Alginate Films as Macromolecular Imprinted Matrices

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Abstract
Macromolecularly imprinted polymers have been developed to mimic the non-covalent interactions driving molecular recognition in nature. The creation of an engineered antibody mimic would allow for the development of customizable films for biomolecular sensing. To demonstrate this principle, a cross-linked alginate film has been imprinted with bovine serum albumin (BSA) using aqueous biocompatible gelation methods. The imprinting efficiency of the synthesized films imprinted with BSA was determined and compared to the non-specific uptake of complementary proteins which were not imprinted in the alginate matrix. It was found that the recognition of the BSA using an alginate film was 6.4 mg/g polymer, which compares favorably to previously reported macromolecularly imprinted networks. The absorption of non-imprinted cationic proteins by the alginate matrix demonstrates that overcoming non-specific binding needs to be a focus of future work in order to successfully employ these materials towards biomolecular sensing within a physiological environment.

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Keywords
Molecularly imprinted polymer, alginate, hydrogel, bovine serum albumin

1. Introduction
Molecularly imprinted polymers (MIPs) are polymeric networks designed to specifically recognize a template molecule [1–3], often through non-covalent interactions and the formation of physical cavities (vacuoles) within the network which enhance the uptake of the template molecule. These engineered materials could be used to re-

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produce antibody–antigen interactions [4] and, therefore, would be useful to replace natural antibodies in biomolecular sensing applications. While small molecule imprinting has been demonstrated with high affinity, imprinting macromolecules, such as proteins, remains a challenge. The large size of macromolecular templates limits their diffusion through polymeric matrices. Protein conformation may be unstable, and non-covalent interactions may be significantly changed in organic solvents typically used for the synthesis of MIPs [5, 6]. Conversely, synthesis of imprinted polymer matrices in aqueous environments may impede the non-covalent interactions between the polymer matrix and the template molecule [7, 8]. Despite the challenges involved in imprinting polymeric matrices with protein templates, many successes have been reported and reviewed [9–13]. A wide variety of proteins have been imprinted, including human serum albumin [14], hemoglobin [15], ribonuclease A and lactalbumin [11]. Hydrogel materials, either synthetic or natural, are often selected since they present synthesis conditions which are advantageous for maintenance of the native protein conformation, can be customized to present non-covalent function groups, and it is possible to vary the hydrogel porosity to optimize the cavity formation and diffusion of the macromolecules through the gel. Typical bulk hydrogel imprinting with protein macromolecules results in differential absorption, also termed recognition, on the order of 1–100 mg/g polymer, as reported for bovine serum albumin and bovine hemoglobin, respectively [15, 16].

In this research, water-soluble alginate was used as a protein imprinting matrix. Alginates are linear, non-branched polysaccharides that can be ionically cross-linked into physical polymer networks [17]. Alginate is a natural material, harvested from brown algae, then processed to varying grades depending on the purity required for a given application. This natural biomaterial has found widespread use as a food additive, in pharmaceutical formulations, in wound healing and in tissue engineering [18].

The chemical structure of sodium alginate, shown in Fig. 1, consists of 1,4′-linked β-D-mannuronic acid and α-L-guluronic acid residues, in varying amounts arranged in blocks along the chain backbone. Physical networks are formed by the exchange of sodium ions associated with the guluronic acid residues with divalent cations in the cross-linking solutions. The guluronic residues stack to form a characteristic egg-box structure. Dimerization of the alginate chains occurs through the divalent cations, as illustrated in Fig. 2, causing junctions between many chains to create a network structure [19].

Specific properties of alginates enable their use as biomaterials. Alginates are cross-linked in conditions which are mild, not requiring heat, oxidants, or organic solvents [19]. These mild conditions permit the incorporation of macromolecules such as proteins, and even cells [20], into the network, without modification of the biological activity of the proteins or cells. Alginates are generally considered biocompatible and non-immunogenic due to their chemical composition [18], although mitogenic impurities found in commercial grade alginate can cause an immune response, and therefore an ultra-pure grade of alginate should be used in applications.
where biocompatibility is critical [21]. The formation of matrix blocks, large beads and microbeads is easily accomplished, and the materials allow for some control of the pore size, degradation rate and mechanical properties, for example, by using an alginate with specific ratios of mannuronic acid to guluronic acid.

Alginate networks incorporating proteins have been studied as controlled-release drug delivery systems [19, 22]. Small molecules diffuse freely through the material, since it contains up to 95% water, but the diffusion of larger molecules may be affected by the material porosity, the erosion of the material and non-covalent interactions between the proteins and the alginate.

Although the biocompatibility and biodegradability of alginates are extensively documented, only limited literature reports the use of alginate capsules to achieve the macromolecular imprinting. The reported imprinting methods use an inverse suspension method which involves the use of organic chemicals such as chloroform and hexane, which are incompatible with medical applications [6, 7]. The work of Zhang et al. [23] was the first attempt at macromolecular imprinting using calcium-alginate-based microcapsules via an inverse suspension method using hydroxyethyl
cellulose to improve the mechanical properties of the microspheres [23]. Subsequent research in this area included the introduction of an emulsion technique to generate surface imprinted alginate microspheres [24], the use of calcium phosphate to cross-link the alginate [25] and the addition of polyacrylate to the polymer [26] to further improve mechanical stability and thereby increase the imprinting of BSA. The highest BSA equilibrium absorption capacity, \( Q_e \), obtained using the emulsion template techniques was approx. 10 mg/g polymer for the MIP, in comparison to approx. 3 mg/g non-imprinted polymer synthesized under the same conditions in absence of the template molecule, resulting in a differential recognition of 7 mg/g polymer [26].

Recently, Herrero and Peppas have demonstrated protein imprinting by means of calcium alginate-based polymer capsules via ionic gelation using biocompatible processing methods. This first attempt at a biocompatible macromolecular imprinting method to form alginate microcapsules, without the use of organic chemicals, was able to achieve recognition of up to 3.0 mg BSA/g capsules [27, 28].

The work presented here makes several modifications to the previously reported systems. First, we are interested in synthesis of a molecularly imprinted alginate film, rather than the formation of microbeads, allowing integration of the films into biomolecular sensors. Second, we find improved binding of BSA to the imprinted film in comparison to previously reported results using an aqueous imprinting method [27, 28]. Finally, proteins varying in charge and size are equilibrated with the imprinted alginate films to elucidate the mechanism of the binding interaction.

The research towards alginate imprinted polymers described here anticipates the non-covalent binding of the carboxyl group of the alginate polymer with the template molecule functional groups. The low pH of the solution during the cross-linking, which is below the pI of BSA, creates a positively charged, hydrophilic molecule, which non-covalently interacts with the anionic alginate. In the case of BSA, at a pH of 4.2 ionic interactions may occur between the alginate and histidine, as shown in Fig. 3. Raising the pH above the pI of BSA will cause a repulsion effect between the negatively charged BSA and the negatively charged alginate and break the ionic interactions, as shown in Fig. 4 for glutamic acid. Alginate may also form hydrogen bonds with a template molecule, which will be unaffected by the modulations in pH.

2. Materials and Methods

2.1. Gelation of Alginate MIPs

Molecularly imprinted alginate hydrogel films were prepared by cross-linking sodium alginate in the presence of the template protein, bovine serum albumin (BSA). Deionized (DI) water was titrated to a pH of 4.2 with HCl (Fisher Scientific). To form the imprinting solution, 200 mg BSA (Sigma-Aldrich) was dissolved
in 20 ml DI water at pH 4.2. Next, 0.4 g sodium alginate (Sigma-Aldrich) was added and stirred until dissolved. To form the control solution, 0.8 g sodium alginate was

Figure 3. Proposed ionic bond between histidine (an abundant amino acid in BSA) and alginate during the imprinting process.

Figure 4. Proposed repulsion between glutamic acid (an abundant amino acid in BSA) and alginate during the extraction process.
added to 40 ml DI water at pH 4.2 and stirred until dissolved. Solutions were stored at 4°C until use.

To cross-link films, 1.5 g of the alginate solutions was dispensed into a Petri dish with a 5-cm diameter. The solution was leveled, and then 5 ml of an aqueous 2% calcium chloride (CaCl2) (Fisher Scientific) solution was pipetted over the alginate. The alginate was allowed to cross-link for 4 min.

2.2. Release of Template from Alginate MIP

After cross-linking, the films were rinsed and the release of BSA from the alginate rinse solutions was monitored. First the films were rinsed in a 0.05 M Tris-HCl (pH 7.4) solution with 1% CaCl2, which was changed hourly for 3 h, and then the films were rinsed in DI water. The water was changed daily until a sample of the rinse solution had an absorbance <0.003 OD, as measured at 280 nm on a Lambda 10 UV-Vis spectrophotometer (Perkin Elmer). A calibration curve developed from a serial dilution of a 1 mg/ml solution of BSA was used to calculate the amount of BSA in each rinse solutions.

2.3. Recognition of Alginate MIP

Recognition studies were performed by incubating cross-linked and rinsed alginate films with BSA solutions. A 1 mg/ml solution of BSA in deionized water was prepared, and 50 ml of the solution was added to a 50 ml polypropylene centrifuge tube with the alginate film. The samples were placed on a rotary mixer and the solution was sampled daily to read the absorbance of the solution at 280 nm using a UV-Vis spectrophotometer (Lambda 10, Perkin Elmer) until equilibration was reached. To calculate the absorption of BSA at equilibrium, equation (1) was used:

$$Q_e = \frac{(C_0 - C_{eq})V}{W}$$

where $Q_e$ is the equilibrium absorption, $C_0$ is the initial protein concentration, $C_{eq}$ is the final concentration of protein at equilibrium, $V$ is the volume of the protein solution and $W$ is the weight of the polymer incubated in the solution. Imprinting efficiency, IE, can then be defined as shown in equation (2):

$$IE = \frac{Q_{MIP}}{Q_{NIP}}.$$ 

The interaction between an imprinted polymer and the template molecule can be compared to an antigen–antibody binding interaction. This association and dissociation of the complex can be symbolized as:

$$[P] + [L] \xrightleftharpoons[k_f]{k_r} [PL],$$

where [P] is the concentration of unbound antibody, [L] is the concentration of unbound ligand, [PL] is the concentration of protein and ligand which are bound, $k_f$ is the forward reaction rate and $k_r$ is the reverse reaction rate of association. A standard
metric for the assessment of the strength of this interaction is the dissociation constant, or $K_D$. One can also define the equilibrium binding constant, $K_{eq}$, as shown in equation (4):

$$K_{eq} = \frac{k_f}{k_r} = \frac{1}{K_D} = \frac{[PL]}{[P][L]}.$$  \hspace{1cm} (4)

The dissociation constant was used to compare the strength of the interaction between the polymer film and the template molecule with traditional antibody–antigen interactions. In our case, $[P]$ becomes the concentration of possible binding sites in the polymer film, $[L]$ is the concentration of template in solution and $[PL]$ is the concentration of template absorbed and presumably bound in the polymer film, all observed at equilibrium.

### 2.4. Specificity of Alginate MIP

Proteins with varying molecular mass and isoelectric points (pI), provided in Table 1, were selected for determining the specificity of the alginate MIP for BSA (all proteins obtained from Sigma-Aldrich). Ovalbumin is a protein found in abundance in egg white, with a pI similar to BSA but with a lower molecular mass. It is expected that ovalbumin would bind non-specifically to any cavities formed in the BSA-imprinted films. Hemoglobin (Hb) is the oxygen-transporting protein found in red blood cells. The bovine hemoglobin selected for these experiments has a molecular mass similar to BSA, but with a higher pI. In water, Hb should be close to neutral in charge. Human serum albumin (HSA) has a similar molecular mass and pI, but a different structure from that of BSA.

Alginate hydrogels used in the selectivity experiments were prepared as described above. Films were then incubated in 1 mg/ml solutions of the competitor proteins selected and the absorbance of each solution was measured daily. The absorbance of the ovalbumin solutions were read on the Lambda 10 UV-Vis spectrophotometer in a cuvette at 280 nm. The absorbance of the Hb solutions was read on the same spectrophotometer at 405 nm. The absorbance of the HSA samples was read using a microplate reader (Synergy HT, Biotek) at a wavelength of 280 nm.

*Table 1.*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular mass (kDa)</th>
<th>pI</th>
<th>Hydrodynamic radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>66</td>
<td>4.9</td>
<td>120</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45</td>
<td>4.7</td>
<td>27.4</td>
</tr>
<tr>
<td>HSA</td>
<td>69</td>
<td>4.8</td>
<td>34.5</td>
</tr>
<tr>
<td>Hb</td>
<td>68</td>
<td>6.8</td>
<td>55</td>
</tr>
</tbody>
</table>
To analyze the selectivity of the alginate hydrogels for the template protein, the selectivity, $\alpha$, was calculated using equation (5):

$$\alpha = \frac{K_{a,\text{template}}}{K_{a,\text{competitor}}},$$

where the equilibrium affinity constant is calculated using equation (4).

2.5. Swelling of Alginate MIP

Since the degree of swelling and, therefore, the pore size, of the alginate hydrogels may be affected by the imprinting process, the swelling of the films was monitored after synthesis. The cross-linked polymer films, imprinted as described above, using non-imprinted polymer films cross-linked without BSA as controls, were weighed immediately after synthesis. The films were incubated in deionized water, removed from the incubation solution each day, blotted to remove excess solvent and weighed.

The degree of swelling, $Q$, was calculated using equation (6):

$$Q = \frac{W_0 - W_t}{W_0},$$

where $W_0$ is the as-synthesized initial weight, and $W_t$ is the weight at time $t$.

3. Results and Discussion

3.1. Gelation of Alginate MIP

Upon exposure to the divalent CaCl$_2$ solution, a semi-opaque, mechanically stable film quickly formed. This film could be transferred with tweezers to the Tris buffer rinse solution upon completion of the cross-linking. The thickness of the resulting films was approx. 1 mm.

3.2. Release of Template from Alginate MIP

The release of BSA from the rinse solutions is shown in Fig. 5. Consistently, approx. 10 mg BSA was detected in the rinse solutions, while 15 mg BSA was in the polymer solution prior to cross-linking. A significant portion of the BSA is likely removed during the cross-linking process, since cross-linking induces significant de-swelling of the gel. As the solvent is expelled from the polymer when it cross-links, the template molecule is also pushed out of the film.

3.3. Recognition of Alginate MIP

Preferential absorption of the imprinted BSA template was demonstrated through equilibrium recognition studies. The calculated values for $Q_e$ (in units of mg/g polymer) are shown in Table 2. The amount of BSA absorbed was 6.4 mg/g polymer, which compares favorably to previously reported values of approx. 0.3 mg/g polymer [23]. Using the equation for the dissociation constant, the $K_{eq}$ of the BSA
imprinted alginate polymer was found to be 6 mM. This is several orders of magnitude above the dissociation constants found for small molecule imprinted polymers [9], and above the reported binding affinity of an epitope approach to BSA imprinting [29], which indicates a weaker interaction between the polymer matrix and the protein. However, these results are similar to the results of other work demonstrating polymeric imprinting BSA in aqueous media, which have demonstrated adsorption capacities on the order of 5 mg/g polymer [16], and surface imprinted microbeads, with demonstrated adsorption capacities of 1.4 mg/g polymer [14]. The absorption of BSA is shown as a function of time in Fig. 6. A single replicate (Fig. 6b) attains equilibrium within 6 days. Since the recognition experiment occurred in water, Ca^{2+} ions will be exchanged and the film will slowly degrade [30], causing the loss of recognition sites for BSA over time. This gradual degradation accounts for the decrease in absorption of BSA at longer time points. The time to reach equilibrium varied between 3 and 6 days.

Table 2.

$Q_e$ of alginate films imprinted for BSA (MIP) and non-imprinted (NIP), average of four recognition experiments ($n = 3$ for each experiment)

<table>
<thead>
<tr>
<th>Sample</th>
<th>$Q_e$ (mg/g polymer)</th>
<th>Imprinting efficiency (IE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP</td>
<td>6.4</td>
<td>64</td>
</tr>
<tr>
<td>NIP</td>
<td>0.1</td>
<td>--</td>
</tr>
</tbody>
</table>

Figure 5. Cumulative amount of BSA released from the imprinted alginate film during 0.05 M Tris-HCl (pH 7.5) with 1% CaCl$_2$ rinse (hours 1, 2 and 3, shown in detail in inset) and during deionized water rinse (remaining timepoints) ($n = 3$). Error bars represent ±1 SD. Exponential fit to data is shown.
Table 3.
Selectivity comparison

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\alpha$</th>
<th>IE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>$1.6 \times 10^{-6}$</td>
<td>1.1</td>
</tr>
<tr>
<td>Hb</td>
<td>$1.4 \times 10^{-7}$</td>
<td>1.0</td>
</tr>
<tr>
<td>HSA</td>
<td>$-1.0 \times 10^{-5}$</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Films imprinted with BSA, exposed to either BSA or a competitive molecule ($n = 3$).

3.4. Specificity of Alginate MIP

The results of the specificity experiments reveal that the imprinting efficiency of the BSA imprinted alginate films is significantly lower for the selected proteins than for the BSA itself, meaning that the amount protein absorbed by the MIP is similar to the amount of protein absorbed by the NIP for ovalbumin, Hb and HSA, as shown in Table 3. The selectivity of the films, calculated using equation (6), is insufficient for ovalbumin and Hb. In the case of ovalbumin, precipitation of the polymer occurred during the course of the experiment, making the standard specificity calculation inaccurate — a significant amount of the protein was simply precipitated out of solution, rather than absorbed into the imprinted alginate film. The imprinting efficiency results demonstrate this more clearly, in that the MIP does not absorb more OVA than the NIP. In the case of Hb, it is likely that the predominance of positively charged residues on the protein, at the pH under test, allows the Hb to non-specifically bind to the anionically charged alginate. This experiment demonstrates that though the molecular imprinting appears to be a real effect, the application of the films in physiological conditions may be limited due...
to significant non-specific binding of positively charged species in physiological conditions.

3.5. Swelling of Alginate MIP

The swelling behavior of the imprinted alginate films was similar to that of the non-imprinted alginate films. As shown in Fig. 7, the MIP and NIP films present similar the degree of swelling and swelling kinetics. The existence of similar swelling kinetics in both the imprinted and non-imprinted films indicates that the mesh size of the alginate films is sufficiently large that the presence of imprinted cavities within the film does not enhance the transport of solvent into the film, and the effect of the imprinting process upon the swelling behavior of the alginate films is minimal.

4. Conclusions

We have found that the binding of BSA to alginate films is comparable, and in some cases improved, over large macromolecular imprinted results previously cited in the literature [23, 26, 30, 31]. Although in the experiments presented here the equilibration was reached after 6 days, this is due to the considerable thickness of the films. If the films were reduced in thickness from 1 mm to 1 µm, the time scale should be reduced from days to minutes, as has been demonstrated with alginate microbeads [25]. Reduction of the film thickness can be achieved through spin-coating and other micro and nanofabrication techniques.

Although the imprinted versus non-imprinted films absorb the competitive proteins equally, some proteins exhibit high degrees of non-specific binding to the alginate films. This is due to the ionic interactions between a positively charged molecule and the negatively charged alginate — despite the presence of binding cavities preferential for BSA, the bulk alginate attracts a considerable amount of Hb, which has a higher pI. In addition to a careful design of the functional interactions, it is clear from these results there must also be some consideration for avoidance of non-specific binding, perhaps by incorporation of poly(ethylene gly-
col) (PEG), which could shield the bulk polymer from non-specific binding. Little attention has been paid to this issue in literature, possibly since often these materials will be used for chromatography applications where the composition of the solution is more controlled in comparison to biomedical applications where the physiological environment is quite complex.

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