

# Novel Method for the Isolation of Proteins and Small Target Molecules from Biological and Aqueous Media by Salt-Assisted Phase Transformation of Their PEGylated Recognition Counterparts

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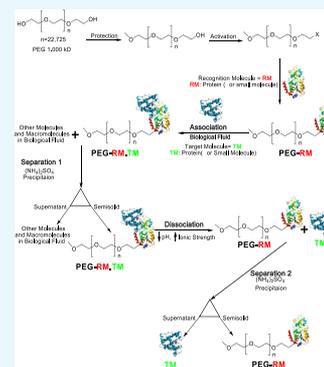
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**ABSTRACT:** An efficient and simple method for the application of PEGylated affinity ligands in precipitative isolation of protein target molecules (TMs) from a biological fluid such as blood serum or small target molecules from an aqueous medium is presented for the first time. This approach is based on the high binding specificity of PEGylated recognition molecules (PEG-RMs) to their TMs and the unique physicochemical properties of PEG that result in their salt-assisted phase transformation. Addition of PEG-RM to blood serum results in the formation of an RM-specific macromolecular complex (PEG-RM + TM  $\rightarrow$  PEG-RM.TM) that undergoes facile salt-assisted phase transformation to a separable semisolid with ammonium sulfate. PEG-RM.TM is then dissociated into its components by pH reduction or an increase of ionic strength (PEG-RM.TM  $\rightarrow$  PEG-RM + TM). PEG-RM is salted out to afford pure TM in solution. The same phenomenon is observed when RM or TM are small molecules. The general applicability of the method was validated by PEGylation of two proteins (protein A, sheep antihuman IgG) and a small molecule (salicylic acid) used as model RMs for the isolation of Igs, IgG, and serum albumin from blood serum. The isolated protein TMs were shown to be pure and aggregate-free by gel electrophoresis and dynamic light scattering (DLS). IgG isolated by this method was further characterized by peptide mass fingerprinting. PEGylated protein A was used to demonstrate the recyclability and scale-up potential of PEG-RM. IgG isolated by this method from blood serum of a hepatitis C-vaccinated individual was tested for its binding to sheep antihuman IgG by UV spectroscopy, and its bioactivity was ascertained by comparison of its enzyme-linked immunosorbent assay (ELISA) result to that of a blood sample from the same individual. Reciprocity of RM and TM was ascertained using PEGylated salicylic acid to obtain pure serum albumin, and PEGylated serum albumin was utilized for near-exclusive isolation of one drug from an aqueous equimolar mixture of three drugs (salicylic acid, 91%; capecitabine, 6%; and deferiprone, 3%). Advantages of this approach, including target specificity and general applicability and celerity, over other affinity methods for the isolation of proteins are discussed at a molecular level.



## 1. INTRODUCTION

Isolation of proteins remains at the cutting edge of biotechnology after over a century of research since the pioneering work of Hofmeister in 1889. This is due to great advances in biochemistry and molecular biology of proteins in the past few decades<sup>1–4</sup> that have resulted in their application in the treatment of various diseases from atopic eczema<sup>5</sup> and cancer<sup>6</sup> to the potential nonaddictive treatment of drug abuse.<sup>7</sup> In fact, the biopharmaceutical market valued at \$ 269 billion in 2019 is expected to reach \$ 446 billion by 2025.<sup>8</sup> At the present time, 86 monoclonal antibodies (mAbs) have been approved by the FDA and 17 new applications are under review.<sup>9</sup>

Separation methods have been broadly divided into nonchromatographic and chromatographic methods.<sup>10</sup> The former methods are efficient and inexpensive and used extensively in the chemical industry (e.g., extraction, crystallization, precipitation, filtration, dialysis, distillation,

derivatization). On the other hand, chromatographic methods (e.g., thin-layer, column, paper, gas, high-performance liquid, ultrahigh-performance, and affinity chromatography) are costly and limited to high-value, low-volume products such as biopharmaceuticals or natural products (e.g., affinity chromatography for mAbs<sup>3</sup> and antisense oligonucleotides<sup>11</sup>). Even so, because of time and costs associated with chromatography “Anything But Chromatography” has been coined as ABC of isolation of biopharmaceuticals.<sup>12</sup>

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In contrast to chromatographic methods, extraction and precipitation are efficient and inexpensive. However, they suffer from a lack of selectivity for the isolation of proteins.<sup>13</sup> This shortcoming was addressed in the 1980s by applying the concept of affinity, first developed in the 1950s for chromatography, to extraction and precipitation. The common feature in affinity methods (chromatography, extraction, and precipitation) is the use of a target-specific recognition molecule (RM) attached to a polymer for the isolation of a target molecule (TM). The platform is a water-insoluble polymer (resin) in affinity chromatography and a water-soluble polymer in affinity extraction and affinity precipitation. In the two later cases, the polymers can undergo phase transformation in aqueous media from soluble to insoluble in response to stimuli such as temperature, pH, ionic strength, chelating agents, polyelectrolytes, surfactants, low-molecular-weight substances, and light.<sup>14–17</sup> Phase transformation of polymer-RM.TM, its separation from the aqueous mixture, and subsequent dissociation to its components allow for the isolation of the TM.

On the other hand, the most important difference between affinity chromatography and the latter two affinity methods at the molecular level is heterogeneity vs homogeneity of the systems in the important steps of absorption (polymer-RM + TM → polymer-RM.TM) and desorption (polymer-RM.TM → polymer-RM + TM). The water-insoluble platform in affinity chromatography causes the process to be heterogeneous in its entirety. This will no doubt affect the specificity of absorption and desorption through exerting limitations on the molecular motions of RM and TM by restricting their freedom of movement. The problem is somewhat, but not completely, remedied in affinity chromatography by the use of spacer arms of an appropriate length to afford some freedom of movement, reduce steric hindrance, and improve capacity and specificity as well as flow.<sup>18</sup>

In affinity extraction and precipitation, however, water solubility of the polymer allows the critical steps of absorption and desorption to be homogeneous, affording freedom of movement of RM and TM and a flexible molecular environment that could improve specificity. However, optimum separation is contingent upon the use of appropriate polymers and stimuli for specific types of proteins (mAbs, enzymes, protein A, immunoglobulins, plant proteins, avidin, etc.).<sup>14,19,20</sup> Accordingly, an assortment of polymer and stimulus combinations has been reported for affinity extraction and precipitation. However, while PEG has been used in affinity extraction, there is no example of its application in affinity precipitation.<sup>20</sup> The variety of polymers (other than PEG) and stimuli combinations in affinity precipitation attests to the lack of a precipitation method with general applicability to the isolation of biomolecules. In fact, a recent review on various methods for the isolation of proteins with emphasis on “Anything But Chromatography”, while including affinity extraction, does not address affinity precipitation.<sup>12</sup>

In light of the above, affinity extraction and precipitation have been mostly used in the early capture of dilute proteins from biological fluids<sup>14,15,20</sup> and the isolation of pharmaceutical-grade mAbs is still heavily dependent on late-stage protein A affinity chromatography, which is the most important cost center in the titer-dependent production process (45% for a low titer of 0.1 g/L to 80% for a high titer of 1.0 g/L).<sup>21,22</sup> Moreover, the isolation of mAbs, which can be as many as 14 steps, must be designed with minimum physical or chemical

stress to prevent the formation of protein aggregates,<sup>23–25</sup> which are immunogenic, can lead to an anaphylactic response, and even cause death.<sup>26</sup> Aggregates must be removed from pharmaceutical-grade mAbs, usually by size exclusion chromatography, which increases the cost of production.<sup>26,27</sup> Additionally, the use of various materials (membranes, resins, etc.) and large volumes of buffers with no or limited possibility of recycling imposes considerable environmental burden and adversely affects the affordability of mAbs.<sup>28</sup> In spite of the well-known difficulties of heterogeneous chromatographic methods, which are more pronounced in large-scale applications<sup>29,30</sup> and have resulted in the idiom “Anything But Chromatography”,<sup>12</sup> research on downstream processing of mAbs has been focused on improvements of the existing affinity chromatographic methods.<sup>31–34</sup> There is therefore a need for a simple protocol with broad applicability to the isolation of proteins from biological fluids that is devoid of the difficulties of heterogeneous affinity chromatographic processes and the lack of general applicability of reported affinity extraction and precipitation methods.

In this paper, we describe a novel and highly efficient four-step precipitative protocol utilizing high-molecular-weight PEG-RMs capable of affording pure proteins directly from blood serum as well as nearly exclusive separation of a drug from an aqueous equimolar mixture of three different drugs. The advantages of this method over reported affinity methods are discussed at the molecular level.

## 2. MATERIALS AND METHODS

Chemical reagents and solvents were obtained from commercially available sources such as Sigma-Aldrich, Fluka, or Merck and used without further purification. PEG 1000 kD was obtained from Aldrich (lot number MKBW2578V). Saturated ammonium sulfate (4.1 M) was used for affinity precipitation. Protein A and sheep antihuman IgG were obtained from Sina Biotech. Co. (Lot Nos.: 960218 and S90918, respectively). Human serum albumin (HSA) was obtained from Octapharma Co. (Batch No.: K748B6661). Human blood was obtained from the peripheral vein of a healthy donor with a sterilized disposable plastic syringe. Blood sera were prepared by preserving blood samples without an anticoagulant in sterilized test tubes in the vertical position until clots were formed. The clots were then gently removed from the test tubes with a glass rod or a swab and the samples were centrifuged at 1000g for 10 min followed by decantation of the serum. Matrix-assisted laser desorption/ionization mass spectrometry (Applied Biosystems 4800 matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF)/TOF, Nd:YAG 200 Hz laser) was used for peptide mass fingerprinting. Dynamic light scattering measurements were performed using a HORIBA DLS Analyzer. High-performance liquid chromatography (HPLC) was performed using an in-house validated method for deferasirox. Enzyme-linked immunosorbent assay (ELISA) measurements were performed in Kimiagar Serology Laboratories using a validated standard operating procedure provided by the ELISA Kit supplier (Pishtaz Teb Zaman Diagnostics Co.).

**2.1. Isolation of Igs or IgG with PEGylated Protein A or PEGylated Sheep Antihuman IgG.** **2.1.1. PEGylation of Protein A or Sheep Antihuman IgG.** PEG 1000 kD (20 g, 0.02 mmol) was activated using the process reported by Gonzalez et al.<sup>35</sup> with CDI affording 19 g of product (95%). To a solution of protein A (1 mL, 0.5 mg/mL,  $1.19 \times 10^{-5}$  mmol) or sheep

antihuman IgG (1 mL, 0.5 mg/mL,  $3.3 \times 10^{-6}$  mmol) in 4 mL of phosphate-buffered saline (PBS) buffer pH 7.2 was added activated PEG (66 mg,  $6.66 \times 10^{-5}$  mmol). The resulting solution was gently swirled at 0–10 °C for 48 h. Completion of the coupling was monitored by native PAGE. A saturated solution of ammonium sulfate was then added to the reaction mixture. The semisolid material was separated by centrifugation and washed with a saturated solution of ammonium sulfate to remove any unbound protein A or sheep antihuman IgG. The washing procedure was repeated three times until the supernatant was negative to the ninhydrin test (lack of protein). The semisolid residue (PEGylated protein A or PEGylated sheep antihuman IgG) was used for the isolation of IgG or Igs from the blood serum.

**2.1.2. Isolation of Igs from Blood Serum with PEGylated Protein A.** The following general procedure for isolation of Igs from blood serum was used: Blood was withdrawn from a healthy volunteer and diluted with 1 mL of PBS buffer, pH 7.2. PEG-RM from the experiment in Section 2.1.1 (200  $\mu$ L,  $3.3 \times 10^{-3}$   $\mu$ mol protein A) was then added to 60  $\mu$ L of diluted sample of human serum containing approximately  $6.7 \times 10^{-3}$   $\mu$ mol Igs (for PEGylated protein A). This approximate value is based on the average content of Igs provided by Dati et al.<sup>36</sup> The mixture was gently swirled for 45 min at room temperature. A saturated solution of ammonium sulfate (0.5 mL) was added to the mixture, which resulted in salt-assisted phase transformation of PEG-RM.Igs to a semisolid in about 2 min. The semisolid was separated by centrifugation at 3000 RPM for 5 min. PBS buffer (0.5 mL) was added to the semisolid, and the solution was gently swirled to wash away unbound serum components, followed by centrifugation and decantation. The wash procedure was repeated twice by the addition of ammonium sulfate, separation of the semisolid, and the addition of 0.15 M phosphate-buffered saline pH 7.2 (PBS, 1 mL). PEG-RM.Igs was then dissociated into its components (PEG-RM + Igs) by the addition of 0.15 M glycine–HCl, pH 2.5. PEG-RM was removed as a semisolid by the addition of ammonium sulfate and centrifugation as above. The final solution contained pure Igs and ammonium sulfate. The salt was removed by dialysis in 0.15 M PBS buffer, pH 7.2, twice for 2 h at a ratio of 1 vol of sample to 100 vol of buffer. The protein concentration was determined using UV spectroscopy (see Results). The final solution was 5 mL due to PEG-RM.TM formation, dissociation, and dialysis.

**2.1.3. Isolation of IgG from Blood Serum with PEGylated Sheep Antihuman IgG.** The general procedure provided in the experiment in Section 2.1.2 was followed. It should be noted that the quantity of IgG in 60  $\mu$ L of serum used in this experiment is  $3.3 \times 10^{-3}$   $\mu$ mol. This approximate value is based on the average Igs content provided by Dati et al.<sup>36</sup> PEG sheep antihuman IgG was obtained after the addition of a saturated solution of ammonium sulfate as a semisolid in about 2 min. Finally, IgG was separated and purified according to the experiment in Section 2.1.2.

**2.1.4. Scale-Up of Isolation of Igs from Blood Serum with PEG-RM (L = Protein A).** The experiment in Section 2.1.2 was scaled-up using 1000  $\mu$ L of diluted sample of blood serum containing approximately  $1.11 \times 10^{-1}$   $\mu$ mol Igs (for PEGylated protein A). The scaled-up process afforded identical protein concentration and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis to that of the 60  $\mu$ L sample.

**2.1.5. Recycling of PEGylated Protein A.** PEGylated protein A was recovered by washing 3 times with 0.15 M PBS buffer, pH 7.2, and stored in the buffer containing 0.01% sodium azide at –20 °C. The recovered PEG-RM was recycled at least three times to repeat the experiment in Section 2.1.2 and afforded identical results.

**2.1.6. Peptide Mass Fingerprinting of Isolated IgG.** A 0.5 mg/mL purified sample of IgG (experiment in Section 2.1.3) was characterized with peptide mass fingerprinting (PMF) using the method of Shevchenko et al. (see Results)<sup>37</sup> Samples were spotted on a MALDI plate mixed with an equal volume of matrix solution of *a*-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid, air-dried, and analyzed with a MALDI-TOF/TOF mass spectrometer, operated in reflector positive mode. The data were then interpreted and processed using Data Explorer software version 4.0 (Applied Biosystems).

## 2.2. Isolation of Salicylic acid from an Equimolar Mixture of Drugs with PEGylated Human Serum Albumin.

**2.2.1. PEGylation of Human Serum Albumin.** The process of the experiment in Section 2.1.1 (PEGylation of protein A) was repeated using human serum albumin.

**2.2.2. Isolation of Salicylic Acid from an Equimolar Mixture of Drugs with PEGylated Human Serum Albumin.** An equimolar mixture of salicylic acid (0.05 mg, 0.375  $\mu$ mol), deferiprone (0.051 mg, 0.375  $\mu$ mol), and capecitabine (0.134 mg, 0.375  $\mu$ mol) was prepared in 0.5 mL of 0.15 M PBS buffer pH 7.2. PEGylated human serum albumin (375 mg, 0.375  $\mu$ mol) was added to the drug mixture, and the solution was gently swirled for 1 h at room temperature. A small quantity of a saturated solution of saturated ammonium sulfate (ca. 0.5 mL) was then added to the solution, causing immediate formation of a PEG-HSA.“Drug” as a semisolid, which was centrifuged at 4000 RPM for 5 min and decanted. The semisolid was washed twice with a saturated solution of ammonium sulfate (1 mL) to ensure removal of unbounded drugs and other potential impurities. The final semisolid (PEG-HSA.“Drugs”) was dissolved in 5% acetic acid to disrupt molecular interactions involved in high specificity and binding (*vide supra*). The mixture was swirled for 10 min to cause dissociation of PEG-HSA.“Drugs” to PEG-HSA + “Drugs”. Addition of a small quantity of a saturated solution of ammonium sulfate resulted in salt-assisted phase transformation of PEG-HSA to a semisolid, leaving the “Drugs” in solution. The “Drugs” were extracted into ethyl acetate, dried over sodium sulfate, and filtered. The solvent was removed under reduced pressure to afford a dry solid, which was subjected to HPLC chromatography using an in-house validated method for deferiprone, which affords good separation of the drugs: column, RP-C18, 300 mm  $\times$  4.0 mm, 10  $\mu$ m; mobile phase, 550 mL of 0.02 molar  $\text{KH}_2\text{PO}_4$  buffer, pH 2.5, and 450 mL acetonitrile; and flow rate, 1.0 mL/min (Figure 4).

## 2.3. Isolation of Human Serum Albumin with PEGylated Salicylic Acid.

**2.3.1. PEGylation of Salicylic Acid.** PEG 1000 kD (50 g, 0.05 mmol) was brominated using the process of Bückmann et al.<sup>38</sup> with  $\text{PBr}_3$  affording 49 g of a pale yellow product (98%). To a solution of salicylic acid (1.0 g, 7.24 mmol) in 100 mL of *N,N*-dimethylformamide (DMF) was added potassium carbonate (2.0 g, 14.48 mmol), followed by activated PEG (10 g, 0.01 mmol). The resulting solution was heated to 80–90 °C for 10 h. Reaction completion was monitored by thin-layer chromatography (TLC) for the



hydrodynamic diameter of each sample was measured using a HORIBA DLS analyzer (Figure 6).

### 2.5. Measurement of Binding of Isolated IgG to PEGylated Sheep Antihuman IgG by UV Spectroscopy.

Binding to PEGylated sheep antihuman IgG to IgG isolated by our method (experiment in Section 2.1.3) was determined by UV spectroscopy at 280 nm. A 1 mL sample of isolated IgG (0.5 mg/mL,  $3.33 \times 10^{-6}$  mmol) was mixed with a 2 mL sample of PEGylated sheep antihuman IgG (0.5 mg/mL,  $6.66 \times 10^{-6}$  mol/mL based on sheep antihuman IgG) and the procedure of the experiment in Section 2.1.3 was repeated to separate the semisolid obtained from the addition of saturated ammonium sulfate (PEGylated sheep antihuman IgG.IgG). The quantity of IgG from combined supernatants was determined by UV spectroscopy to be 0.12 mg/mL,  $8 \times 10^{-7}$  mmol.

**2.6. Evaluation of Bioactivity of Isolated IgG With Enzyme-Linked Immunosorbent Assay (ELISA).** The IgG titer from the blood serum of a hepatitis C-vaccinated individual was measured by ELISA (21 mIU/mL). IgG isolated from the blood serum of the same individual by our method (experiment in Section 2.1.3) was also used in the ELISA experiment to afford a titer of 9 mIU/mL.

## 3. RESULTS

The general concept for the isolation of target proteins from a biological fluid such as blood serum or target small molecules from an aqueous equimolar mixture of three different small molecules is shown in Scheme 1. It is composed of four steps of association (absorption), separation 1, dissociation (desorption), and separation 2.

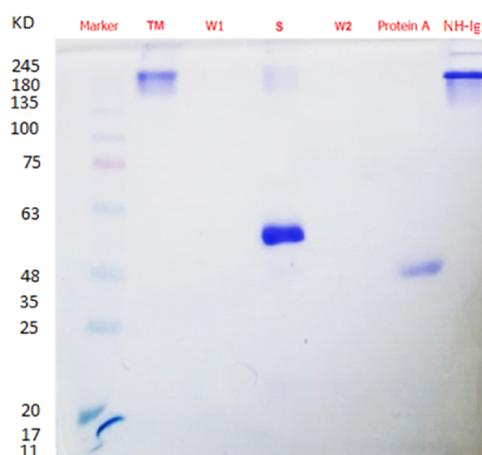
PEG-RMs were synthesized using chemically activated large-molecular-weight PEGs (1000 kD) followed by condensation with RMs. Binding of PEG-RM with a target protein in blood serum or with a target drug from an aqueous equimolar mixture of drugs occurred because of the high specificity of binding between PEG-RMs and the target molecules (TMs), resulting in the formation of a PEG-RM.TM macromolecular complex (association). Imparting unique physicochemical properties of PEG to PEG-RM.TM results in salting out of the macromolecular complex as a separable semisolid at low ammonium sulfate concentrations (separation 1). PEG-RM.TM was then dissolved in an appropriate buffer to afford a homogeneous mixture and converted to its components (PEG-RM.TM  $\rightarrow$  PEG-RM + TM) by decreasing the pH or increasing the ionic strength of the solution (dissociation). PEG-RM was salted out as a separable semisolid by the addition of ammonium sulfate, leaving pure TM in solution (separation 2), which can be isolated as a solid by diafiltration or dialysis of the added salt followed by lyophilization.

Proof of principle for the utility of this method was obtained using various models including PEGylated protein A and PEGylated sheep antihuman IgG for the isolation of Igs and IgG from blood serum, respectively, and PEGylated human serum albumin for the isolation of salicylic acid from an aqueous equimolar mixture of three drugs. Also, reciprocity of RM and TM was ascertained by reversing the roles of serum albumin and salicylic acid wherein PEGylated salicylic acid was used for the isolation of human serum albumin from blood serum. Furthermore, PEGylated protein A was used as a model to demonstrate the recyclability and scale-up potential of PEG-RMs.

Proteins and PEG can be easily detected in separation 1 and separation 2 (Scheme 1) using ninhydrin and iodine, respectively.<sup>39,40</sup> As expected, PEG-RM and PEG-RM.TM showed the presence of both protein and PEG, while the supernatants and washes were positive for proteins but negative for PEG. This shows a lack of binding of PEG-RM to untargeted proteins or peptides in blood serum. These detection methods were also used in PEGylation reactions of proteins.

Permutations of this method for the isolation of proteins and small target molecules are described below in more detail.

**3.1. RM and TM are Proteins.** Isolation of Igs (IgA, IgD, IgE, IgG, IgM) and IgG from blood serum as TMs was examined with PEGylated protein A and PEGylated sheep antihuman IgG, respectively (experiments in Sections 2.1.2 and 2.1.3). The semisolid from separation 1 was washed three times (Scheme 1,  $n = 3$ ) to remove any trace of unbound protein. The semisolid from separation 2 was dissociated in glycine buffer and precipitated with ammonium sulfate twice (Scheme 1,  $n = 2$ ) to ensure complete removal of any residual TM in PEG-RM.TM. The supernatants containing the TM were combined and identified using SDS-PAGE electrophoresis (Figures 1 and 2). The binding efficiency was

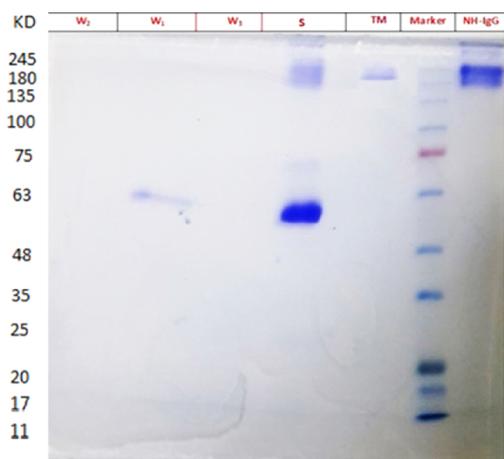


**Figure 1.** SDS-PAGE gel electrophoresis for the isolation of Igs as TM from blood serum using PEGylated protein A. S = supernatant, W<sub>1</sub>, W<sub>2</sub> = washes, and NH-Igs = normal human Igs.

calculated based on the initial approximate quantity of the TM used. The final quantity of TM isolated in solution was measured using UV spectroscopy (90% for protein A and 51% for sheep antihuman IgG). It should be noted that each protein A binds to two Igs and each sheep antihuman IgG binds to one IgG.<sup>41</sup>

Figure 1 demonstrates that Igs (TM) are properly separated (TM lane, as compared to the reference NH-Igs) and very little TM is observed in the supernatant (lane S), which is due to the high capture efficacy of protein A for TM (90%).<sup>41</sup> The protein concentration was determined on the final volume of 5 mL (2.97 mg/mL, see the experiment in Section 2.1.2). The washes do not show any Igs (lanes W<sub>1</sub> and W<sub>2</sub>). Also, no protein A is observed in the TM lane due to leaching, demonstrating the chemical stability of PEG-RM under the experimental conditions. Moreover, no protein aggregate was detected in the TM lane.

Figure 2 indicates that IgG (TM) is properly separated (TM lane, as compared to the reference NH-IgG lane). Some TM is



**Figure 2.** SDS-PAGE gel electrophoresis for the isolation of IgG as TM from blood serum using PEGylated sheep antihuman IgG as PEG-RM. S = supernatant,  $W_1$ ,  $W_2$ ,  $W_3$  = washes, and NH-IgG = normal human IgG (150 kD).

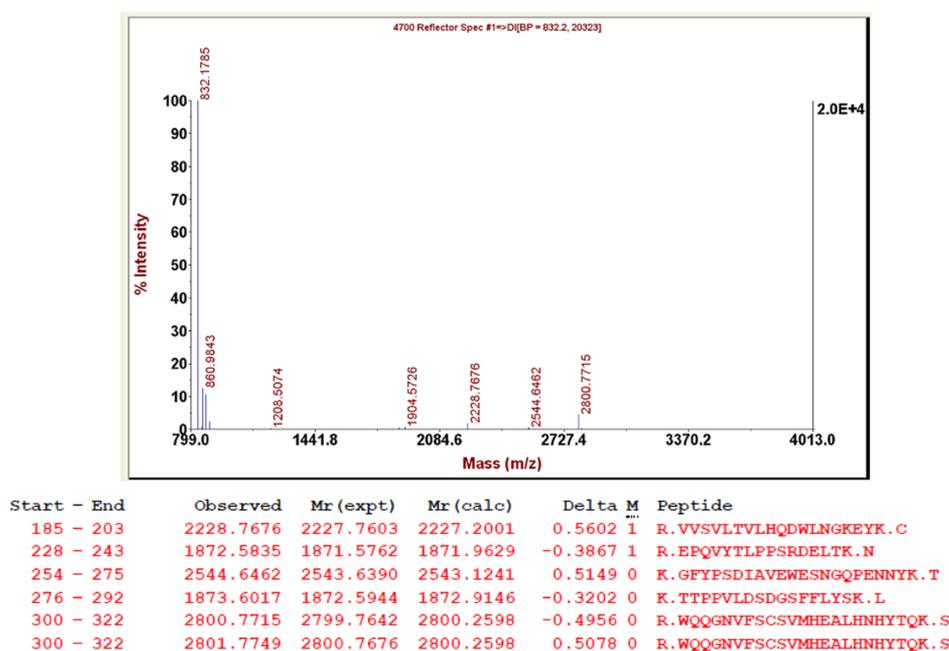
observed in the supernatant (lane S) IgG for TM (51% binding), which is in line with previously reported data by other investigators on lower capture efficacy of sheep antihuman IgG for IgG as compared with that of protein A.<sup>41</sup> Therefore, using a molar excess of PEGylated sheep antihuman IgG to TM in the experiment in Section 2.1.3 may result in complete removal of IgG. In affinity chromatography also, an excess amount of affinity resin is used for improved removal of the TM and a considerable fraction of the total resin capacity (50% or more) remains unused.<sup>42</sup> No IgG is observed in the washes (lanes  $W_1$  and  $W_2$ ). However, a small quantity of serum albumin, which is not the TM, is observed in the wash 1 ( $W_1$ ) lane. Also, no sheep antihuman IgG is observed in the T lane due to leaching, demonstrating the chemical stability of PEG-RM under the experimental

conditions. Moreover, no protein aggregate was detected observed in the TM lane due to unfolding.

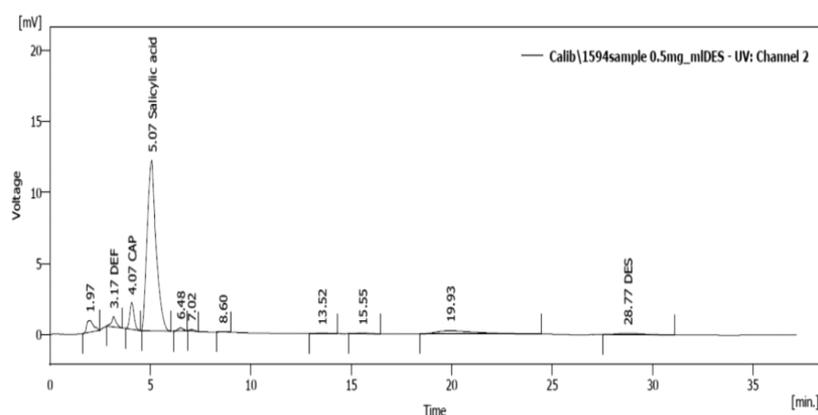
To examine the scale-up potential of the isolation method, the process was repeated using a 16.7-fold increase in the volume of blood serum (experiment in Section 2.1.4, from 60  $\mu$ L to 1000  $\mu$ L). The scaled-up process afforded identical protein concentration and SDS-PAGE gel electrophoresis to that of the 60  $\mu$ L sample (experiment in Section 2.1.2).

To ascertain the identity of the isolated IgG, it was subjected to peptide mass fingerprinting (PMF), as described in the experiment in Section 2.1.6.<sup>43</sup> The mass spectra of the trypsin digest of IgG and MASCOT search results are shown in Figure 3 and demonstrate good correspondence between the observed and calculated values for immunoglobulin heavy constant gamma 1 (IGHG1). Peaks in the 800–1400  $m/z$  are due to keratin as well as trypsin autocatalysis. This contamination is known and has been described in FindPept.

**3.2. RM is a Protein and TM is a Small Molecule.** Compared to other lipoproteins such as  $\alpha$ -1-acid glycoprotein or  $\alpha$ ,  $\beta$ , and  $\gamma$  globulins, human serum albumin is known to have a very high affinity for small molecules with carboxylic acid function.<sup>44</sup> It is therefore reasonable to assume that in an equimolar mixture of three different drugs, PEGylated human serum albumin would preferentially bind to a drug containing a carboxylic acid function. PEGylated human serum albumin (experiment in Section 2.2.2) was evaluated for preferential binding to salicylic acid in an excess equimolar mixture of salicylic acid, capecitabine, and deferiprone (experiments in Sections 2.2.1 and 2.2.2). The semisolid from separation 1 was washed twice to remove any unbound drugs (Scheme 1,  $n = 2$ ). The semisolid from separation 2 was dissociated with acetic acid and precipitated with ammonium sulfate twice to ensure complete removal of any residual TM in PEG-RM.TM (Scheme 1,  $n = 2$ ). The supernatants containing the TM were combined and identified by HPLC chromatography. Figure 4 demonstrates the preferential binding of salicylic acid to PEG-HSA (77% salicylic acid, 5% of capecitabine, and 3%

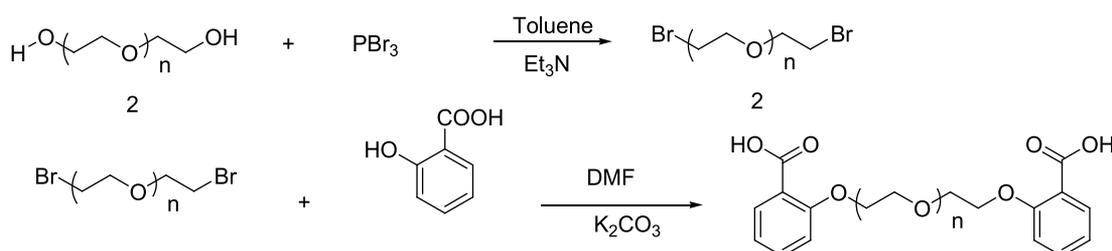


**Figure 3.** MALDI mass spectrum of IgG trypsin digest.



**Figure 4.** HPLC for the separation of salicylic acid as TM from an equimolar mixture of deferiprone (DEF), capecitabine (CAP), and salicylic acid.

### Scheme 2. PEGylation of Salicylic Acid

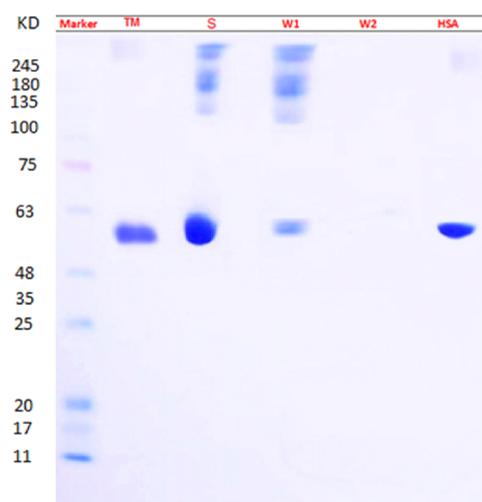


deferiprone). No attempt was made to identify the peaks corresponding to the remaining 15% since this experiment was qualitative and conducted as a proof of principle. Nonetheless, the unidentified peaks are close to the three active pharmaceutical ingredient peaks and could be their degradation products. Corrected to 100%, the percentages of the drugs will be 91, 6, and 3%, respectively. Details are provided in the experiment in Section 2.2.2.

**3.3. RM is a Small Molecule and TM is a Protein.** The reciprocity of RM and TM was examined by switching their roles in the above protocol (experiment in Section 2.2.2). Accordingly, salicylic acid was PEGylated at its hydroxyl function to evaluate its preferential binding serum albumin in blood serum (Scheme 2).

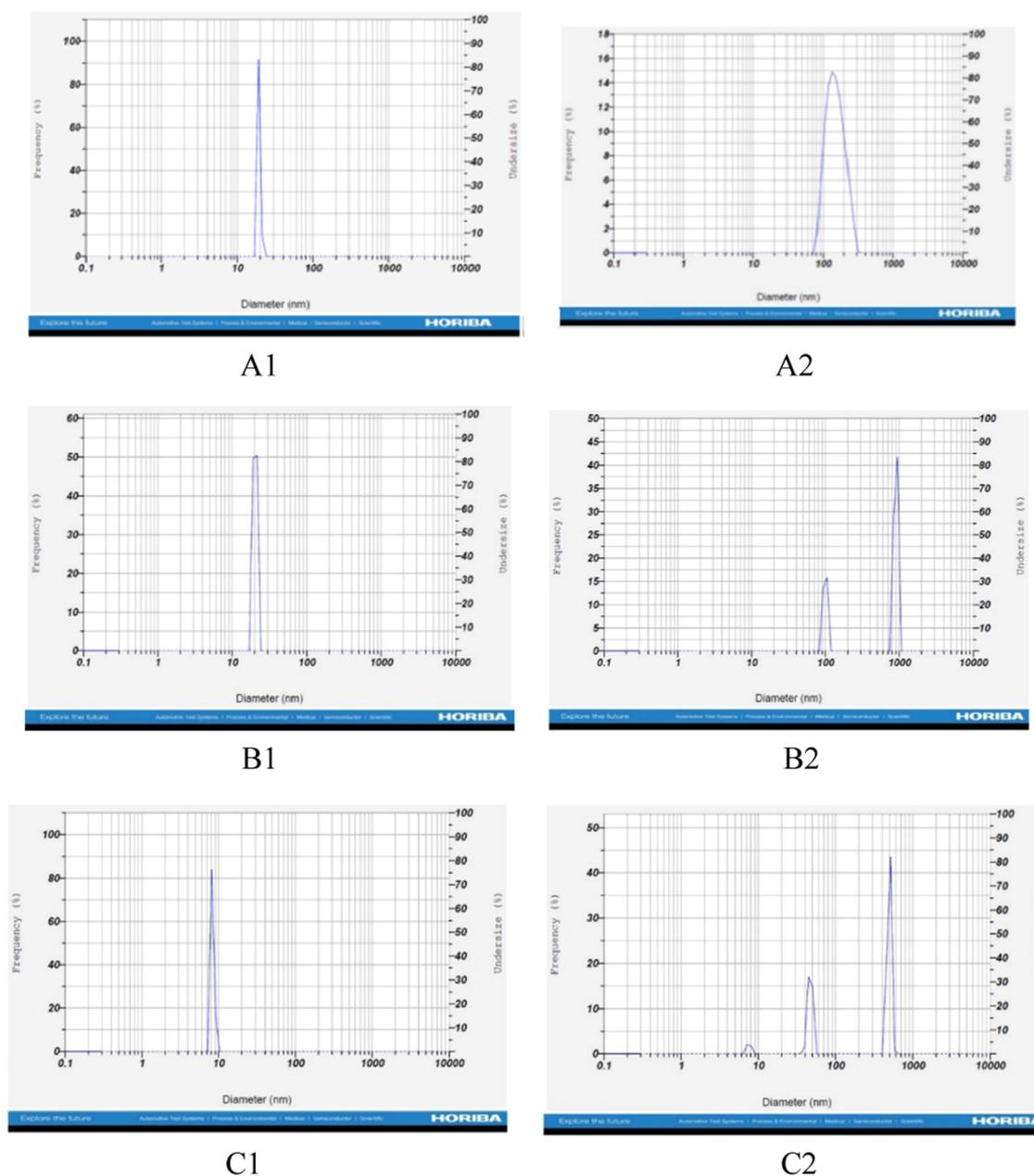
Binding of the resulting PEGylated salicylic acid to serum albumin was then validated (experiment in Section 2.3). The semisolid from separation 1 was washed twice to remove any unbound protein (Scheme 1,  $n = 2$ ). The semisolid from separation 2 was dissociated with acetic acid twice to ensure complete removal of any residual TM in PEG-RM.TM (Scheme 1,  $n = 2$ ). The supernatants containing the TM were combined and identified by SDS-PAGE electrophoresis (Figure 5). As shown below, human serum albumin is properly separated (TM lane, as compared to the reference human serum albumin lane). Since the purpose of this experiment was qualitative rather than quantitative, a large excess of blood serum was used simply to demonstrate the preferential binding of serum albumin to PEGylated salicylic acid. Therefore, a noticeable quantity of TM is observed in the supernatant (lane S) and after the first wash ( $W_1$  lane).

Figures 1, 2, and 5 demonstrate that the protocol reported here for the isolation of proteins from blood serum does not result in aggregate formation. This was further validated by dynamic light scattering (DLS) in the experiment in Section 2.4.<sup>45</sup> DLS spectra of freshly isolated and aged proteins (6–12



**Figure 5.** SDS-PAGE gel electrophoresis for the isolation of human serum albumin (TM) from blood serum using PEGylated salicylic acid. No aggregate of human serum albumin was detected in the isolated TM. S = supernatant,  $W_1$ ,  $W_2$  = washes, and HSA = human serum albumin.

months at  $-20\text{ }^\circ\text{C}$ ) are shown in Figure 6 and demonstrate the hydrodynamic diameters of human serum albumin (A1), normal human Igs (B1), and human IgG (C1) isolated by our method to be 18, 19.1, and 7.8 nm, respectively. The hydrodynamic diameters of the same proteins aged for 6 or 12 months at  $-20\text{ }^\circ\text{C}$  are compared on the right. Human serum albumin showed a wide band at 144 nm after 12 months at  $-20\text{ }^\circ\text{C}$  (A2). Normal human Igs showed hydrodynamic diameters of 93.6 and 847.8 nm (B2) and IgG showed hydrodynamic diameters of 7.3, 44.3, and 467.1 nm (C2) after 6 months at  $-20\text{ }^\circ\text{C}$ .



**Figure 6.** Dynamic light scattering spectra: A1 = freshly isolated human serum albumin; B1 = freshly isolated normal human Igs; C1 = freshly isolated IgG and the same proteins aged 6–12 months at  $-20^{\circ}\text{C}$ : A2, human serum albumin, 12 months; B2, Igs, 6 months; C2: IgG, 6 months.

UV spectroscopy was used to measure the binding of IgG isolated by our method for PEGylated sheep antihuman IgG (experiment in Section 2.5). The fact that very low IgG isolated by our method was found in the combined supernatant of the isolation procedure with PEGylated sheep antihuman IgG ( $0.12\text{ mg/mL } 8 \times 10^{-7}\text{ mmol}$ ) compared to the original quantity of IgG used in the experiment in Section 2.5 ( $3.33 \times 10^{-6}\text{ mmol}$ ) demonstrates that the isolation process did not adversely affect the binding of IgG to PEGylated sheep antihuman IgG. Also, as presented in the experiment in Section 2.6, bioactivity of isolated IgG was determined by comparing the ELISA titer of blood serum of a hepatitis C-vaccinated individual ( $21\text{ mIU/mL}$ ) with IgG isolated by our procedure from blood serum of the same individual ( $9\text{ mIU/mL}$ ). Considering the washes of the semisolid in separation 2 (Scheme 1) for complete separation

of IgG from the PEGylated sheep antihuman IgG semisolid, the above values demonstrate that our isolation protocol does not adversely affect the bioactivity of the isolated protein.

#### 4. DISCUSSION

We recently reported a highly efficient and simple method for the resolution of racemic mixtures using PEGylated resolving agents.<sup>46</sup> The target enantiomers were obtained through binding to the PEGylated resolving agent, salt-assisted phase transformation of the resulting complex to a separable solid which was and then dissociated to its components to afford the target enantiomers in very good yields. Simplicity and efficiency of the method, our previous experience with chemically modified matrices for immobilization of proteins,<sup>47–52</sup> and the lack of precedence for the use of PEG as the polymer in affinity precipitation of biomolecules prompted

us to examine the applicability of PEGylated recognition molecules (PEG-RMs, RM = proteins or small molecule) to the isolation of model protein TMs from blood serum and a model small TM from an aqueous equimolar mixture of three drugs.

A review of the literature revealed that proteins and small molecules have been PEGylated with relatively low molecular weight PEG (8 kD) and used for *affinity extraction* to increase the partition coefficient of target proteins in aqueous two-phase systems (ATPs). For example, Andrews et al.<sup>53</sup> used an aqueous mixture of pure IgG, PEGylated protein A (8 kD PEG), and dextran to demonstrate a 10–12-fold increase in the partition coefficient of IgG in the upper layer of an aqueous two-phase system containing PEGylated protein A. Silvério and co-workers<sup>54</sup> used PEGylated phenylalanine (8 kD PEG) to increase the partition coefficient of lysozyme or bovine serum albumin. However, we could not uncover any report in which PEG was used as a polymer for *affinity precipitation* of proteins.<sup>17,19</sup>

In light of the above, we evaluated the applicability PEG-RMs including two model proteins (protein A, sheep antihuman IgG) and a model small molecule (salicylic acid) for the isolation of Igs, IgG, and serum albumin from blood serum respectively. Our results demonstrate that PEGylated protein RMs can be utilized in a simple four-step process with high specificity and efficiency for precipitative isolation of various biomolecules from a complex biological fluid such as blood serum.

It must be emphasized that the reported PEG-based *affinity extraction* was carried out on very simple three-component mixtures to increase the partition coefficient of the target proteins.<sup>53,54</sup> On the other hand, in the method reported here, PEG-RMs afford pure protein TMs from blood serum, which is a highly complex mixture of innumerable molecules and macromolecules. In the present method, the observed specificity of binding (Association) is a consequence of the fundamental interactions between the tertiary structures of the two proteins (*vide infra*). There are therefore considerable differences between the specificity and efficiency of the PEG-based *precipitation* methods reported here for the first time and PEG-based *extraction* methods reported previously.<sup>53,54</sup>

PEG and ammonium sulfate have been used traditionally for the precipitation of proteins.<sup>55,56</sup> This could, at least in theory, interfere with the precipitation of protein TMs. However, in addition to binding specificity, another key to the isolation of a single protein from blood serum and near-exclusive isolation of a drug from an equimolar mixture of three drugs in our method is the use of PEG with its unique physicochemical properties, which are imparted to PEG species (PEG-RM.TM and PEG-RM). Among these properties are entirely different effects of salt on the water solubility of PEG as compared to proteins. Equally important is the inverse relationship between the molecular weight of PEG and the quantity of the salt required to cause its phase transformation from a solution to a semisolid. In combination, these unique properties provide an ideal condition for the precipitation of PEG species (PEG-RM.TM and PEG-RM) without coprecipitation of untargeted proteins or drug molecules (*vide infra*).

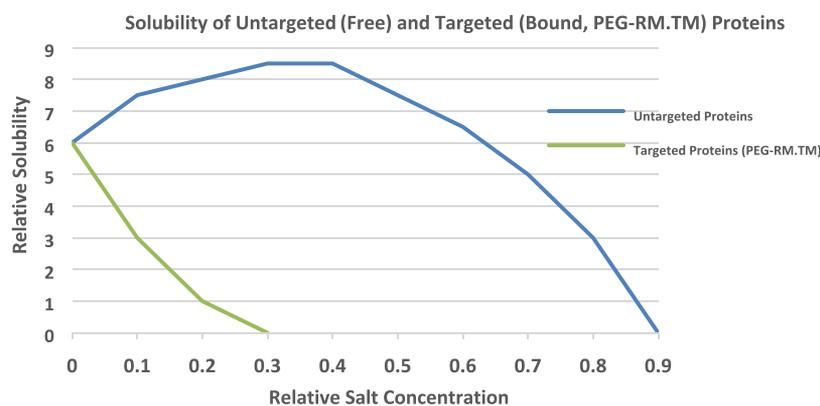
Similar to the resolution of enantiomers alluded above,<sup>46</sup> two basic phenomena are involved in our method for the isolation of biomolecule TMs from blood serum. The specificity of binding (Scheme 1, association) is caused by inimitability of RM, and salt-assisted phase transformation of

PEGylated species is affected by unique physicochemical properties of PEG (Scheme 1, PEG-RM.TM separation 1 and PEG-RM separation 2). A discussion of these phenomena is necessary to fully appreciate their contributions to the utility of the method reported here.

Specificity is the underlying reason for binding (association) of two molecules. It is a result of the fundamental interactions (electrostatic, ionic and H-bonding; van der Waals: dipole–dipole; hydrophobic; and  $\pi$  interactions) between two molecules. These are universal parameters and apply to all chemical entities with various sizes, surface characteristics, and charge distributions. Collectively, they result in the lowest free energy of complex formation.<sup>57</sup> PEGylation will undoubtedly affect the availability of chemical functional groups involved in fundamental interactions of RM and thereby influence its specificity for binding to TM. Accordingly, there is no single strategy that can unambiguously define the binding properties of PEGylated proteins to their TMs.<sup>58,59</sup> However, a large number of fundamental interactions between biological molecules appear to afford a remarkable level of binding specificity in the PEGylated protein,<sup>60</sup> which has resulted in the approval of numerous PEGylated therapeutic entities.<sup>9</sup>

In the case of PEGylated proteins, Pai<sup>61</sup> and Gokarn<sup>62</sup> have reported that PEG assumes a random coil conformation extending away from the protein in aqueous solutions. Other investigators have demonstrated that PEGylation does not drastically affect the conformation of many proteins and in fact stabilizes them.<sup>58,63</sup> Cattani et al. have shown the interaction between the PEG and the protein to be relatively negligible.<sup>64</sup> Other reports have shown that PEGylation minimizes protein unfolding; expedites refolding; and reduces denaturation, disulfide exchange, and aggregate formation.<sup>58,59</sup> Also, many PEGylated proteins used as drugs maintain their therapeutic properties and exhibit increased conformational stability compared to their native counterparts.<sup>65,66</sup> Effects of PEGylation on *in vivo* and *in vitro* activities of therapeutic proteins by branched<sup>67</sup> and linear<sup>58</sup> PEGs have been reported by various investigators. In fact, relative quiescence of PEG has afforded 26 FDA-approved PEGylated drugs<sup>68</sup> and 125 sponsored clinical trials for new PEGylated therapeutic entities.<sup>69</sup> In combination, the available data imply that the interactions between proteins and PEGs are much less than what would be expected from PEG random coil volume alone, which has been calculated to be ten times the volume of the crystalline form.<sup>64</sup> In short, our results on the use of model PEGylated proteins and salicylic acid also demonstrate an astounding level of the binding specificity of PEG-RMs for their TMs.

The second important phenomenon in this report is salt-assisted phase transformation of PEGylated species (Scheme 1, PEG-RM.TM separation 1 and PEG-RM separation 2). The effect of salt on the solubility of PEG and proteins is very different. Protein solubility increases at low salt concentrations (slating-in) because of salt interference with fundamental interactions, which in turn results in a decrease in nonspecific protein–protein binding.<sup>70</sup> High salt concentrations, on the other hand, reduce water–protein interactions and result in salting out of proteins.<sup>58</sup> PEG, On the other hand, salts out at low salt concentrations, and equally important, there is an inverse relationship between the molecular weight of PEG and the quantity of salt required for its salt-assisted phase transformation.<sup>71</sup> Therefore, the use of high-molecular-weight PEG in our method guarantees phase transformation of PEG



**Figure 7.** Schematic presentation of salt-assisted phase transformation of untargeted (free) and targeted (bound) proteins (PEG-RM.TM) using high-molecular-weight PEGs.

species (PEG-RM.TM and PEG-RM) at low salt concentrations with a concomitant increase in the solubility of untargeted (free) proteins. Furthermore, using PEG (1000 kD) ensured dominance of the precipitative character of PEG over solubility contribution of the two proteins (RM and TM) in PEG-RM.TM at low salt concentrations. Moreover, high-molecular-weight PEG prevents multi-PEGylation, which would inadvertently involve additional heteroatoms (SH, NH, OH) of the RM that are also involved in fundamental affinity interactions with its TM.<sup>72,73</sup>

Salting out of PEG is believed to involve different types of interactions between PEG, water, and salt.<sup>74</sup> Each oxyethylene unit of PEG forms hydrogen bonds with 3–5 water molecules, increasing its hydrodynamic volume by 5–10 folds.<sup>72</sup> Therefore, salt interferes with water–oxyethylene electrostatic interactions by disrupting ionic and hydrogen bonding. At the point of salting out, the salt–water interactions become more favorable than PEG–water interactions.<sup>73</sup> This process is entropy-driven and causes an increase in the local concentration of PEG in the aqueous medium, ultimately resulting in phase transformation of PEG from a solution to a semisolid.<sup>74</sup> The unique physicochemical properties of PEG are imparted to PEG species PEG-RM and PEG-RM.TM that salt-out accordingly. In combination, these characteristics provide an ideal situation for precipitation of PEG-RM.TM with a simultaneous increase in solubility of untargeted (free) proteins, providing a better opportunity for binding of PEG-RM to the target protein (TM) in the homogeneous system.<sup>58,70,71</sup>

An additional advantage for the use of PEG is its neutrality. The use of charge-responsive polymers that undergo phase transformation in aqueous media (soluble to insoluble) in reported affinity precipitation is disadvantageous since charged stimuli (acid, base, electrolytes, small molecules, etc.) can also disrupt electrostatic interactions in the native conformation of proteins.<sup>13,17</sup> This can result in nonspecific binding interactions that reduce the efficiency of the association step. However, high-molecular-weight PEG salts out from the solution with a minimum quantity of ammonium sulfate with a concomitant increase in the solubility of unbound proteins. Moreover, ammonium sulfate has been used traditionally in very high concentrations for protein isolation and is therefore not expected to disrupt the tertiary structure of proteins.

IgG isolated by this method showed excellent binding to PEGylated sheep antihuman IgG as established by UV spectroscopy. Equally important, the bioactivity of IgG,

isolated by this method from the blood serum of a hepatitis C-vaccinated individual, was ascertained by comparison of its ELISA result to that of a blood sample from the same individual. These results demonstrate that the native conformation of IgG isolated by our protocol is retained.

Dissociation of the macromolecular complex to its components (PEG-RM.TM  $\rightarrow$  PEG-RM + TM) by decreasing the pH or increasing the ionic strength of the aqueous solution is brought about by the interference of the ionic species with electrostatic and van der Waals interactions between PEG-RM and TM. This in turn can affect hydrophobic and  $\pi$  interactions, which are essential for high specificity and binding between PEG-RM and TM in the case of proteins.<sup>75</sup>

It should be noted that the binding of PEG-RM to TM is an equilibrium process in which specificity is the prime determinant in the association step.<sup>76</sup> Separation 1 and separation 2 (Scheme 1), on the other hand, are gradual processes and are completed when salt–water interactions become more favorable than PEG–water interactions. Therefore, according to the Le Chatelier Principle, the gradual addition of ammonium sulfate shifts the equilibrium forward as PEG-RM.TM departs from the aqueous medium in the form of a semisolid, affording pure PEG-RM.TM after a few washes (Scheme 1). In light of the above, salting out of untargeted (free) and targeted (bound) proteins is expected to follow the general trend shown in Figure 7.

Finally, oligonucleotide aptamers hold great promise as potential replacements for protein biopharmaceuticals.<sup>77,78</sup> However, the target molecule in various SELEX methods is attached to water-insoluble platforms, affording heterogeneous systems with known limitations<sup>78,79</sup> and making pH, ionic strength, and buffer selections crucial factors in aptamer absorption to and desorption from affinity-modified matrices (pH shifts).<sup>80</sup> The affinity precipitation method presented here has the potential to provide homogeneous SELEX in binding (association) and desorption (dissociation) steps for both small molecules as well as macromolecules. Accordingly, we are at the present time evaluating the applicability of the precipitative method to the isolation of nucleic acids and oligonucleotide aptamers. This, along with the application of PEG-RM (RM is a protein or a small molecule) for the isolation of aptamers in homogeneous SELEX systems, will be reported in the near future.

## 5. CONCLUSIONS

In this paper, we present the first time use of a number of PEGylated recognition molecules as models for the isolation of target proteins from blood serum and selective isolation of the target drug from an aqueous equimolar mixture of three different drugs by affinity precipitation. Simplicity, efficiency, and applicability of the present method were demonstrated in model systems used in this paper.

PEG-RMs were stable under the experimental conditions used to isolate their TMs, and their recyclability and scale-up potential was established using PEGylated protein A. Binding specificity between RM and TM and the use PEG are the keys to exclusive isolation of a single protein from the blood serum and preferred separation of a drug from an aqueous equimolar mixture of three drugs. Also, homogeneity of this system in the critical steps of association and dissociation (Scheme 1) may be the reason for its celerity and perhaps the rationale for the absence of aggregates in isolated protein TMs. Moreover, binding of IgG isolated by the present protocol and its biological activity were demonstrated by US spectroscopy and ELISA experiments, respectively.

The precipitative isolation protocol reported here provides thought-provoking possibilities for its potential applications in medicinal chemistry. For example, it may be possible to use a PEGylated drug receptor, transmembrane active transporter, etc. to identify lead drug molecule(s) obtained from combinatorial chemistry. In the reverse scenario, PEGylated small molecules can be used to capture a target protein (a drug receptor, transmembrane active transporter, etc.). In combination, our results support the contention of applicability of this method for the isolation of proteins and small-molecule TMs from complex biological fluids such as blood serum. Clearly, infinite variations in the structures of biological macromolecules mandate evaluation of the efficacy of the protocol to each case by experimentation.

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## Author Contributions

All authors contributed to the study. Material preparation, chemistry, and data analysis were performed by J.M. Biochemistry experiments were performed by B.Z., M.N., and L.F. Conception, design, and the first draft of the manuscript were written by K.K., and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Notes

The authors declare no competing financial interest.

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