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Dye Ligand Affinity Nanoparticles for the Depletion of Biomolecules in Proteomics

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ABSTRACT

Objective: Serum proteins are indicators for certain diseases. However, detection of the biomarkers is difficult because the more abundant proteins mask the less abundant ones. The depletion of abundant serum proteins will help in the discovery and detection of less abundant proteins that may prove to be informative disease markers. Dye ligands have attracted great attention because of their cost-effectiveness, easy immobilization, stability, and high binding capacity. Due to these advantageous properties, dye ligands have also been chosen as an alternative for biological ligands such as antibodies, enzymes, etc.

Materials and Methods: Poly (ethylene glycol dimethacrylate) [p (EGDM)] nanoparticles were prepared using the surfactant-free emulsion polymerization technique. Then, Reactive Red 120 (RR 120) dye was immobilized to nanoparticles in a nucleophilic substitution reaction. The RR120 attached nanoparticles were characterized.

Results: The size/size distribution of p (EGDM) nanoparticles was measured with a Zeta sizer. The scanning electron microscope (SEM) images were found to support a measurement of around 100 nm. The maximum adsorbed amount of albumin was observed at pH 6.0. The maximum depleted albumin concentration was found to be 453.9 mg/g nanoparticles according to the experimental results. Desorption studies were carried out by addition of 0.5 M of KSCN to the albumin solutions. The desorption results showed that the binding of albumin to the nanoparticle was reversible.

Conclusion: Our results demonstrated that dye attached nanoparticles have the potential for depleting albumin from serum in proteomics.

Keywords: Proteomics, dye ligand, nanoparticles, Reactive Red 120, biomolecules, albumin

INTRODUCTION

Proteomics is focused on the study of proteins, particularly their structures and functions. Serum proteins are indicators for certain diseases and these proteins have been used as biomarkers for these disorders (1).

Serum contains 60–80 mg of protein/mL, in addition to various molecules, including lipids, electrolytes, etc. (2). The total number of proteins is approximately ten thousand, but this number can reach several times this amount in normal and diseased cells as each protein undergoes many post-translational modifications. However, the more abundant proteins make proteome analysis very challenging because these proteins mask the less abundant ones (1, 3, 4). Indeed, one major challenge of plasma protein analysis is that as few as 0.2 % of the proteins make up 99 % of the total protein content of serum. Albumin, for example, represents more than half of the whole blood protein mass in serum (5). Nevertheless, the timely detection of misexpressed proteins in the early stages of a given disease is extremely challenging (6). This is especially important in proteomic studies where potential biomarkers are investigated. Therefore, the first step when examining serum proteome analysis in general is to reduce sample complexity (3).

It is very important that any methods developed in the depletion of highly abundant proteins should be low-cost and involve high-throughput techniques. There have been

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many techniques developed for the depletion of albumin in the literature. Among these techniques, affinity technologies draw much attention.

Dye ligands have attracted great attention because of their cheapness, easy immobilization properties, stability, and high adsorption capacity. Due to these advantageous properties, dye ligands have also been chosen as an alternative for the biological ligands such as antibodies, enzymes, etc. (6-11). These dye ligands are structurally very similar to some biological molecules (cofactor, substrate, etc). Because of this similarity, dye ligands can easily interact with the active center of some enzyme structures. Therefore, these dye ligands are called affinity ligands. Many reactive dyes which are also used as textile dyes have been used for the adsorption of proteins. These dyes generally contain a reactive group (mono- or dichloro triazine ring) with a chromophore group (azo, anthroquinone or phthalocyanine). Due to the complex structures of dye molecules and protein molecules, the interactions between them are usually secondary interactions. In general, hydrophobic interactions, electrostatic bonds and hydrogen bonds are quite dominant among these interactions (12).

Adsorption capacity of polymeric support material increases when the particle size of the support is decreased (13-15). In the case of the surface adsorption concept, when the particle size decreases, its surface area tends to be wider and therefore shows higher adsorption capacities (16). Synthesis and development of new nano-sized materials is a way to increase the adsorption capacity. Also, the physical and chemical properties of the nano-sized polymer demonstrate atypical change because of their small size and large surface area (17-19).

Various sorbent systems containing different dye ligands were used for the depletion, purification, and separation of albumin. Reactive Green HE-4BD containing hollow fibers (20); Cibacron Blue F3GA immobilized poly (GMA) microbeads and chitosan microspheres (21,22); and Reactive Green 19 immobilized p (HEMA) cryogel disks could be listed as examples (23).

In this study, firstly, RR 120 dye was attached to poly(ethylene glycol dimethacrylate) [p (EGDM)] nanoparticles. At the end of the synthesis procedure, characterization studies were completed. Then, reusability of the RR 120 attached p (EGDM) nanoparticles was also investigated.

MATERIALS AND METHODS

RR 120 Attachment to the p (EGDM) Nanoparticles

P (EGDM) nanoparticles were synthesized using a surfactant-free emulsion polymerization technique (24). Dye ligand RR 120 was covalently attached to the synthesized nanoparticles. For this, 70.0 mL of RR 120 solution was added on 0.1 g p (EGDM) nanoparticles. (3.0 mg/ml; in 4.0 g of NaOH) for 4 h. After this incubation period, dye attached nanoparticles were washed with distilled water and methanol several times in order to remove physically attached RR 120 molecules. RR 120-attached poly (EGDM) nanoparticles were stored at 4°C.

Characterization of RR 120 Attached Nanoparticles

Fouirer transform infrared spectroscopy (FTIR) analysis was performed to show the binding of RR 120 onto p (EGDM) nanoparticles. For this purpose, 0.1 g nanoparticles were mixed with 0.1 g of KBr homogeneously, and then pressed into pellets. The FTIR spectra of dye ligand-bound and non-dye ligand-bound nanoparticles were examined using an FTIR (Perkin Elmer Spectrum 100, USA).

Assessment of surface morphology and size of the synthesized nanoparticles was carried out using a scanning electron microscope (SEM). The SEM images were obtained by coating the dry p (EGDM) nanoparticles with a thin gold layer (Philips, XL-30S FEG, The Netherlands). Particle size of the synthesized nanoparticles was analyzed with a nano zetasizer (Nanos, Malvern Instruments, London, UK).

Adsorption of Bovine Serum Albumin (BSA) onto RR 120 Attached Nanoparticles

In our study, BSA binding studies were carried out in a batch system. In this system, synthesized nanoparticles were mixed with BSA solution, and the adsorption experiment was conducted at 25°C for 2 h with a 100 rpm stirring rate. The effects of time, medium pH, BSA concentration, and temperature on the BSA binding capacity of RR 120 attached nanoparticles were also investigated. For these purposes, some adsorption conditions were changed. For example, binding experiments were performed for different adsorption times (5-150 minutes) to show the effect of time for BSA adsorption. In addition, the pH of the solution was changed between 4.0 and 8.0 using various buffer systems. The effect of BSA concentration on adsorption was investigated by changing the BSA concentration in the range of 0.2-8.0 mg/mL and performing binding experiments. Adsorbed BSA amount was calculated by the determination of the initial and final BSA concentration using the Bradford method (25).

Desorption Studies

To evaluate the reusability of the RR 120 attached p (EGDM) nanoparticle, first the adsorbed BSA was removed. To do this, a 0.5 M KSCN solution was used as a desorption agent. The nanoparticles loaded with BSA were incubated with the 0.5 M of KSCN solution for 1 h at 25°C with constant shaking at 150 rpm. BSA desorption ratios from the dye attached nanoparticles were calculated by the following equation:

$$\text{Desorption ratio } (\%) = \frac{\text{Desorbed BSA amount}}{\text{Adsorbed BSA amount}} \text{ x } 100$$

When the reusability of the RR 120 bound nanoparticles were evaluated, experiments were performed over five adsorption/desorption cycles with the same nanoparticles.

RESULTS AND DISCUSSION

Recently, nanotechnology has become an important research area and very significant developments have occurred in various areas of science and technology. When considering the

biotechnology fields, collaboration between nanomaterials and biomolecules has attracted great interest (26). Their wide surface areas present great binding spaces for biomolecules and they can also be easily derivatized with a number of ligands. Even with their small particle size, nonporous nanoparticles can bind over 100 mg of biomolecules per gram of wet particles (27).

Especially in recent years, when the literature is examined, it was seen that nano-sized polymeric materials have frequently been used for the isolation and purification of biomolecules. This is due to their extremely high surface area, nano-sized materials that offer large surface area for increased adsorption capacity of biomolecules. Functional surface groups of the nano-sized polymers allow easy derivatization with various ligands which have different character and functionalities. Dye ligands have been used as an alternative for biological ligands such as antibodies, enzymes due to cost-effective, easy immobilization, stability and high binding capacity.

After the nanoparticles were synthesized by emulsion polymerization technique, RR 120 was covalently bonded to the surfaces of nanoparticles in a nucleophilic substitution reaction between the triazine chloride of RR 120 and hydroxyl groups of EGDM. The hypothetical representation of p (EGDM) nanoparticles with RR 120 attached is shown in Figure 1.

The dye ligand adsorption capacity of p (EGDM) nanoparticles increases with increasing the dye concentration. Nanoparticles

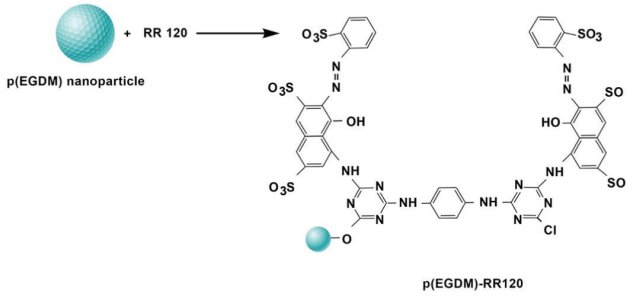

Figure 1. Schematic presentation of RR 120 attached p (EGDM) nanoparticles.

reach the maximum adsorption capacity whereas free –OH groups on the surface of nanoparticles reach saturation. Figure 2 demonstrates the FTIR spectra of RR 120, RR 120 attached nanoparticles and p (EGDM).

The O-H stretching vibration band was observed to be 3200–3600 cm^{-1}. With the addition of RR 120 to p (EGDM), absorption was significantly increased because of the existence of N-H bending in the RR 120 structure. At p (EGDM) polymer spectrum, the stretching vibrations band of carbonyl (C=O) groups was observed around 1720. Characteristic stretching vibrations bands of ester (C-O) were around 1000-1200 cm^{-1}. The bands were seen at 1080 cm^{-1} and 1160 cm^{-1} in the p (EGDM)-RR 120 and RR 120 spectrums were due to the symmetrical stretching of the S=O bond and the asymmetric stretching of the S=O bond in RR 120. As a result, the obtained spectra supports that RR 120 participates in the p (EGDM) nanoparticles.

The size and shape of RR 120 attached nanoparticles were determined by examining the SEM photographs (Figure 3). When

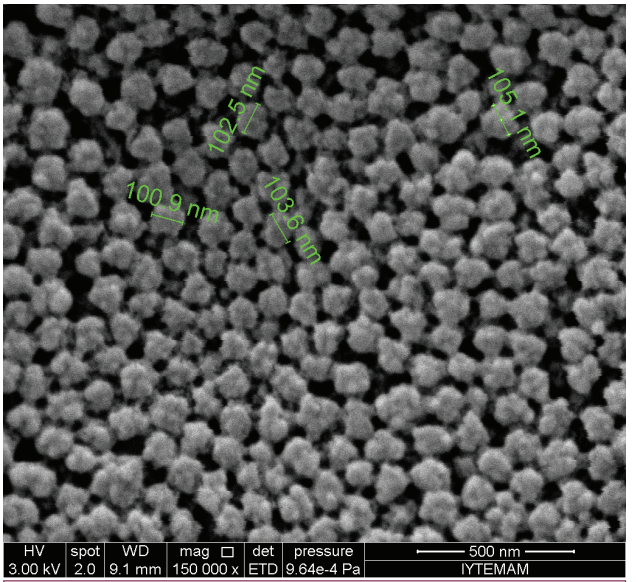

Figure 3. SEM images of p (EGDM)-RR 120 nanoparticles.

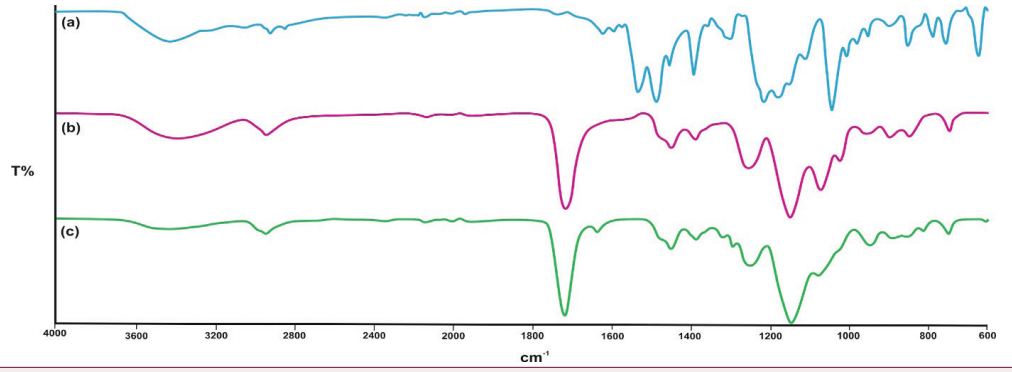

Figure 2. (a) RR120, (b) p (EGDM)-RR120, (c)p (EGDM) spectrum of FTIR.

the SEM photographs were investigated, it was seen that the nanoparticles were relatively spherical, non-porous and approximately 100 nm in width.

In addition, the size/size distribution of p (EGDM) nanoparticles was measured with a Zeta sizer. A measurement of approximately 100 nm was found to support the size recorded in the SEM images (Figure 4).

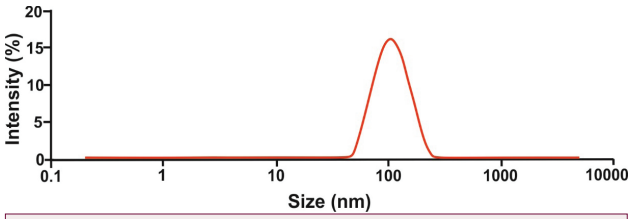

Figure 4. Zeta-size analysis of the RR 120 attached p (EGDM) nanoparticles.

To determine the effect of time on albumin binding, adsorption studies on dye-attached nanoparticles were performed between 0 and 150 minutes. When Figure 5 is examined, it is seen that albumin adsorption increases over time and reaches a plateau value at 120 minutes. Since it reached the plateau value in 120 minutes, all experiments were carried out for 120 minutes.

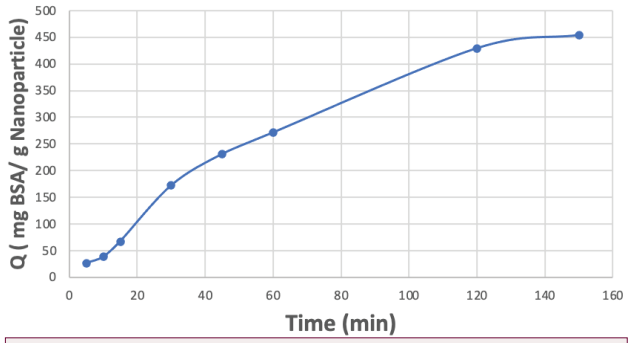

Figure 5. BSA adsorption and time curve. (BSA initial: 2mg/mL, pH:6, T=25°C).

As shown in figure 5, BSA adsorption and time studies were done at 5, 10, 15, 30, 45, 60, 120 and 150 minutes (BSA initial: 2 mg/mL, pH:6, T=25°C). Adsorption capacity values Q increased with time and reached a maximum value (453.9 mg/g) in these conditions.

Albumin adsorption experiments were performed with various pH and the pH effect on albumin binding is shown in Figure 6.

Figure 6 shows the pH effect on BSA adsorption to dye attached p (EGDM) nanoparticles. According to the graph, from the acidic to the neutral region, the adsorption capacity of p (EGDM-RR120) nanoparticle increases. Maximum BSA adsorption was found at pH: 6 to be 453.9 mg/g. The important point here is the non-specific adsorption to the nanoparticles. According to Figure 6, the non-specific adsorption to the p (EGDM) nanoparticles was negligible. The interactions between albumin and RR 120 molecules attached on the nanoparticle surface are weak interactions. When the structure of albumin and RR 120 molecules is examined, hydrophobic, electrostatic and/or hydrogen bonds may occur between these two molecules. These interactions may increase further due to the presence of sulfonate, amine, and cyclic hydrophobic groups on the dye molecules and a few ionizable groups on the amino acid side chains on the albumin molecule.

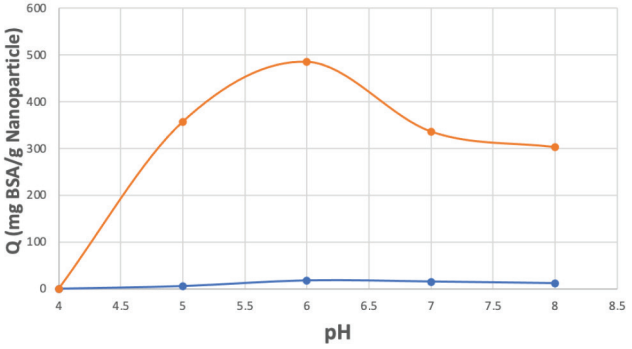

Figure 6. pH effect on BSA binding (Concentration of BSA: 2mg/mL, adsorption time: 2 hours) (Blue line: non-specific adsorption; red line: RR 120 attached p (EGDM)).

The effect of albumin concentration on the binding capacity of RR 120 attached p (EGDM) nanoparticles is shown in Figure 7. When the initial albumin concentration was increased, the adsorbed amount of albumin onto dye-attached nanoparticles increased rapidly. The saturation value was 6.0 mg/mL of albumin concentration and generally takes place in affinity adsorption studies because all active biomolecules binding regions are busy with previously bound biomolecules. It can also be concluded from Figure 7 that affinity between the RR 120 and albumin molecules was very high.

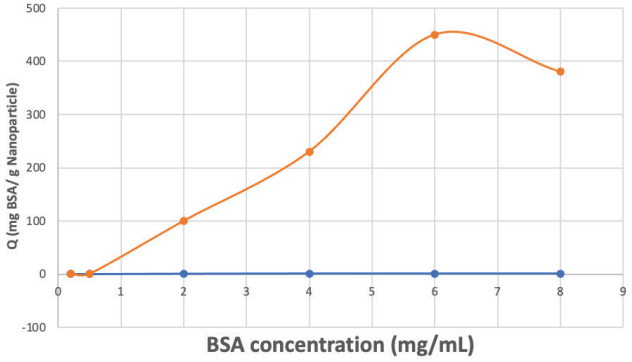

Figure 7. Effect of concentration on BSA binding (pH: 6, adsorption time: 2 hours) (Blue line: non- specific adsorption; red line: RR 120 attached p (EGDM)).

Figure 8 presents temperature effect on the albumin binding onto RR 120 attached p (EGDM) nanoparticles. As can be seen

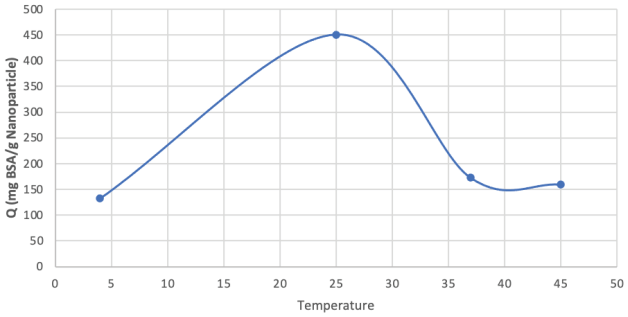

Figure 8. Temperature effect on the BSA adsorption (Concentration of BSA: 1mg/mL, pH: 6, adsorption time: 2 hours).

in Figure 8, the amount of adsorbed albumin increased with increasing temperature. This adsorption behavior can be explained by the hydrophobic interaction between the albumin and RR 120 attached nanoparticles. In hydrophobic interaction chromatographic techniques, the adsorbed amount of adsorbent generally increases with increasing temperature. Albumin adsorption capacity of the RR 120 attached nanoparticles decreased at temperatures higher than 25°C. When considering the protein adsorption, the adsorbed amount of proteins generally decreases because of the three dimensional conformational changes occurred at high temperatures.

The results of the presented study show that RR 120 attached nanoparticles are very valuable polymeric materials which can be used for adsorption of BSA and therefore can be applied in the depletion of albumin. The high albumin adsorption capacity of the prepared nanoparticles can increase its usage for biotechnological and biomedical applications.

As we mentioned before, in the experiments in which the reusability of RR 120 bound nanoparticles were evaluated, the experiments were performed by performing five adsorption/desorption cycles with the same nanoparticles. The albumin adsorbed RR 120 attached nanoparticles were desorbed with 0.5 M KSCN. Desorption rates were found to be between 70-85 %. It should be noted that at the end of the applied five adsorption/desorption cycles, there was no significant decrease in the adsorption capacity of albumin to dye-attached nanoparticles (Figure 9).

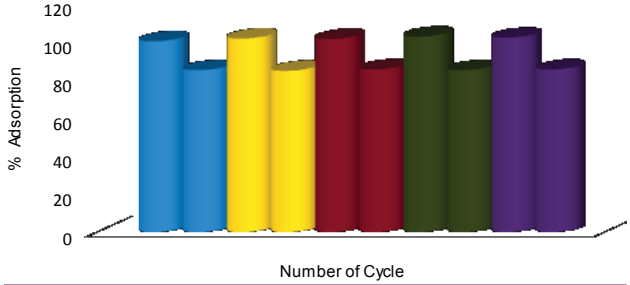

Figure 9. Reusability of p (EGDM)-RR 120 nanoparticle (Desorption agent: 0.5 M KSCN, concentration of BSA: 1mg/mL, pH: 6, adsorption time: 2 hours, desorption time: 1 hours).

CONCLUSIONS

In our study, RR 120 was used for the affinity adsorption of albumin which is a highly abundant protein in serum. p (EGDM) nanoparticles were synthesized as a depletion material and derivatized with RR 120. The effect of pH, albumin concentration, and temperature on albumin adsorption onto RR 120 attached nanoparticles was investigated. Our findings from this study indicate that RR 120 attached nanoparticles can be easily used for the adsorption and depletion of albumin from plasma/serum. This nanoparticle, which was developed for the first time in our study for albumin depletion, has the advantages of high binding capacity and relatively low cost compared to other systems.

Ethics Committee Approval: Ethics committee approval is not required because of no material or experimental animal that would require permission.

Peer-review: Externally peer-reviewed.

Conflict of Interest: The author have no conflict of interest to declare.

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