Separation and purification of hyaluronic acid by glucuronic acid imprinted microbeads

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Abstract

The purification of hyaluronic acid (HA) is relatively significant to use in biomedical applications. The structure of HA is formed by the repetitive units of glucuronic acid and N-acetyl glucosamine. In this study, glucuronic acid-imprinted microbeads have been supplied for the purification of HA from cell culture (Streptococcus equi). Histidine-functional monomer, methacryloylamidohistidine (MAH) was chosen as the metal-complexing monomer. The glucuronic acid-imprinted poly(ethylene glycol dimethacrylate–MAH–Copper(II)) [p(EDMA-MAH-Cu2+)] microbeads have been synthesized by typical suspension polymerization procedure. The template glucuronic acid has been removed by employing 5 M methanolic KOH solution. p(EDMA-MAH-Cu2+) microbeads have been characterized by elemental analysis, Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) images and swelling studies. Moreover, HA adsorption experiments have been performed in a batch experimental set-up. Purification of HA from cell culture supernatant has been also investigated by determining the hyaluronidase activity using purified HA as substrate. The glucuronic acid imprinted p(EDMA-MAH-Cu2+) particles can be used many times with no significant loss in adsorption capacities. Also, the selectivity of prepared molecular imprinted polymers (MIP) has been examined. Results have showed that MIP particles are 19 times more selective for glucuronic acid than N-acetylglucose amine.

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1. Introduction

Hyaluronic acid (HA) is a naturally occurring linear polysaccharide composed of glucuronic acid and N-acetylglucosamine repeats via a β-1,4 linkage. HA-based biomaterials secure wide-range applications in the healthcare industry. The materials derived from HA are exploited to relieve the pain of knee osteoarthritis, in cataract surgeries, in abdominal and pelvic surgeries, and as a dermal filler to erase facial wrinkles. Moreover, an aging population, several product innovations, and underserved end-user segments are creating a huge market for HA-based biomaterials [1–4].

Hyaluronan was biochemically purified in 1934 by Karl Meyer and John Palmer [5] from the highly viscous vitreous humor of bovine eyes. It was also isolated from certain strains of bacteria, namely hemolytic streptococci [6]. Various methods have been described for the separation of hyaluronan oligomers. Most of these involve the digestion of polymeric HA with the endohydrolase, testicular hyaluronidase, followed by purification by size-exclusion chromatography [7,8], ion-exchange chromatography [9–11] and reversed-phase ion pair high performance liquid chromatography [12]. These methods result in even-numbered oligosaccharides for which the minimal size is HA4 (i.e., 2 disaccharide units in length). The separation and purification of HA involves the precipitation of HA from fermentation broth by repeatedly using large amounts of organic solvents such as ethanol, acetone, isopropanol, etc. [13,14]. However, the complicated and time consuming process involved leads to high cost. Although membrane technology was used in some purifying processes [15,16], it was solely operated to concentrate HA solution or to remove small soluble molecules. Hence, as the case stands it can be argued that further processes are needed.

Molecular imprinting is a method for making selective binding sites in synthetic polymers using molecular template. Target molecules (i.e. glucuronic acid) can be used as templates for imprinting crosslinked polymers. After the removal of the template, the remaining polymer seems to be more selective. The selectivity of the polymer depends on various factors be it the size and shape of the cavity and the rebinding interactions. Covalent interactions, noncovalent interactions and metal ion coordination can be exploited to organize the functional monomers around the template. In terms of strength, specificity and directionality, the metal coordination interaction resembles more a covalent interaction than hydrogen bonding or electrostatic interactions in water [17]. These features make metal coordination a promising binding for the preparation of highly specific templated polymers for the recognition of proteins, via the arrangements of metal coordinating ligands on their
D-Glucuronic acid, hyaluronidase and metacryloylchloride were supplied by Sigma (St. Louis, MO, USA) and used as received. Ethylene glycol dimethacrylate (EDMA) and azobisobutyronitrile (AIBN) were purchased from Fluka A.G. (Buchs, Switzerland). All other reagents, unless specified, were of reagent grade and were used without further purification. Laboratory glassware was kept overnight in a 5% HNO₃ solution. Before employed in the experimental process, the glassware was rinsed with deionized water and dried in a dust-free environment. Buffer and sample solutions were prefiltered through a 0.2 μm membrane (Sartorius, Göttingen, Germany). All water utilized in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731). This was followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system.

Functional monomer, MAH, and metal chelate monomer, [MAH-Cu(II)], were synthesized according to the previously published procedures [21,22].
where $W$ was recorded. The swelling ratio was calculated by using the following expression (Eq. (1)):

$$\text{Swelling ratio} \% = \left( \frac{W_s - W_0}{W_0} \right) \times 100$$

where $W_0$ and $W_s$ are the weights of particles before and after swelling respectively.

2.4. Equilibrium binding analyses and selectivity studies

The capacity of the adsorbent for HA was determined in batch mode. Adsorption capacity of DGA-imprinted microbeads was determined with pure HA in the equilibration buffer. The equilibration buffer was prepared at pH 6.0 in 200 mM NaCl and 50 mM NaOAc buffer. This buffer was adjusted to the desired pH by adding 0.1 M NaOH and 0.1 M HNO3.

The pH was maintained in a range of ±0.1 until the equilibrium was attained. In all experiments, polymer concentration was kept constant at 50 mg/10 ml. The adsorption experiments were conducted at 25 °C for 1 h with magnetic stirring. Total concentration of HA was determined after a minimum of 15 min., the SDS culture was grown on M17 agar (Fluka) for 24 h at 37 °C. For the production of HA, firstly, a test tube containing 10 ml of M17 broth (Fluka) with 5 mg of glucose ml$^{-1}$ was inoculated from agar plate. After a 24 h incubation period at 37 °C, 2 ml of this culture was transferred to 100 ml of fermentation broth which embodied the following (grams per liter of distilled water): glucose, 20.0; yeast extract, 10.0; Na2HPO4.12H2O, 2.5; and MgSO4.7H2O, 0.5 in a 250-ml erlenmeyer flasks. Glucose solutions were autoclaved separately and mixed aseptically with the other components on cooling. The microorganisms were developed and HA production was initiated at 37 °C and 250 rpm for 20 h.

2.5. Bacterial strain and production of HA

*S. equi* RSKK 679 was obtained from Refik Saydam National Type Culture Collection (RSKK), Ankara, Turkey. The culture was grown on M17 agar (Fluka) for 24 h at 37 °C. For the production of HA, firstly, a test tube containing 10 ml of M17 broth (Fluka) with 5 mg of glucose ml$^{-1}$ was inoculated from agar plate. After a 24 h incubation period at 37 °C, 2 ml of this culture was transferred to 100 ml of fermentation broth which embodied the following (grams per liter of distilled water): glucose, 20.0; yeast extract, 10.0; Na2HPO4.12H2O, 2.5; and MgSO4.7H2O, 0.5 in a 250-ml erlenmeyer flasks. Glucose solutions were autoclaved separately and mixed aseptically with the other components on cooling. The microorganisms were developed and HA production was initiated at 37 °C and 250 rpm for 20 h.

2.6. Purification of HA using of DGA imprinted microbeads

For the pre-purification of the fermentation medium, at least 0.01% sodium dodecyl sulfate (SDS) was added to the culture in order to release HA from the cells. After a minimum of 15 min., the SDS culture is titrated for flocc formation after addition of varying amounts of a 10% solution of hexadecyltrimethylammonium bromide. This precipitate
was then solubilized in 2 M CaCl₂ of approximately 1/10 to 1/20 the original volume. The resulting suspension was centrifuged and supernatant was collected.

After the pre-purification of the fermentation medium, HA was separated from supernatant. The supernatant solution was used as a HA source. DGA imprinted particles (100 mg) were treated with 10 ml of supernatant solution for 2 h by magnetic stirring at 150 rpm. The particles were then centrifuged and washed to remove unbound molecules. The amount of adsorbed HA on the DGA imprinted particles was determined by measuring the initial and final concentrations of HA within the adsorption medium. Then, DGA imprinted particles were placed in the desorption medium and stirred for 2 h at 25 °C, at a stirring rate of 600 rpm. The desorption of HA from p(EDMA-MAH-Cu²⁺) microbeads was performed with 1 M NaOH. The determination of desorbed HA was maintained spectrophotometrically at 232 nm by adding hyaluronidase enzyme. One unit hyaluronidase activity was defined as the amount of enzyme causing an increase of 0.001 optical density value per minute at 25 °C and pH 7.0.

3. Results and discussion

3.1. Characterization of DGA microbeads

The elemental analysis results showed that the p(EDMA-MAH-Cu²⁺-DGA) contains 24.00% C; 6.536% H and 0.031% N before the removal of template, DGA. After template removal, the elemental composition of p(EDMA-MAH-Cu²⁺) stood at 41.46% C; 6.680% H and 0.059% N. The removal of template can be confirmed by the increasing ratio of C, N and H.

3.2. Adsorption isotherm of HA to DGA imprinted microbeads

Adsorption of HA from aqueous solutions was investigated in batch experiments. As can be observed from Fig. 4, the amount of adsorbed HA per unit mass of glucuronic acid imprinted beads increased with

![Fig. 3. SEM images of DGA imprinted microbeads.](image)

![Fig. 4. The effect of HA concentration on the amount of adsorbed HA.](image)

![Fig. 5. Langmuir and Freundlich adsorption isotherms of binding of HA by the imprinted microbeads.](image)

![Fig. 6. Scatchard’s plot of HA rebinding by the imprinted microbeads.](image)

The functional groups of DGA imprinted microbeads were characterized by FTIR spectroscopy. The bands correspond to the amide II absorption band at 1528 cm⁻¹, carbonyl band at 1659 cm⁻¹, Cu–O stretching band at ~536 cm⁻¹ and stretching band for carboxyl at 1727 cm⁻¹. The peaks at 536 and at 1727 cm⁻¹ belonging to DGA disappeared after the removal of DGA.

Furthermore, SEM images of microbeads were obtained. As can be seen clearly in Fig. 3, microbeads have very porous structure. The MIP particles are crosslinked hydrophilic matrices. In water, the equilibrium swelling ratios of the NIP and MIP particles used in this study are 30% and 42% in, respectively. Compared with NIP particles, the swelling ratio of the MIP particles increases in swelling media. Formation of template cavities in the polymer structure introduces more hydrodynamic volume into the polymer chain, which can adsorb more solvent molecules. It should be noted that these polymers are quite rigid, and durable enough due to cross-linked structure. As such, these MIP particles are suitable for possible packed-bed applications.
the initial concentration of the HA. The maximum adsorption capacity was 810 mg g\(^{-1}\) of glucuronic acid imprinted beads.

When adsorption data of HA were fitted against Langmuir and Freundlich isotherms, satisfactory fits were found with the Langmuir isotherm. The Langmuir isotherm is a valid monolayer sorption on a surface containing a finite number of binding sites. The Langmuir constant was calculated as 5.51 × 10\(^{-3}\) 1 mg\(^{-1}\) from this plot and the \(R^2\) value of Langmuir plot was found to be 0.9882 (Fig. 5).

The association constant (\(K_a\)) for the specific interaction between the template imprinted beads and the template (HA) itself was determined by Scatchard’s plots using glucuronic acid imprinted beads. Fig. 6 shows the Scatchard plot including two different lines. This situation suggests that the DGA imprinted beads have two binding sites for HA. The \(K_a\) for the first binding of HA to DGA imprinted beads is 7.50 × 10\(^3\) M\(^{-1}\) and the maximum number of ligand-exchange interaction sites, \(Q_{\text{max}}\), is 1.26 μmol g\(^{-1}\). The \(K_a\) for the second binding of HA to MIP receptor is 9.49 × 10\(^6\) M\(^{-1}\) and the maximum number of ligand-exchange interaction sites, \(Q_{\text{max}}\), is 0.56 μmol g\(^{-1}\). The values of \(K_a\) suggest that affinity of the binding sites is very durable as well as biological receptors (10\(^{-5}\)–10\(^{-3}\)). \(R^2\) values of these plots were found to be 0.9976 and 0.9997 for the first and second binding of HA to MIP receptor, respectively.

### 3.3. Selectivity of DGA imprinted microbeads

The adsorption capacity of glucuronic acid imprinted polymers for the adsorption of HA was evaluated as well as a related molecule N-acetylglucosamine. N-acetylglucosamine and glucuronic acid are two repetitive units of HA. But also glucuronic acid and N-acetylglucosamine have different molecular structure. According to experimental studies; \(Q_{\text{max}}\) values of glucuronic acid and N-acetylglucosamine were determined as 232.8 and 12 mg g\(^{-1}\) for MIP beads, respectively. As shown from the above data, p(EDMA-MAH-Cu\(^{2+}\)) microbeads were 19 times selective for glucuronic acid than N-acetylglucosamine. Consequently, the results indicate that p(EDMA-MAH-Cu\(^{2+}\)) microbeads have higher adsorption capacities for glucuronic acid than for N-acetylglucosamine. This situation did not cause as a surprise since imprinting creates shape selective cavities within the polymer structure.

### 3.4. Regeneration of DGA imprinted microbeads

Regeneration is a crucial step in all affinity chromatography techniques. It was thus necessary to evaluate the regeneration efficiency of the affinity adsorbents after each cycle. In this study, more than 90% of the adsorbed HA molecules were eliminated easily from the p(EDMA-MAH-Cu\(^{2+}\)) microbeads in all instances when 0.10 M NaOH was used as a desorption agent. It has to be noted that there was no Cu\(^{2+}\) release in desorption cycle which shows that metal–chelate monomer MAH-Cu(II) are polymerized on the beads surface by strong chelate formation.

In order to show the reusability of the glucuronic acid imprinted beads, the adsorption–desorption cycle was repeated 10 times using the same imprinted beads from aqueous HA solution. It emerged that there was no significant loss in the adsorption capacity of the microbeads. As seen from Fig. 7, the results showed that the adsorption capacity of microbeads was decreased by only 11% after a 10 adsorption–desorption cycles. These results demonstrated the stability of the present solid support as an imprinted affinity beads.

### 3.5. Purification of HA by using glucuronic acid imprinted beads

The classical HA purification method required several steps, such as precipitation, centrifugation and affinity adsorption. The p(EDMA-MAH-Cu\(^{2+}\)) microbeads provided an efficient single step method to purify HA from bacterial culture supernatant, showing high adsorption capacity (810 mg g\(^{-1}\)) and high selectivity for HA. The amount of the purified HA with imprinted particles was 2268 ± 33 mg l\(^{-1}\) in supernatant of bacterial cell culture.

### 4. Conclusion

In this study, HA was purified from S. equi by using of glucuronic acid imprinted polymer [p(EDMA-MAH-Cu\(^{2+}\))] based on metal–chelate interactions. The predominant advantage of p(EDMA-MAH-Cu\(^{2+}\)) microbeads lies in its simplicity, stability and cheapness of the support. Furthermore this prepared support can be used in aqueous solution. The maximum HA adsorption capacity was found as 810 mg g\(^{-1}\) of DGA imprinted microbeads. The association constant (\(K_a\)) was determined as 7.50 × 10\(^3\) M\(^{-1}\) for the first binding of HA and 9.49 × 10\(^6\) M\(^{-1}\) for the second binding of HA to DGA imprinted microbeads. These values suggest that affinity of the binding sites is very durable as well as biological receptors (10\(^{-5}\)–10\(^{-3}\)). The selectivity of p(EDMA-MAH-Cu\(^{2+}\)) microbeads for glucuronic acid was estimated by using N-acetylglucosamine and it was observed that MIP particles were 19 times more selective for glucuronic acid than N-acetylglucosamine. It can be concluded that based on our findings HA was adsorbed onto MIP sorbent by glucuronic acid unit. Also, it was observed that DGA imprinted microbeads can be used several times without loss in adsorption capacity. Finally, the results showed that MIP beads designed for HA purification have high selectivity, low cost and are compatible with biological systems.

### References