure-area isotherms of selected biopolymers on SSE solution*

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

302 The isotherm of BSA monolayer over SSE at time zero was similar in shape to that observed on
303 water with an increase of only $\sim 7\%$ in the limiting area per molecule (of note, in a separate
304 experiment aiming to determine the amount of biomolecule residing at the interface following
305 the film deposition over SSE, BSA was found not to dissolve in this subphase). The increase in
306 limiting area per molecule may be explained by the proteins' conformational changes and/or
307 immediate adsorption of ions from the SSE solution to the BSA film. The BSA monolayer
308 incubated for 18 hours on SSE prior to compression showed a further increase in the apparent
309 limiting area per molecule, $\sim 23000 \text{\AA}^2$, which is 44% larger than the limiting area detected at
310 time zero. This increase corroborates with the accumulation of minerals detected by the BAM
311 and the PM-IRRAS measurements. The overall shape of the isotherm on SSE solution (after 18

312 hours of incubation) was appreciably different than the corresponding isotherm obtained at time
313 zero. The isotherm exhibits two compressed phases separated by a plateau region, the first one
314 with $k = 30$ m/N and a second (at $\sim 8000 \text{ \AA}^2$) with $k = 8$ m/N characterizing "solid-like"
315 monolayers. The isotherm of the BSA monolayer incubated over water for 18 hours (control
316 experiment, see Figure 3A and Table 1) exhibited essentially the same pattern as that obtained at
317 time zero over water, indicating that the increase in BSA surface pressure on SSE solution was
318 due to the mineral accumulation at the interface.

319 The limiting area per molecule of fibrinogen film was found to be at $\sim 8000 \text{ \AA}^2$ (Figure 3B).
320 Fibrinogen which is composed of 225 amino acids, may hypothetically show a limiting area per
321 molecule of $225 \times 16 \text{ \AA}^2 = 3600 \text{ \AA}^2$. Hence, similarly to consideration discussed above for the
322 BSA film, the fibrinogen molecules according to the isotherm do not form a closely packed film
323 at this state. The fibrinogen isotherm on SSE at time zero showed a limiting area per molecule
324 even larger, $\sim 15000 \text{ \AA}^2$, almost double the corresponding one detected on water. BAM images of
325 fibrinogen on SSE (Figure 1) showed a hollow network of the protein most probably enhanced
326 by adsorbed ions definitely not closely packed. Analyses of the fibrinogen film at the interface
327 indicated that it does not dissolve in the SSE subphase (Table 1). Hence, the increase in the
328 limiting area per molecule on SSE points to possible conformational changes of the protein as
329 well as to strong and immediate binding interactions between the protein and ions originating
330 from the SSE solution. The immediate mineralization did not have a strong effect on the
331 compressibility of the fibrinogen film, with values of $k = 30$ m/N and 47 m/N on water and SSE
332 solution, respectively.

333 After 18 hours of film incubation over SSE, the isotherm was of the same shape obtained on SSE
334 at time zero, however, with an offset of ~ 10 mN/m in surface pressure for compression ($k = 44$
335 m/N) up to $\sim 7000 \text{ \AA}^2/\text{molecule}$, an area per molecule which is quite close to that starting the
336 compressed state of the film on water. The curve then showed a second steep increase in surface
337 pressure, indicative of a "solid-like" resistant film, $k = 16$ m/N, pointing to extensive
338 mineralization process and film reorganization that took place along the incubation period
339 (Figure 3B and Table 1). The curve obtained for the control experiment with fibrinogen film
340 incubated on phosphate-deprived-SSE solution was similar to that obtained over SSE for time
341 zero, yet with a small offset in surface pressure. Hence, the main interactions and precipitation

342 obtained on the interface were formed by calcium phosphate minerals (discussed further for the
343 alginate system below).

344 Alginic acid spread over the interface of water appeared to be fully dissolved in the subphase
345 (Figure 3C). Alginic acid film was generated on SSE solution by spreading 1 mL of 0.1 mg/mL
346 alginate solution. This amount of spread material showed a low surface pressure region
347 extending down to $\sim 550 \text{ \AA}^2/\text{molecule}$ (Figure 3C), followed by a compressed phase ($k = 22$
348 m/N). The area per molecule is based on the fraction of the polysaccharide remaining at the SSE
349 interface, which was found in a separate set of experiments to be 21% (Table 2). The
350 compression curve obtained for the alginate film that was incubated for 18 hours over SSE
351 solution resembled in shape that on SSE at time zero, however, with a surface pressure higher by
352 $\sim 10 \text{ mN/m}$ throughout the compression range, indicative of the more extensive mineralization
353 that occurred over this period of time.

354 The reference isotherm, of alginate incubated over phosphate derived SSE solution, exhibited a
355 constant surface pressure of $\sim 5 \text{ mN/m}$ throughout the whole range, with a transition to a
356 somewhat less compressible film in an area lower than $250 \text{ \AA}^2/\text{molecule}$. These isotherms
357 highlight phosphate-calcium interactions that contribute to the mineralization process. It is
358 possible that calcium ions that adsorb to the alginate at the interface may further interact with
359 phosphate ions in the subphase and these together can create crystallization nuclei. It may also be
360 that calcium-phosphate clusters or nuclei would form first within the SSE solution and then
361 become adsorbed to the interface.

362 Lysozyme spread over water could not be stabilized as a film at the interface (Figure 3D) yet,
363 over SSE solution a Langmuir (π - A) isotherm could be obtained with a limiting area per
364 molecule at $\sim 200 \text{ \AA}^2/\text{molecule}$ (Figure 3D), and a compressed state characterized by $k = 31 \text{ m/N}$
365 (Table 1). Calculating the hypothetical area per molecule in a similar manner to that described
366 for BSA and fibrinogen showed that the fully unfolded state of lysozyme occupies an area of 163
367 $\times 16 = 2608 \text{ \AA}^2/\text{molecule}$. Unlike the BSA and fibrinogen films, in this case the isotherm as well
368 as the measurement of protein residing at the interface, 13.5% (Table 2), indicate that most of the
369 lysozyme film dissolved in the SSE subphase. Incubation of the lysozyme film over SSE solution
370 for 18 hours prior to compression resulted in an isotherm which already at $400 \text{ \AA}^2/\text{molecule}$

371 appeared in the compressed state, $k = 39$ m/N, indicative of the continuous accumulating
372 minerals at the interface (Figure 3D and Table 1).

373 *3.4 Surface pressure-area isotherms of extracellular polymeric substances*

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

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

394 *3.5 Comparison of biopolymer tendencies to induce mineralization*

395 In order to compare the tendencies to induce mineralization by the studied biopolymers, for each
396 of the biopolymers' isotherms we extracted a value representing the increase in surface pressure
397 over the 18 hour incubation time, denoted $\Delta\pi_5$. This increase was measured at the area per
398 molecule where the biopolymer film on SSE at time zero was $\pi = 5$ mN/m. $\Delta\pi_5$ for BSA

399 isotherms is indicated by the arrow in Figure 3A. This increase in surface pressure was also
400 normalized to the actual amount of material per unit area remaining at the interface denoted $\overline{\Delta\pi_5}$.
401 The results are summarized in Table 2.

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

412 *3.6 Biopolymer comparison by chemical composition analysis of mineralized films*

413 The mineralized films formed during incubation of the biopolymers over SSE solution were
414 collected, acidified, and analyzed by ICP for the elemental ratio between calcium and
415 phosphorus (Ca/P). It should be noted that the Ca/P ratio for the SSE solution is 89. The Ca/P
416 ratio of hydroxyapatite (HAP), which is considered the most stable form of calcium phosphate
417 minerals, is 1.67. The ratios found for the mineralized films (Table 3) are much lower than that
418 of SSE, yet slightly higher than the expected ratio in HAP. Ca/P values of BSA, fibrinogen,
419 lysozyme and EPS were between 4 and 8, indicating a large extent of mineralization; the
420 exceptional ratio of ~ 14 found for alginic acid may be explained by high concentration of
421 calcium ions due to sorption interactions with the negatively charged carboxylic groups of the
422 polysaccharide. Lysozyme which has a pI value of 11.3 and is positively charged in the SSE
423 solution, might adsorb phosphate ions and increase phosphate local concentration at the
424 interface, as indeed indicated by the smallest observed Ca/P ratio (i.e. high phosphate content).
425 Mineralization occurred to a larger extent at prolonged incubation times; the Ca/P ratio of BSA
426 that was incubated for 48 hours with SSE was lower than that of the BSA incubated for 18 hours.

427 Mineralization experiments longer than 48 hours could not be carried out with BSA since the
428 mineralized films appeared to sink to the bottom of the trough, as a result of their increased
429 weight.

430 **4. Discussion**

431 The biopolymer films and the EPS were found to induce calcium-phosphate mineralization.
432 Using surface pressure - area isotherms the extent of mineralization could be characterized
433 qualitatively and also quantified by several parameters. The mineralization started immediately
434 probably through attractive electrostatic interactions, as could be deduced from the higher
435 surface pressure, or larger limiting area per molecule, on the SSE solution at time zero compared
436 to the water system. The electrostatic charge of the biopolymer (i.e. its pI value) can be referred
437 to in order to estimate the tendency of the biopolymer to interact with mineralizing ions. BSA
438 and fibrinogen have pI values of 4.7 and 5.8 respectively and in the SSE mineralization solution
439 (pH 7.0) both are negatively charged and hence may form electrostatic interactions with calcium
440 ions and other cations from the subphase. BSA and fibrinogen contain a high percentage (16.5%
441 and 13.1%, respectively) of anionic amino-acids (Asp and Glu) which are reported as the most
442 active residues for inducing mineralization of calcium salts [38]. We found that the mineralizing
443 tendencies of the BSA and fibrinogen as deduced by the normalized increase in surface pressure,
444 $\overline{\Delta\pi_5}$, were different, with fibrinogen inducing mineralization to a larger extent than BSA.
445 Further studies, beyond the scope of this manuscript, are needed to elucidate more details on the
446 factors that lead to such differences. This study revealed that there are factors other than pI that
447 could influence the mineralization process, such as the structure of the biomolecules, their
448 conformation and interactions with other biomolecules, within the film at the interface.
449 Interestingly, these insights, in particular of conformational changes and mineralization
450 processes at interfaces may potentially be related to recent studies aiming at unraveling the links
451 between protein composition and conformations and their role in controlling biomineralization
452 [39].

453 Alginic acid was used in this study as a model polysaccharide foulant because acidic
454 polysaccharides were identified as major components of organic matter in wastewater effluents

455 [40,41]. Alginic acid showed the least extent of mineralization, as compared with the other
456 biopolymers.

457 All biopolymers exhibited increased compressibility when spread over SSE or after the
458 incubation, as a result from the accumulated calcium-phosphate minerals at the interface.
459 However, a pronounced decrease in compressibility with increasing incubation time was noted
460 for EPS, indicating increased resistance to compression in comparison to the other biopolymers
461 tested. Noteworthy, this finding may be related to the composition of the EPS film, which is
462 mainly of fatty acid-like molecules (56.6% humic substances) whereas the other films were
463 polymeric (biopolymer) materials.

464 As mineralization evolves over time the biopolymers may exhibit different tendencies, as for
465 example: Alginic acid and lysozyme may have an inhibitory role in the nucleation stage of
466 mineralization by sequestering calcium ion species [39] but at later stages induce mineralization
467 similar to the other biopolymers. Indeed in the experiments carried out here both alginic acid and
468 lysozyme showed no signs of mineralization in BAM measurements at time zero, but after 18
469 hours showed extensive mineralization as the other biopolymers, according to BAM and to Ca/P
470 ratios measured by ICP. The Ca/P ratios of films collected over the SSE solution after 18 hours
471 of incubation were much lower than the ratio in the SSE solution. The Ca/P ratio of the BSA film
472 which was incubated for 48 hours was substantially lower in comparison to that of the 18 hour
473 incubation (4.2 and 7.0, respectively), hence more minerals are accumulating at the interface
474 with time, resulting in much higher P concentrations relative to the SSE value (Ca/P = 89).
475 Another possible cause for lower Ca/P values in 48 hour is that the calcium-phosphate minerals
476 accumulated at the interface may be transformed into the more stable hydroxyapatite phase over
477 time, as the value obtained is closer to the value of hydroxyapatite (Ca/P = 1.67). Other
478 experiments with such long incubation times were not successful as the minerals accumulating
479 on the interface became heavy and films sunk into the subphase. Another reason for the deviation
480 of the detected Ca/P ratio from the theoretical value of hydroxyapatite can be the simultaneous
481 mineralization of calcium carbonate, as can be seen in the PM-IRRAS spectra of the different
482 model biopolymers, where a peak around 1460 cm^{-1} (representing carbonates) was detected. It is
483 worth mentioning that both calcium phosphate and calcium carbonate have positive saturation
484 indices in SSE solution: 6.68 and 0.79 respectively [12].

485 Although amorphous calcium phosphate (ACP) is below saturation in SSE solution (saturation
486 index -3.21) ACP prenucleation centers of 180 Å diameter and 8×10^{-6} volume fraction were
487 observed in four days old SSE solution using small-angle neutron scattering (SANS) as
488 published previously [12]. Stabilization of these particles by magnesium and other ions of the
489 SSE solution as proposed in [42] cannot be excluded.

490 This study showed for the first time that various biopolymers representing foulants in wastewater
491 treatment systems, as well as EPS, induce heterogeneous mineralization, mainly of calcium-
492 phosphate, at the interface of SSE solution. We showed that once both calcium and phosphate
493 became adsorbed and created nucleation sites, the mineralization continued to evolve. The
494 methodology presented here could be further utilized to characterize and quantify the extent,
495 phases and rates of mineralization by different biofoulants and different types of EPS. The
496 importance of this work lies in understanding the influence of EPS and biofouling-born
497 substances on calcium phosphate scaling in membrane based wastewater desalination. The
498 results support the previously reported notion [12-13] in desalination processes that pre-treatment
499 by removal of biopolymers as well as phosphate from the feed effluents before reverse osmosis
500 desalination, may be highly efficient in minimizing membrane fouling and scaling.

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510

511 **Appendices: Supplementary data**

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

515

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