
Selective binding of carcinoembryonic antigen using imprinted polymeric hydrogels

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Abstract: A poly(allylamine hydrochloride) carcinoembryonic antigen-imprinted hydrogel was synthesized using a water-soluble crosslinker, ethylene glycol diglycidyl ether, to investigate its viability for protein recognition. The imprinting factor of the imprinted hydrogel toward carcinoembryonic antigen was found to be ~ 5 , while the imprinting factor of the imprinted hydrogel toward α -feto-protein was determined to be ~ 2 , suggesting selectivity

and specificity toward the template protein. This work lays the foundation for the development of a novel line of imprinted hydrogel systems capable of protein recognition for diagnostic and therapeutic applications. © 2008 Wiley Periodicals, Inc. *J Biomed Mater Res* 87A: 359–363, 2008

Key words: carcinoembryonic antigen; hydrogel; molecular imprinting; protein recognition

INTRODUCTION

Carcinoembryonic antigen (CEA) is a 180 kDa glycosylated surface protein overexpressed on most human adenocarcinomas including colon, rectum, pancreas, and breast.¹ CEA is a globular protein, soluble in water, with an isoelectric point of ~ 4.7 .² The low isoelectric point of CEA suggests that the protein has an overall negative charge in its native environment within the body. Although the overall charge of the protein is negative above pH 4.4, there are no concentrated regions of charge.

CEA is believed to function as a homotypic intercellular adhesion molecule, vital in organizing the structure of the fetal colon.³ Recent evidence suggests that deregulated expression of CEA has an instrumental role in tumorigenesis through the inhibition of terminal cell differentiation and the disruption of tissue architecture.^{4,5} In the past, CEA has been used as a marker to monitor cancer development, yet recently research has begun investigating the protein's potential within cancer therapy.^{6–9}

Polymers with a high affinity to specific proteins in aqueous media have the potential to be used as substrates in medical diagnostic and clinical applications. A hydrogel synthesized using the technique of

molecular imprinting, having a high affinity to CEA antigen and loaded with a therapeutic drug, could be used to effectively target cancer cells.

Molecular imprinting is a technique that creates synthetic materials containing specific receptor sites having a high affinity for a target molecule. Three-dimensional cavities are created within a polymer matrix complementary to the size, shape, and functional group orientation of the target molecule. The size and shape of the cavity allow the target molecule or similar molecules to occupy the cavity space, while the functional group orientation within the cavity will preferentially bind in specific locations to only the target molecule and not to similar molecules. We refer to the term "molecular imprinting" only as a technique and do not imply that imprinting has been achieved on the resulting hydrogel at the molecular level.

Research in the past decades has focused on the imprinting of low molecular weight molecules rather than large molecules, that is proteins, due to the many challenges facing protein imprinting such as poor mass transport, cross-reactivity of sites, fragile nature of proteins, and solvent problems.^{10,11} By using a cationic hydrogel, synthesized using water-soluble monomers and crosslinker, several of these challenges may be overcome. The hydrogel exhibits slight swelling (10–20%) around pH of 7, which could allow for better mass transport within the polymer matrix. Since the polymer, crosslinker, and protein template are all soluble in water, the entire imprinting process can be completed under conditions, which

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maintain the integrity of the protein. If successful, this research will lay the foundation for the development of polymers capable of selective and specific recognition of biomacromolecules for diagnostic and therapeutic applications. In this respect, a hydrogel created using the technique of molecular imprinting is unique in having the ability to recognize target proteins and the ability to release molecules, that is therapeutics, in an intelligent manner based on pH, temperature, ionic concentration, or time.

We have developed an imprinted poly(allylamine hydrochloride) (PAA-HCl) hydrogel, using a water soluble crosslinker, ethylene glycol diglycidyl ether (EGDE), specific for CEA based on noncovalent interactions. Electrostatic interactions between the positively charged amine groups on the polymer and negatively charged amino acids, that is aspartate and glutamate, form the basis of the selective interaction. Furthermore, PAA-HCl-based hydrogels have the ability to selectively bind sugar residues, which should be beneficial when imprinting glycosylated proteins like CEA.¹² All these characteristics make PAA-HCl hydrogel an ideal choice for CEA imprinting. The main purpose of this experiment was to investigate the potential for a primary amine-based hydrogel, and cationic hydrogels in general, for use in protein imprinting. The synthesis of MIPs, which exhibit high affinity, selectivity, binding capacity, and low nonspecific binding, may provide researchers with a low-cost, easily obtainable method for studying the fundamental interactions, which occur during biological recognition processes.

EXPERIMENTAL

Materials

Poly(allylamine hydrochloride) ($M_w = 15,000$) and ethylene glycol diglycidyl ether (50%, technical grade) were purchased from Sigma-Aldrich (Milwaukee, WI). Sodium hydroxide (99.998%) and hydrochloric acid (36–38.5%) were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Carcinoembryonic antigen (>98% purity, sterile filtered) and α -fetoprotein (AFP) (>98% purity, sterile filtered) were purchased from Fitzgerald Industries International (Concord, MA). Puradisc 25AS polyethersulfone membrane filters were purchased from Whatman (Florham, NJ). Deionized water (DI water) was obtained using a Millipore Super-Q water system. All chemicals were used as received.

Methods

Ultraviolet-visible light spectrophotometer calibrations

All ultraviolet-visible light (UV-vis) spectrophotometric measurements were obtained using a Perkin-Elmer

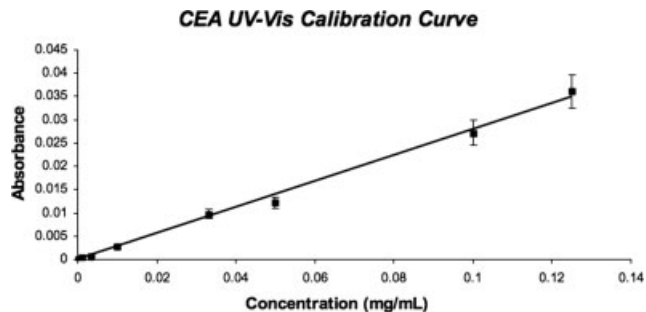


Figure 1. UV-vis calibration curve for CEA converting sample UV-vis absorbance to protein concentration. A linear regression was fit to the data points.

Lambda 25 UV-vis spectrophotometer purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). A CEA calibration curve was constructed using 8 concentrations of protein, in DI water, varying sequentially from 0.125 to 0.0005 mg/mL (Fig. 1). An AFP calibration curve was constructed using 6 concentrations of protein, in DI water, varying sequentially from 0.1 to 0.005 mg/mL (Fig. 2).

Hydrogel synthesis

The CEA-imprinted hydrogel was synthesized using the following protocol: 700 μ L of PAA-HCl, 50% (w/v) in DI water, was placed in a 5.7 mL glass vial; 178.8 μ L of DI water was then added to the vial and the solution was mixed for \sim 60 s. Next, 10 μ L of 10M sodium hydroxide (NaOH) was added to adjust the pH to 7. Then, 100 μ g (11.2 μ L) of CEA was added to the solution, which was gently mixed for 5 min and allowed to stand for 5 min to allow association. Finally, 100 μ L (10% v/v) of ethylene glycol diglycidyl ether was added to the solution, which was gently mixed to ensure homogeneous crosslinking. The 10% v/v concentration refers to the concentration of crosslinker within the solution composed of polymer and crosslinker. The solution was allowed to stand for 24 h.

Protein removal

Subsequent to synthesis, the hydrogel was washed with DI water to remove all impurities. First, the hydrogel was cut into small pieces, and 4 mL of water was added to the vial. The vial was then mixed on a Barnstead International Labquake for 24 h. Successive washes were completed with only 2 mL of DI water. Each sample went through approximately four 24-h wash cycles.

After the final DI water wash, 2 mL of 1M NaOH was added to deprotonate the amine groups on the hydrogel thus disrupting the electrostatic interactions between the polymer and protein. The vial was then mixed for 24 h. Each sample went through approximately four 24-h NaOH wash cycles. UV-vis Absorbance measurements were taken to verify the effectiveness of the base wash in

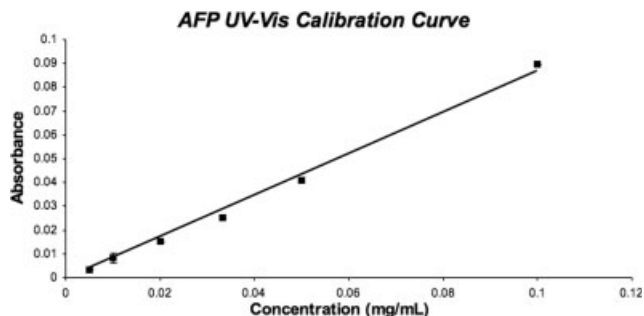


Figure 2. UV-vis calibration curve for AFP converting sample UV-vis absorbance to protein concentration. A linear regression was fit to the data points.

removing the protein. All samples were filtered using Puradisc 0.22 μm polyethersulfone membrane filters.

After the 1M NaOH washes, approximately four to six 24-h water washes were completed with various concentrations of hydrochloric acid (HCl) to adjust the pH to 7.2. All pH measurements were obtained using a Fisher Scientific accumet excel XL15 pH meter.

CEA rebinding experiment

After the final pH adjustment wash, 2 mL of DI water was added to each hydrogel. Next, 100 μg (11.2 μL) of CEA was added to the vial and the solution was mixed for 1 s. An initial baseline absorbance of the solution was recorded, and then the vial was rotated for 24 h. Subsequent absorbance measurements were made at 24 and 48 h.

AFP selectivity experiment

AFP was used in a rebinding experiment to investigate the specificity of the CEA-imprinted hydrogel. After the final pH adjustment wash, 100 μg (50 μL) of AFP was added to the vial and the solution was mixed for 5 s at which time an absorbance measurement was taken. The vial was then rotated for 24 h. Subsequent absorbance measurements were made at 24 and 48 h. The imprinting factor was calculated by dividing the total amount of protein, either CEA or AFP, bound by the imprinted hydrogel by the total amount of protein bound by the nonimprinted hydrogel.

RESULTS AND DISCUSSION

The selectivity of the imprinted hydrogel is based on the specific coordination of noncovalent interactions, mainly electrostatic in nature, between the protonated amine groups on the polymer and negative groups, mainly aspartate and glutamate residues, on the protein. Other noncovalent interactions that play minor roles in the association between the polymer

and protein include van der Waals interactions and hydrogen bonding.

UV-vis spectrophotometry was chosen to determine protein concentrations, since it is a direct form of measurement. Protein absorbance measurements were taken at 280 nm, because aromatics, a major constituent of amino acid residues, absorb at this wavelength. Since concentration can be linearly correlated to absorbance, via Beer's law, a linear regression of the data points was used to convert absorbance directly to protein concentration. The equation for the linear regression of the CEA calibration was determined to be $C = 3.571 \times A$, where C is concentration (mg/mL) and A is absorbance (Fig. 1). The equation for the linear regression of the AFP calibration was determined to be $C = 1.154 \times A$ (Fig. 2). The r^2 values for each regression, CEA and AFP, were 0.996 and 0.994 respectively, which suggest a close linear correlation in both cases.

The data obtained seem to corroborate the potential for an imprinted PAA-HCl hydrogel to effectively rebind a protein and specifically CEA (Fig. 3). The imprinting factor of the imprinted hydrogel, with respect to the nonimprinted control, was ~ 5 , suggesting a high degree of recognition capability. As expected, the nonimprinted hydrogel (control) did bind some protein due to nonspecific interactions, yet these nonspecific interactions were not nearly as effective at rebinding the protein as the specific coordinated cavities formed within the imprinted polymer. Although the results suggest that the protein was able to effectively diffuse into the matrix and rebind to the imprinted sites, the process took quite a long time (24–48 h). This is most likely due to the low diffusion coefficient of the protein within the polymer matrix. The large difference in binding capacity, between the control and the imprinted hydrogel, after 48 h suggests that at this

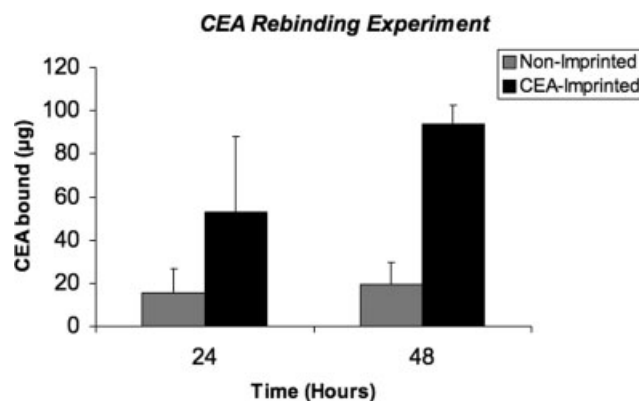


Figure 3. CEA rebinding experiment. Values correspond to amount of protein (CEA) bound by the hydrogels at specific time intervals.

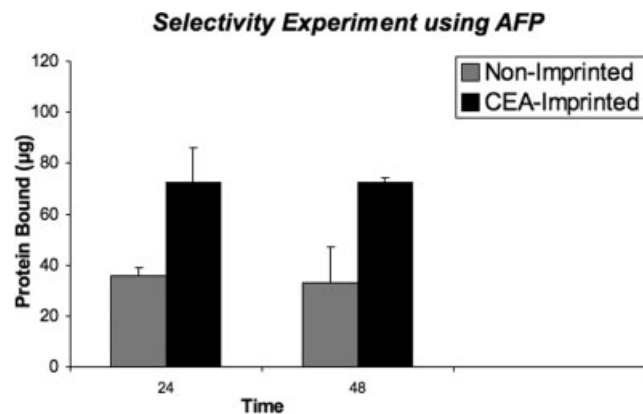


Figure 4. AFP selectivity rebinding experiment. Values correspond to amount of protein (AFP) bound by the hydrogels at specific time intervals.

time the majority of the protein was able to effectively diffuse within the polymer matrix and find a coordinated cavity for binding.

To investigate the specificity of the CEA-imprinted hydrogels, a rebinding experiment was conducted using a similar, yet distinct tumor antigen, AFP. AFP is a 70 kDa, globular protein with an isoelectric point of ~ 4.4 .¹³ Similar to CEA, AFP is an embryonic antigen whose overexpression in adults is suggestive of oncogenic growth. The protein has been shown to bind a variety of ligands including bilirubin, retinoids, steroids, along with a variety of lipids and is believed to stimulate neoplastic growth via an AFP specific cell surface receptor, which undergoes internalization upon binding.^{14,15}

AFP was chosen to investigate the specificity of the CEA-imprinted hydrogel due to the structural and electrostatic similarities between the two proteins. Furthermore, both proteins are embryonic-derived tumor antigens used for monitoring of oncogenic growth. If a CEA-imprinted hydrogel is used for diagnostic applications, its efficacy would depend on its ability to selectively recognize CEA among other antigens, such as AFP.

Although the CEA-imprinted hydrogel did bind an appreciable amount of AFP, the imprinting factor was only two compared to an imprinting factor of five obtained with CEA (Fig. 4). Based upon imprinting factors, the CEA-imprinted hydrogel was ~ 2.5 times more specific for CEA than AFP.

The nonimprinted control hydrogel adsorbed slightly more AFP than CEA, yet the amount was still fairly low (30 μg). The control hydrogel also reached its maximum absorbance within 24 h, which eventually decreased after 48 h. This is probably due to the fact that AFP is a much smaller molecule than CEA, thus much more capable of diffusing into the hydrogel. Yet since the control had no cavities, spe-

cific for the protein, and capable of binding it, some of the protein that initially entered the gel was washed out. Similar to what was observed with the control hydrogel, the CEA-imprinted hydrogel also reached its maximum absorbance within 24 h of rebinding and remained the same after 48 h. The data seem to suggest that the AFP quickly entered the hydrogel and bound to the CEA-imprinted cavities. Although the cavities were not specific for AFP, the interactions within the cavity were strong enough to retain at least some of this protein.

CONCLUSION

The results obtained from the experimentation clearly indicate the capability of an imprinted PAA-HCl hydrogel, synthesized using EGDE water soluble crosslinker, to bind carcinoembryonic antigen (CEA) with a high affinity when compared to a non-imprinted polymer control. The imprinted hydrogel also showed selectivity toward CEA compared to a similar tumor antigen, AFP. The research demonstrates the viability of a cationic hydrogel for protein recognition. Future work will focus on the development of a multivalent, multifunctional hydrogel system capable of more specific protein recognition for diagnostic and therapeutic use.

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