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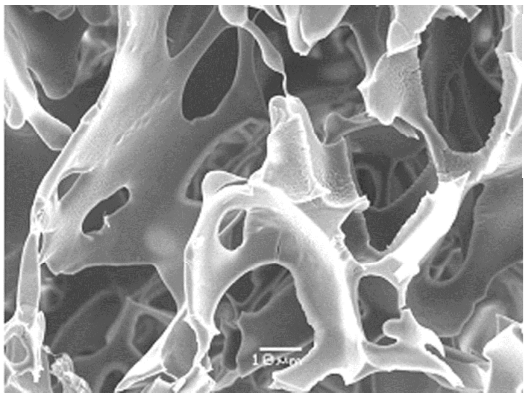

Supermacroporous cryogels can be used for high abundant protein depletion prior to proteome investigations.

Affinity recognition based polymeric cryogels for protein depletion studies

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Abstract

Several affinity recognition based polymeric materials have been developed for the depletion of highly abundant proteins in the serum samples. Among these, cryogels with their macroporous structure give rise to use them in the field of protein depletion. Particularly, the requirement of depletion of high abundant proteins prior to proteome investigations makes cryogels attractive for researchers. In the present review, the recent developments and applications of affinity recognition based polymeric materials and cryogels are reviewed for protein depletion.

1. Introduction

Proteomics is the quantitative and physical mapping of the total proteins from a particular cell line, tissue, body liquids or organism **[1].** Two-dimensional protein electrophoresis (2-DE) for separating proteins and mass spectrometry (MS) for the identification of separated proteins are the most commonly used experimental techniques in proteomics **[2].**

Human serum is attracting increasing interest in proteomics, which attempts to broadly characterize its protein constituents. The characterization of the thousands of individual serum proteins/peptides enables the discovery of the markers of many diseases, such as infection, inflammation, cancer, diabetes, malnutrition, cardiovascular diseases, Alzheimer and other autoimmune diseases **[3-8]**.

Serum contains more than a million different proteins and peptides in a large dynamic concentration (*i.e.* 60–80 mg/ml), which presents many beneficial attributes for proteomic analysis **[4]**. However, this high protein content in serum makes the proteome analysis very challenging, because serum proteins are present across an extraordinary dynamic concentration range from the common highly abundant proteins (tens to ~2 mg/mL) including albumin, immunoglobulins (IgG and IgA), antitrypsin, haptoglobin and transferrin to extremely low abundant proteins (such as the vasoconstrictor peptide endothelin-1) **[9]**. A major contributing factor to the analytical challenge of characterizing the serum proteome is that a single protein, albumin, comprises 50% of total protein content (Figure 1; www.plasmaproteome.org). Indeed, only 10 proteins constitute 90% of the protein content of serum. Of the remaining 10%, 12 proteins make up 90% of this remaining total. Therefore, only 1% of the entire protein content of serum is made up of proteins that are considered to be in low abundance and of great interest in proteomic studies in search of potential biomarkers. The essential first step in the serum proteome analysis is the reduction of sample complexity (*e.g.* to depletion of high abundant proteins), which reduces their masking effect on the biomarkers **[4, 10-12]**.

Figure 1. The chart represents the relative abundance of proteins within plasma. Twenty-two proteins constitute 99% of the protein content of plasma (www.plasmaproteome.org).

While the depletion of all the proteins listed in Figure 1 considered being highly abundant proteins, removal of albumin and immunoglobulins (IgGs) (constitute over 80% of serum proteins) would have a significant improvement toward analysis of the serum proteome.

It is of great importance to find out cheap and highly efficient techniques for the removal of high abundant proteins. Currently, there are several depletion technologies for the removal of albumin and IgG from serum, including ultracentrifugal filtration, dye affinity, immunoaffinity depletion, immobilized metal affinity chromatography (IMAC) and imprinting methods **[4, 13-21].**

In the case of albumin, depletion can be achieved by either well known dye-ligands such as the Cibacron Blue F3GA (CB) and derivatives, or specific antibodies **[22-24].** Dye affinity resins for depletion of albumin have advantages of high loading capacity and economic properties with compared to antibody-based systems, but have been shown to lack of specificity **[25-29].**

The next challenge in serum protein analysis is the depletion of the IgGs. The removal of IgG is commonly achieved by protein A/G affinity adsorbents, which binds to the Fc region of the IgG, **[30-32]**, but specific antibodies can also be used. Protein A/G and monoclonal antibodies to IgG are available from commercial suppliers. However, IgG is present in serum at concentrations in the range of 8–16 mg/mL, and large quantities of antibody and/or protein A/G are required for its quantitative removal. The high specificity of the bioligands provides excellent selectivity. However, in spite of their high selectivities, protein A/G or antibody carrying adsorbents also have some drawbacks that are worth considering: (i) the cost of these materials tends to be very high; (ii) these bioligands are difficult to immobilize in the proper orientation **[33]** and (iii) ligands may leak from the stationary phase and such contamination cannot, of course, be tolerated in clinical applications. Since, depleted IgG from the source of serum should be in high purity (> 99%), which is employed for the treatment of immune disorders, including systemic lupus erythematosus, rheumatoid arthritis, myasthenia gravis, allo-immunization, and cancer **[34-40].**

Among the methods used in depletion approaches, molecular imprinting has much attention in the recent studies, due to the advantages such as easy to prepare, high selectivity toward target molecules and stable at harsh conditions in comparison to common methods.

In the present review, the depletion approaches prior to proteomic investigations are collected regarding to affinity recognition based polymeric materials. Some specific features of hydrogels, so called cryogels, which have recently found an application area in proteomic studies, are also presented for further investigations.

2. Affinity Recognition Based Polymeric Materials for Protein Depletion Studies

Serum proteins may often serve as indicators of diseases and it is a rich source for biomarker discovery. Collectively, albumin and immunoglobulin constitute over 80% of the human serum proteome **[3]**.

Even the best 2-DE can routinely resolve no more than several thousand proteins; only the most abundant proteins can be visualized by gel electrophoresis if a crude protein mixture is used. In this context, affinity based protein depletion prior to 2-DE may help to reduce sample complexity and permit the analysis of lower-abundance proteins. Increasing the amounts of low-abundance proteins relative to the amounts of other proteins facilitates the analysis by 2-DE and MS **[2].**

In literature, the removal of these proteins represents a fundamental improvement toward characterization of the serum proteome **[41].** Prior to proteome analysis, several affinity recognition based commercial columns have addressed for the depletion of high abundant proteins in the serum samples **[11, 15, 42, 43].** Steel and co-workers employed an immune-affinity resin to remove albumin and IgG from human serum samples in order to simplify the serum proteome. Pieper and co-workers revealed 950 protein spots in 2-DE gel using immuno-affinity subtraction chromatography by the following depletion of 10 proteins from human blood plasma **[44].** Chan and co-workers used an affinity spin tube filter method to remove albumin and IgG in enrichment of low-abundant cancer biomarkers in serum. They identified over 250 potential biomarkers for breast cancer **[22].**

Greenough and co-workers have reported the specific removal of 98% of albumin and 80% of immunoglobulin heavy chain from human plasma by affinity chromatography, and the subsequent improvement in the number of spots detected and their resolution following two-dimensional gel electrophoresis **[43].** They employed commercially available cartridges

containing POROS® perfusion chromatography media coated with affinity purified goat polyclonal antibody against HSA, and Protein G cartridge containing POROS® covalently coupled with recombinant Protein G (Applied Biosystems, Foster City, CA, USA), which provide simple, rapid and reproducible method, for the depletion of albumin and immunoglobulin, respectively. Govorukhina and co-workers also compared commercially available three types of columns for their capacity to remove albumin and/or IgG from human serum: (a) columns that bind human serum albumin (Poros Anti HSA, HiTrap Blue, Merck Albumin Removal column), (b) columns that bind γ-globulins (Poros protein G, HiTrap protein G), and (c) a single column that was designed to bind both albumin and γ-globulins simultaneously (Aurum Serum Protein column) **[42].** They have achieved effective depletion of human serum with respect to albumin and/or the γ-globulins using *dye ligand affinity or immunoaffinity chromatography*. Colantonio and co-workers developed a CB based extraction method for the effective and specific removal of albumin from serum with increased detection of low abundant proteins in electrophoresis **[45]**. Morais-Zani and co-workers performed the depletion of albumin from *Bothrops jaraca* plasma using the HiTrap Blue high-performance column (GE Healthcare Life Sciences, Piscataway, NJ, USA) and the kit Albumin&IgG Depletion SpinTrap column (GE Healthcare Life Sciences) **[46].**

On the other hand, Tirumalai and co-workers emphasized that many low molecular weight (LMW) proteins are poorly recovered, as several prefractionation approaches employing chromatographic adsorbents and immunoaffinity methods have been used to remove albumin **[4, 29, 47]**. Albumin is known to act as a carrier and transport protein within blood and therefore is likely to bind many species of interest such as peptide hormones, cytokines, and chemokines **[48].** They have employed a different prior application, named *centrifugal ultrafiltration*, and have significantly depleted the large, highly abundant proteins such as albumin and enriched for LMW proteins. Taking into consideration the role of albumin as a transport protein in blood, they have conducted centrifugal ultrafiltration using a buffer containing

20% acetonitrile to disrupt any potential protein-protein/peptide interactions. They have analyzed the serum samples by SDS-PAGE before and after ultrafiltration and significantly observed a depletion of albumin in the ultrafiltrate **[4].**

Another approach was applying several types of immobilized metal affinity chromatography (IMAC) columns to proteomics **[19].** One of them was for the enrichment of phosphorylated peptides with Ga^{3+} or Fe^{3+} loaded IMAC column **[49]**. The esterification-IMAC method has been successfully used to characterize more than 200 phosphopeptides in the yeast proteome and human carcinoma cell line **[50, 51].** IMAC-based enrichment of phosphorylated peptides also has been demonstrated to work well on simplified mixtures. For instance, IMAC enrichment of phosphorylated peptides without prior chemical modification was used to characterize nearly 300 phosphopeptides after pre-fractionation with anion exchange chromatography **[52]** in cultured plant cells. In 2005, the first study using IMAC to analyze phosphorylation sites present in mammalian tissue has been published. In this work, phosphorylated proteins and subsequently phosphorylated peptides were enriched with IMAC **[53].** The selection of histidine-containing peptides is an important aspect of proteomics in sample simplification and database search. Immobilized Cu^{2+} affinity chromatography has been used in proteomics to simplify sample mixtures by selecting histidine-containing peptides from proteolytic digests **[54]**. IMAC combined with mass spectrometry has recently been employed for isolation of naturally occuring metal binding proteins from total proteins in human liver cells **[55]**. Cu^{2+} charged alginate beads have been directly used as an IMAC medium for purification of IgG from goat serum **[56].** Recently, the studies employing IMAC to deplete IgG from human serum have also been presented. Karataş and co-workers employed Cu^{2+} coupled magnetic poly(ethylene glycol dimethacrylate-*N*-methacryloyl-(L)-histidine methyl ester), m-poly(EGDMA-MAH-Cu) beads, 50–100μm in diameter, for affinity depletion of IgG **[57].** They introduced efficient mass transfer properties of magnetically stabilized fluidized beds (MSFBs) into IgG depletion studies to provide the separation relatively rapid and easy **[58, 59]**. Incorporation of *N-methacryloyl-(L)-histidine*

methyl ester (MAH) monomer into the polymer structure has minimized the ligand leaching problem and the ability to reuse the affinity beads helps to keep the cost reasonable for research purposes **[60] (Figure 2)** In addition to, when anti-HSA-sepharose adsorbent is used together with metal-chelated magnetic beads, albumin and IgG can be depleted in a single step.

Figure 2. Schematic diagram of Cu^{2+} chelated poly(EGDMA-MAH) magnetic bead **[60]**.

Affinity adsorbents with large specific surface area for adsorption are desirable to attain high binding capacity with fast binding kinetics **[61]**. To increase the surface area to the practically useful level of 100 m^2/g, either highly porous materials with large pore size or nonporous nano-sized beads have to be employed. Due to these reasons, micron-sized affinity adsorbents such as silica and polystyrene-based particles have been gaining more attention for the rapid high-performance liquid chromatography of biomolecules **[62]**. A major advantage of the non-porous adsorbents is that significant intra-particle diffusion resistances are absent; this is particularly useful for the rapid analysis of proteins with high efficiency and resolution **[63].** The rapid separation makes it very useful for quality control, on-line monitoring, and purity check of biomolecules such as peptide mapping of recombinant products. However, silica is unstable in extreme pH, and polystyrene-based particles are hydrophobic, which makes them exhibit pronounced nonspecific protein adsorption **[64].** Therefore, the development of other polymeric adsorbents with low nonspecific protein adsorption is desirable.

As a polymer backbone, epoxy-derived adsorbents can be used almost in ideal systems to develop very easy protocols for biomolecule immobilization **[65].** Currently, poly(glycidyl methacrylate) [poly(GMA)] has attracted much attention for its hydrophilic characteristics. Poly(GMA) have been used as the basic matrix because of its known good mechanical strength, stability at neutral pH values even in wet conditions, and high reactivity of the epoxy groups for surface immobilization. In application for the proteomic studies, Altıntaş and co-workers reported iminodiacetic acid (IDA) modified non-

porous monosize poly(GMA) beads with chelated Cu^{2+} ions as a model adsorbent capable of selective binding of IgG from human plasma **(Figure 3 and Figure 4) [66]**. They have reached selective depletion efficiency of IgG as above 98%.

Figure 3. Schematic diagram for the preparation of poly(GMA) metal-chelated beads **[66]**.

Figure 4. SEM photograph of the non-porous monosize poly(GMA) beads **[66]**.

In another study, due to the high reactivity of the epoxy groups for surface immobilization, dye affinity based poly(GMA) monosize beads were prepared by modifying the surface with a dye ligand Cibacron Blue (CB) for albumin affinity depletion **[67]**. Dye attachment onto the non-porous *monosize beads* significantly increased the albumin adsorption (189.8 mg/g), while the non-specific adsorption was 0.8 mg/g. Based on the evaluations of depletion efficiency, reproducibility and binding specificity of the dye-affinity beads in literature offered the promising depletion approach. CB-attached bacterial cellulose (BC) nanofibers, poly(GMA) nanobeads and poly(HEMA) nanospheres were also developed in order to increase the surface area of depletion matrix for high efficiency albumin removal from human plasma **(Figure 5) [68-70].**

Figure 5. SEM images of CB-attached bacterial cellulose (BC) nanofibers (a) **[68]**, poly(GMA) nanobeads (b) **[69]** and poly(HEMA) nanospheres (c) **[70].**

3. Applications of Cryogels to Protein Depletion Studies

3.1. Cryogels

Cryogels, so called hydrogels are synthesized under semi-frozen conditions when the ice crystals serve as porogens, and a network of inter-connected pores is formed after the ice crystals melted **(Figure 6) [71-74].** In contrast to hydrogels, cryogels have large pores (d>1μm), short diffusion path, usually

good biocompatibility, and high mechanical strength **[75]**. They could widely be used as scaffolds in tissue engineering **[76-79]**, as separation matrices in chromatography **[74, 80-84]**, in bioreactors **[85]** and other applications **[71, 72]**. They can be either used as pure polymeric or filled with solid micro/nano-particles in literature for environmental applications **[86-89]**. To a large extent, the applications of cryogels are defined by their macroporous structure allowing effective mass transport of macromolecular solutes and migration of cells, high biocompatibility, good mechanical properties and water state in the interior of these highly hydrated systems similar to the state of bulk water **[90]**.

Figure 6. Cryogel preparation steps: (i) a monomer solution with a cross-linker, (ii) freezing and cryogelation at subzero temperature (iii) thawing to form (iv) macroporous cryogel (freeze-dried for SEM image).

Cryogels have generally nonporous and thin polymer walls, large continuous interconnected pores (10–100 μm in diameter) that provide channels for the mobile phase to flow through **(Figure 7) [91]**. The transport of solutes inside the cryogel column proceeded mainly due to the convection rather than due to the diffusion. As a result of the convective flow of the solution through the pores, the mass transfer resistance is practically negligible. The interactions between cryogel and whole blood without causing damage to the blood cells, and no requirement of any sample pre-treatment steps get cryogels through the most favorable matrices for the proteome investigations **[92]**. In addition, cryogels are classified as economically favorable adsorbents, which can be used in a disposable manner to avoid cross-contamination between successive batches of a sample **[93, 94]**.

3.2. Affinity Recognition based Cryogels for Protein Depletion Studies

Recently, cryogels have been used for removing high abundant plasma albumin and IgG, which constitutes higher than 80% of the total amount of plasma proteins, prior to detection of low-abundance plasma proteome, in

which most of biomarkers of any possible disease could be found.

Kumar and co-workers generated a systematic library of 96 affinity resins which were generated using novel analogs of CB and tested in a batch binding and elution mode using seven different proteins – four *Aspergillus* enzymes namely, nicotinamide adenine dinucleotide phosphate (NADP)-glutamate dehydrogenase, laccase, glutamine synthetase and arginase, bovine pancreatic trypsin and the two serum proteins, HSA and IgG. Binding capacities up to 50 mg/mL HSA were achieved **[95].** Demiryas and co-workers attached CB to poly(acrylamide-allyl glycidyl ether) [poly(AAm-AGE)] cryogel and used this dye-affinity cryogel for binding of HSA from human plasma. The maximum amount of adsorbed HSA was 27 mg/g from aqueous solution up to 74.2 mg/g from human plasma, respectively **[96]**.

Dye immobilization requires an inert, hydrophilic support, which possesses chemically modifiable groups. Most frequently used matrices are the naturally occurring polysaccharide polymers: dextran, agarose, and cellulose. However, these materials can undergo biological degradation. Therefore, in another study, PHEMA was used as the main matrix for CB immobilization, due to its inertness, mechanical strength, chemical and biological stability, and biocompatibility **(Figure 7) [91].** The maximum binding capacity for CB attached PHEMA cryogel column was obtained as 950 mg/g of dry cryogel for non-diluted serum. The adsorption capacity decreased with increasing dilution ratios while the depletion ratio of albumin remained as 77% in serum sample. The detection of less abundant proteins in electrophoresis that hindered by HSA or IgG was improved by removing highly abundant serum proteins prior to analysis **(Figure 8)**.

Figure 7. SEM picture of CB-PHEMA cryogel **[91].**

Figure 8. SDS-PAGE analysis of human serum before and after treatment with PHEMA-CB cryogel column. 5–12% SDS-PAGE, Lane 1: Standard HSA (1 mg/mL), Lane 2: Standard IgG (1mg/mL), Lane 3: untreated human serum, Lane 4: human serum after three repeated treatment with cryogel column,

Lane 5: Wide range Sigma Marker (Molecular Weight, Da), Lane 6: the elution from the cryogel column **[91].**

The development and application of a dye affinity and IMAC based composite cryogel system was investigated for the simultaneous depletion of albumin and IgG from human serum **(Figure 9) [97]**. Poly(GMA) beads embedded PHEMA composite cryogels aimed to combine the advantages of both small particles and convective media without bringing along the disadvantages of a high pressure drop and low binding capacity.

Figure 9. PGMA beads embedded PHEMA cryogel **[97]**.

In the recent study, PGMA beads were modified with CB and iminodiacetic acid (IDA)-Cu^{2+} for simultaneous albumin and IgG depletion, respectively. Albumin adsorption capacity of the PGMA-CB beads embedded PHEMA cryogel and IgG adsorption capacity of the PGMA-IDA-Cu^{2+} beads embedded PHEMA cryogel was found as 342 mg/g and 257 mg/g, respectively. The composite cryogels depleted albumin and IgG from human serum with 89.4% and 93.6% of efficiency, respectively **[97]**.

In another study, a different affinity composite cryogel approach was investigated. Uzun and co-workers synthesized and characterized proteinous, polymeric nanoprotein A nanoparticles (nanoProA) and immobilized CB onto these nanoparticles (CB-nanoProA) **(Figure 10) [98]**. They used CB-nanoProA nanoparticles to embed into poly(2-hydroxyethyl methacrylate) (PHEMA) based cryogels with three different compositions, as the plain PHEMA cryogel, the nanoProA embedded PHEMA cryogel and the CB-nanoProA embedded cryogel. In these compositions, four important points were addressed: (i) increasing the stability of protein A molecules via nanoparticle generation, (ii) enhancing the affinity of protein A molecules toward albumin molecules using CB immobilization, (iii) integrating the features of cryogels with those of the protein A and CB; and (iv) evaluating the simultaneous depletion performance of the cryogels. The competitive protein adsorption was performed using a protein mixture prepared with albumin and

IgG adhering to their plasma protein ratios. Since, the CB was immobilized onto nanoProA nanoparticles, albumin depletion performance of the composite cryogels increased whereas the IgG depleting performance decreased. The nanoProA embedded composite cryogel removed 99.3% of IgG, while the CB-nanoProA embedded composite cryogel removed 97.5% of albumin.

Figure 10. Optical photo (a) and SEM images (b) of the cryogels. White (Left): NanoProA/PHEMA; Blue (Right): CB-nanoProA/PHEMA **[98]**.

3.3. Molecularly Imprinted Cryogels in Protein Depletion Studies

Recently, the utilisation of *molecularly imprinted polymers* (MIPs) has been attracted interest in depletion strategies. MIPs with specific recognition sites shaped by a template molecule have been developed to remove target molecules out of a medium containing a mixture of many components **[99].** MIPs are less expensive than affinity adsorbents with biological ligands, easy to prepare and stable at harsh conditions (i.e., high temperature, pressure, high pH, and organic solvents). MIPs have received much attention for addressing a variety of bioseparation challenges **[99-104]** due to their high selectivity toward the small analytes, bacteria, viruses, peptides, and proteins **[105-111].** However, in case of proteins, there is a small amount of literature on the development of MIPs due to the limited mobility of these large template molecules within highly cross-linked polymer networks and irreversible binding and/or poor efficiency in binding. Furthermore, when imprinting proteins there are some challenges that are largely absent when targeting small analytes, and these are related to the molecular size, complexity, conformational flexibility, and solubility **[112]**. Therefore, from a thermodynamic and practical perspective, it is difficult to develop successful imprints for such macromolecules. In general, the kinetics and thermodynamics of the recognition of the template molecule by MIPs depend on the nature of the intermolecular interactions (e.g., electrostatic, van der Waals, H-bonding) in the binding site as well as the physical and chemical nature of the polymeric adsorbent (e.g., flexibility, accessibility of the binding sites, materials' shapes)

[113]. In addition to, the MIP approach has its limitation for the imprinting of protein macromolecules mainly due to the difficulty of the template removal and the restricted accessibility of the binding sites **[112, 114, 115].**

A supermacroporous cryogel is a good alternative to traditional protein-binding matrices having the advantages as large pores, short diffusion path, low-pressure drop, and very short residence times for both adsorption and elution. Furthermore, the polymerization at subzero temperatures prevents the protein from conformational changes, which leads to rebind to cavities in high selectivity. Andac and co-workers prepared macroporous cryogels imprinted with human serum albumin (HSA) **[116]**. They synthesized a functional co-monomer of N-methacryloyl-L-phenylalanine (MAPA) **[117]** to complex with HSA via hydrophobic interactions prior to polymerization. This poly(hydroxyethyl methacrylate) (PHEMA) based cryogel was prepared in the presence of MAPA-HSA complex by free radical polymerization at temperatures below 0^{o}C (PHEMAPA-HSA cryogel). The template molecule HSA was removed by a desorption agent to attain imprinted cavities for recognition of HSA **(Figure 11).** The maximum adsorption capacity was obtained as 390.2 mg/g of PHEMAPA-HSA cryogel for non-diluted serum. Although, the depletion ratio of albumin was relatively low (35%), the selectivity of PHEMAPA-HSA cryogel was found obviously high regarding the competitive proteins (transferrin and myoglobin) used in the study. The relative selectivity coefficients of the PHEMAPA-HSA cryogel for HSA/transferrin and HSA/myoglobin pairs were found as 56 and 18 times greater than the non-imprinted PHEMAPA cryogel, respectively.

Figure 11. Schematic illustration of PHEMAPA-HSA cryogel **[116]**.

Gao et al. used *p*-vinylphenylboronic acid (VPBA) as functional monomer to complex with a glycoprotein ovalbumin (OB) using the principle of boronate affinity **[73].** They prepared pH and temperature dual-responsive macroporous imprinted cryogels (OB-imprinted cryogels) that possess the capability to recognize and separate a target glycoprotein from a real sample. They also applied the resulting imprinted cryogels to separate OB from egg

white samples efficiently.

A new generation of molecularly imprinted macroporous cryogels were prepared for depletion of hemoglobin (Hb) from human blood by Derazshamshir and co-workers **[118].** They synthesized N-methacryloyl-L-histidine methyl ester (MAH) as a functional monomer for the interaction with Hb via imidazole group prior to polymerization. The preparation of Hb-imprinted cryogel was organized in three main parts. In the first part, MAH was synthesized by reacting methacryloyl chloride with L-histidine methyl ester. MAH was then complexed with Hb. In the second part; a Hb-imprinted poly[(hydroxyethyl methacrylate)-co-(N-methacryloyl-L-histidine methylester)] (Hb-PHEMAH) cryogel was prepared in the presence of MAH-Hb complex by free radical polymerization below 0^{o}C. In the third part, Hb was removed by a convenient desorption agent to have imprinted cavities for molecular recognition of Hb **(Figure 12)**. By this method, they found the maximum Hb adsorption capacity by the Hb-PHEMAH column as 11.4 mg/g polymer on the average, which represents the saturation of the active binding sites of the MIP column. They also reached Hb selectivity coefficients of Hb-PHEMAH cryogel in the presence of bovine serum albumin (BSA) and myoglobin (Myb) as 12 and 38 fold, respectively. They also confirmed Hb depletion from blood hemolysate using SDS-PAGE.

Figure 12. Schematic illustration of preparation of Hb-PHEMAH cryogel **[118]**.

Surface imprinting has been widely studied as one approach to improving the performance of MIPs, as it solves the problems of limited mass transfer and template removal often associated with the traditional technique of molecular imprinting **[20]**. This improvement is especially valuable when imprinting macromolecules (i.e., human serum albumin [HSA]), for which diffusion limitation is the major issue **[21, 119, 120].**

Composite cryogels are classified as porous polymeric monolith, which is synthesized under partially frozen conditions, where a solution/suspension mixture is polymerized around ice crystals **[121]**. A new composite protein-imprinted macroporous cryogel for depletion of albumin from human serum

have been prepared by Andac and co-workers **[122]**. They prepared HSA surface imprinted PGMA beads, which were embedded into the polyhydroxyethyl methacylate (PHEMA)-based cryogel to obtain high HSA binding capacity with low flow resistance **(Figure 13)**.

Figure 13. SEM photographs of HSA imprinted PGMA beads with 4000 times (1A) and 25000 times magnified (1B), MIP (1C and 1E) and NIP (1D and 1F) composite cryogels with different magnification levels **[122].**

PHEMA was selected as the basic component of cryogel because of its inertness, mechanical strength, chemical and biological stability and biocompatibility. The advantages of PHEMA-based interconnected open macroporous cryogel had been combined with the surface imprinting approach. The specific surface area of PHEMA-based MIP composite cryogel was 232.0 m^2/g in average, which was approximately 10-fold larger than the conventional PHEMA cryogel (25.2 m^2/g). MIP composite cryogel column was successfully applied in the fast protein liquid chromatography system for selective depletion of albumin in human serum. The depletion ratio of HSA was highly increased by embedding beads into cryogel (85%), while the non-embedded cryogel was insignificant. The results showed that all the advantages of both MIP beads and macroporous cryogels could be combined as composite materials in proteome applications.

Some selected affinity recognition and MIP based depletion strategies are summarized in Table 1.

Table 1. Some selected affinity recognition and MIP based depletion strategies.

Interaction Type	**Target/Material**	**Efficiency**	**Selectivity**	**Ref.**
IMAC	Enrichment of phosphorylated peptides and proteins	High	Group specific	49-53
IMAC	IgG depletion	High, low cost	Group specific	57

	under MSFBs			
IMAC	IgG depletion with monosize microbeads	Above 98%	Group specific	66
Dye affinity	Albumin depletion with monosize microbeads	High (189.8 mg/g), low cost	Group specific	67
Dye affinity	Albumin depletion with nanofibers, nanobeads, nanospheres.	High, low cost	Group specific	68-70
Dye affinity	Albumin and IgG depletion with cryogels	High efficiency up to 98%	Group specific	91, 95, 96
Dye affinity and IMAC	Albumin and IgG depletion with composite cryogels	High efficiency up to 97% (albumin) and 99% (IgG)	Group specific	97, 98
MIPs	Albumin depletion	Low (35%)	High selectivity against transferrin and myoglobin	116
MIPs	Ovalbumin depletion	High efficiency (23 mg/g MIP cryogel)	High selectivity against transferrin and horseradish peroxidase	73
MIPs	Hemoglobin depletion	High efficiency (23 mg/g MIP cryogel)	High selectivity against albumin and myoglobin	118
MIPs	Albumin depletion with composite cryogels	High efficiency up to 85%	High selectivity against transferrin and myoglobin	122

4. Conclusions

Serum proteome analysis plays an important role in biomarker discovery. However, albumin and immunoglobulin collectively constitute over 80% of the human serum proteome. The essential first step in the serum proteome analysis becomes the reduction of sample complexity (*e.g.* to depletion of these high abundant proteins), which reduces the masking effect on the biomarkers. Several affinity recognition based depletion approaches were

generated using polymeric materials. Among these, cryogels have become attractive for researchers by their sponge like macroporous structure, which leads to enhanced mass transfer properties. Moreover, embedding micro-sized particles into cryogel structure enhance the surface area and binding capacity of proteins. The promising results showed that cryogels could be used in proteome applications by their desirable physical morphologies and favorable protein adsorption capacity.

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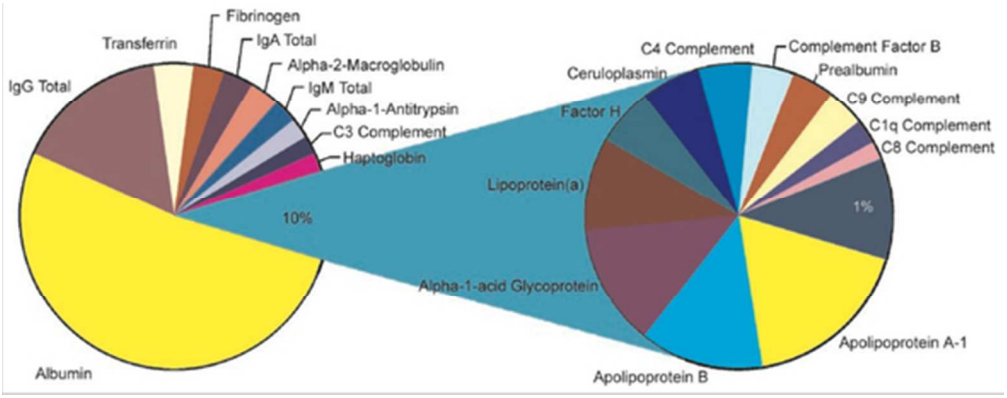

Figure 1. The chart represents the relative abundance of proteins within plasma. Twenty-two proteins constitute 99% of the protein content of plasma (www.plasmaproteome.org).
49x19mm (300 x 300 DPI)

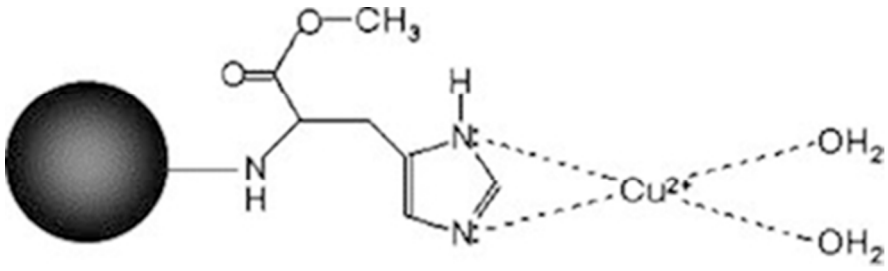

Figure 2. Schematic diagram of Cu2+ chelated poly(EGDMA-MAH) magnetic bead [60].
37x10mm (300 x 300 DPI)

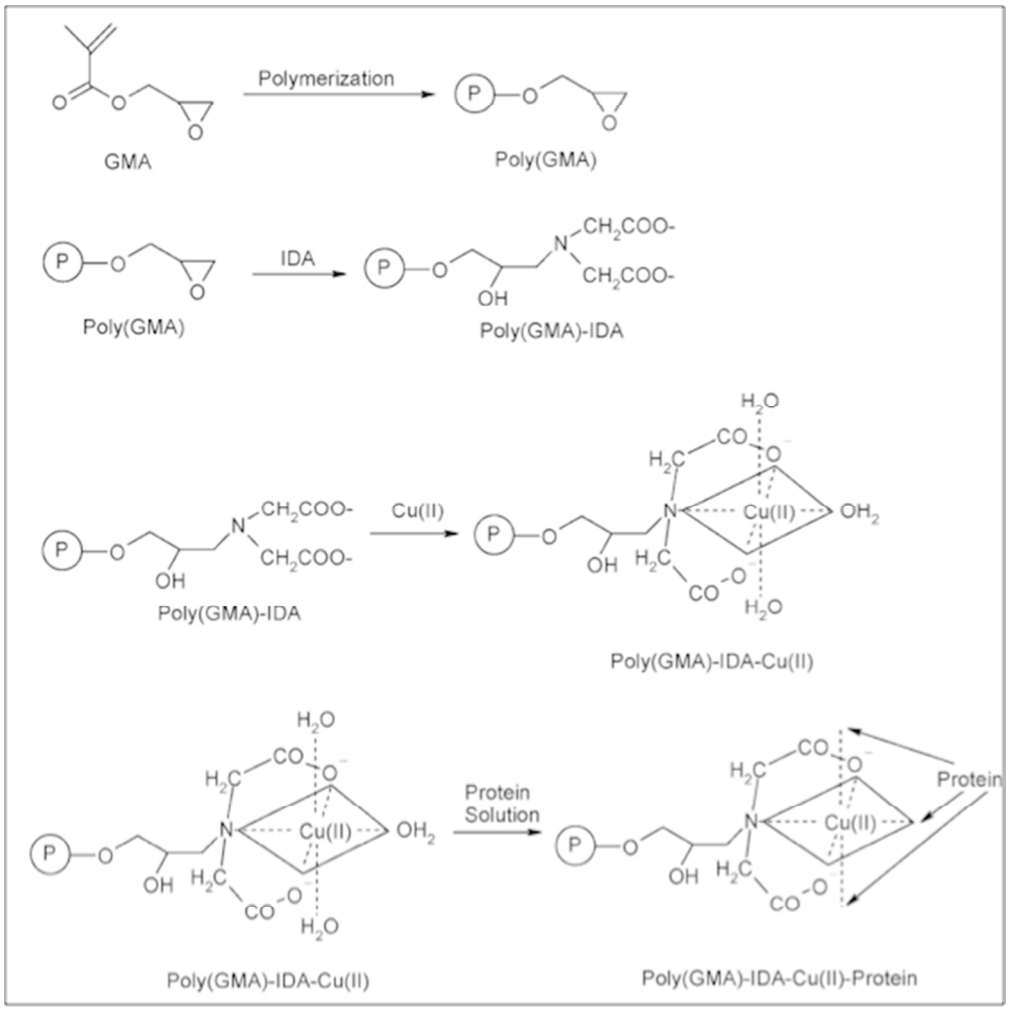

Figure 3. Schematic diagram for the preparation of poly(GMA) metal-chelated beads [66].
76x76mm (300 x 300 DPI)

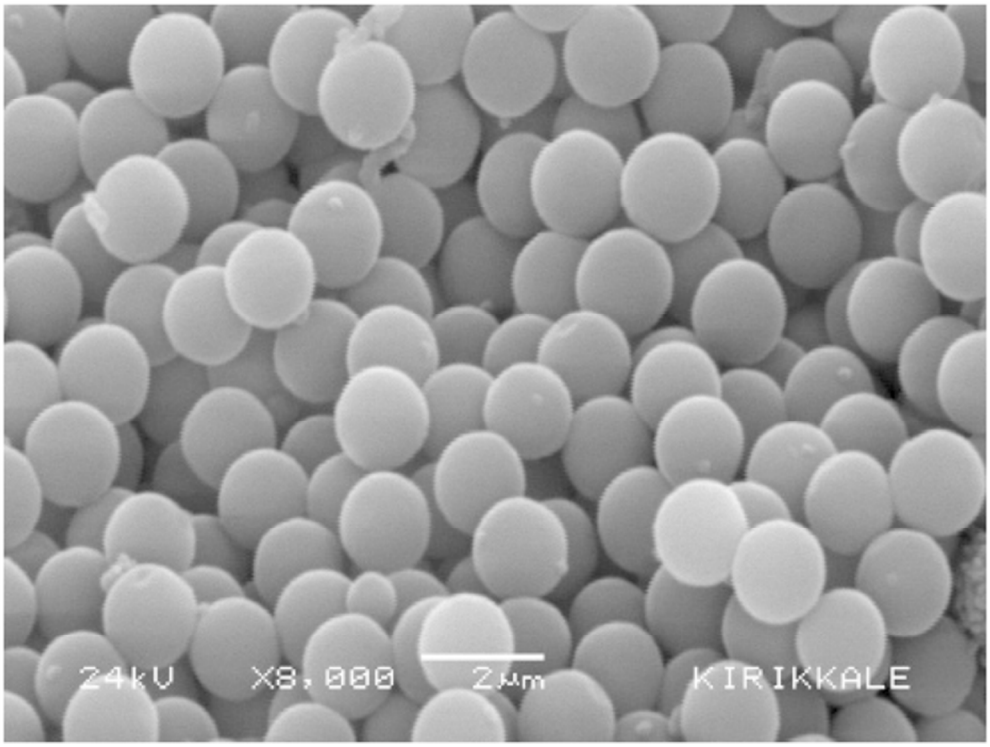

Figure 4. SEM photograph of the non-porous monosize poly(GMA) beads [66].

63x47mm (300 x 300 DPI)

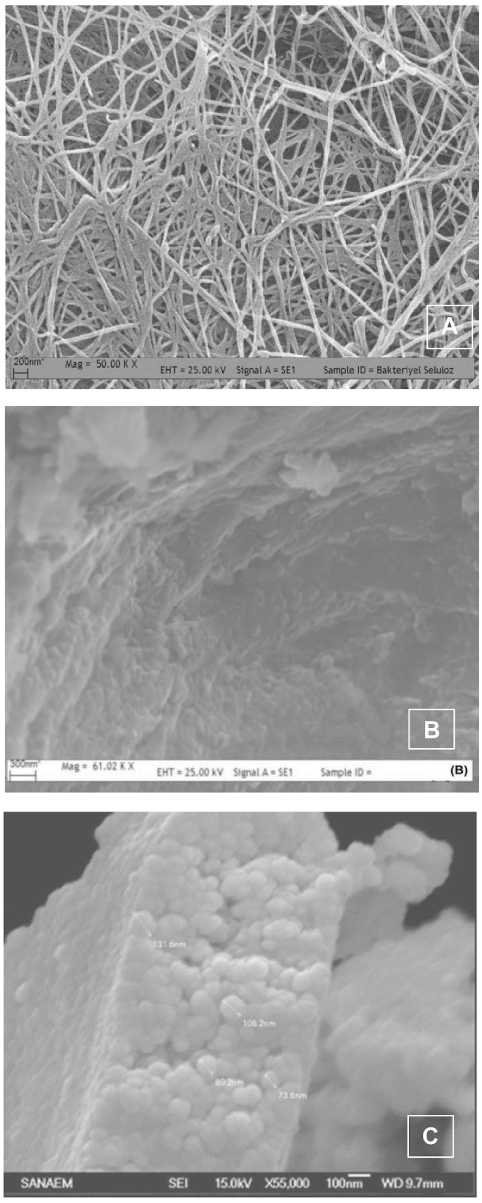

Figure 5. SEM images of CB-attached bacterial cellulose (BC) nanofibers (a) [68], poly(GMA) nanobeads (b) [69] and poly(HEMA) nanospheres (c) [70].
203x513mm (300 x 300 DPI)

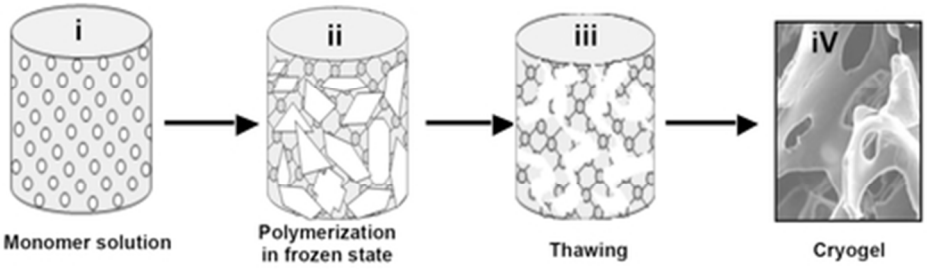

Figure 6. Cryogel preparation steps: (i) a monomer solution with a cross-linker, (ii) freezing and cryogelation at subzero temperature (iii) thawing to form (iv) macroporous cryogel (freeze-dried for SEM image).
41x12mm (300 x 300 DPI)

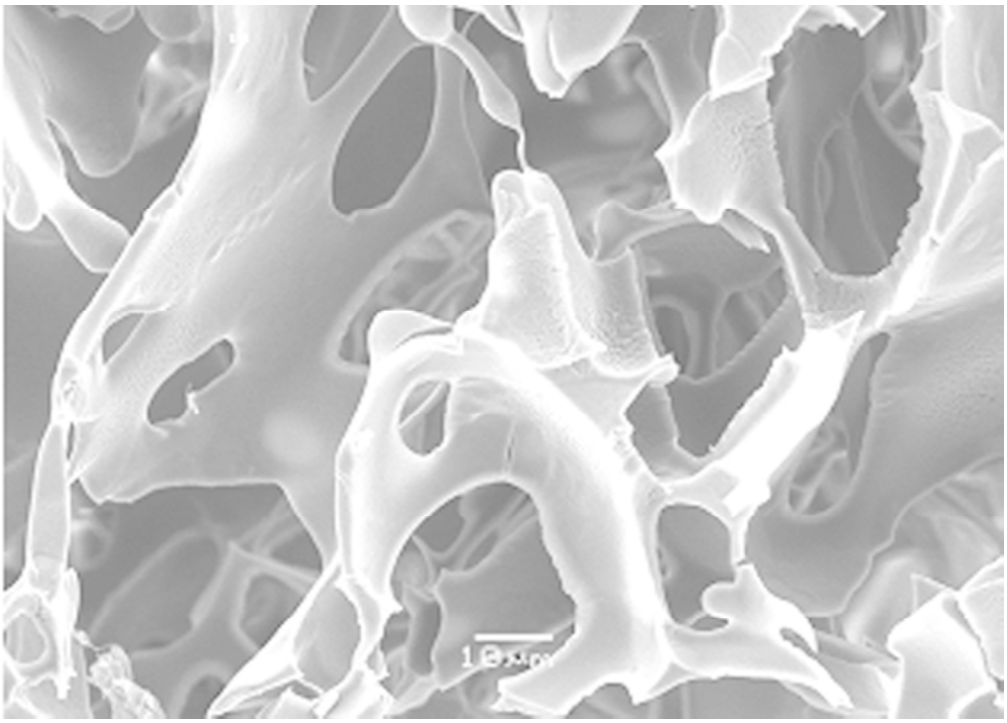

Figure 7. SEM picture of CB-PHEMA cryogel [91].
54x38mm (300 x 300 DPI)

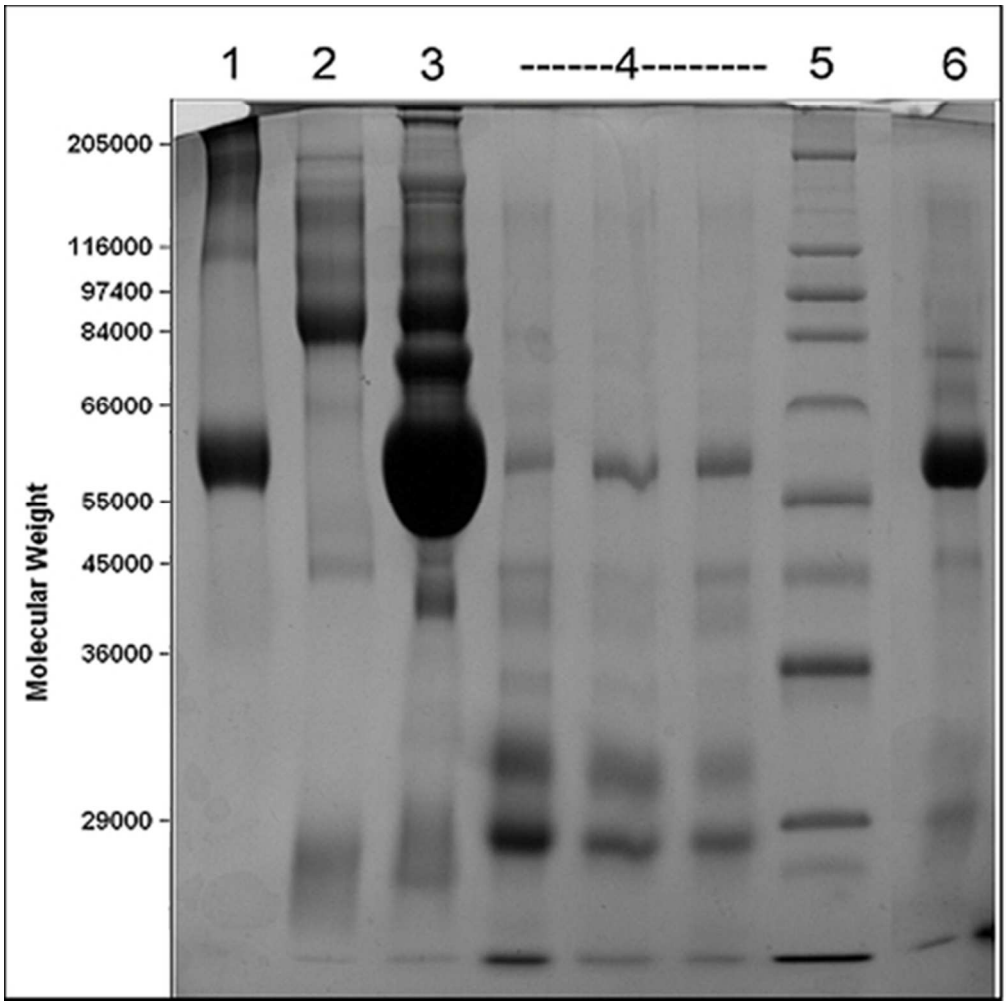

Figure 8. SDS-PAGE analysis of human serum before and after treatment with PHEMA-CB cryogel column. 5–12% SDS-PAGE, Lane 1: Standard HSA (1 mg/mL), Lane 2: Standard IgG (1mg/mL), Lane 3: untreated human serum, Lane 4: human serum after three repeated treatment with cryogel column, Lane 5: Wide range Sigma Marker (Molecular Weight, Da), Lane 6: the elution from the cryogel column [91].
50x50mm (300 x 300 DPI)

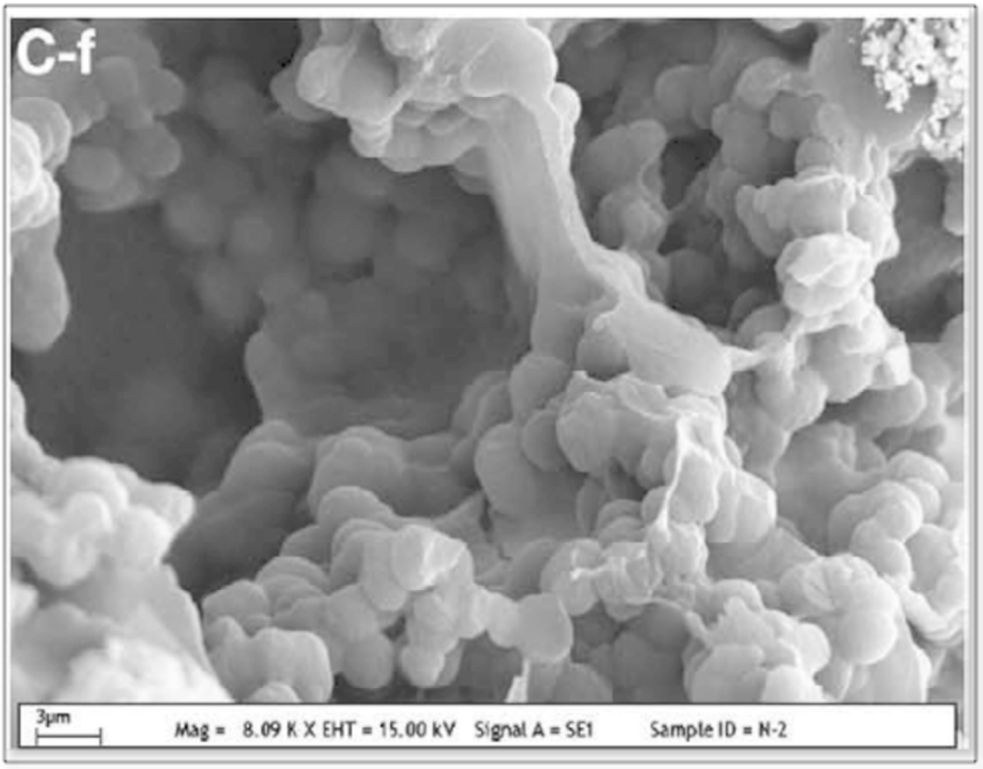

Figure 9. PGMA beads embedded PHEMA cryogel [97].
59x47mm (300 x 300 DPI)

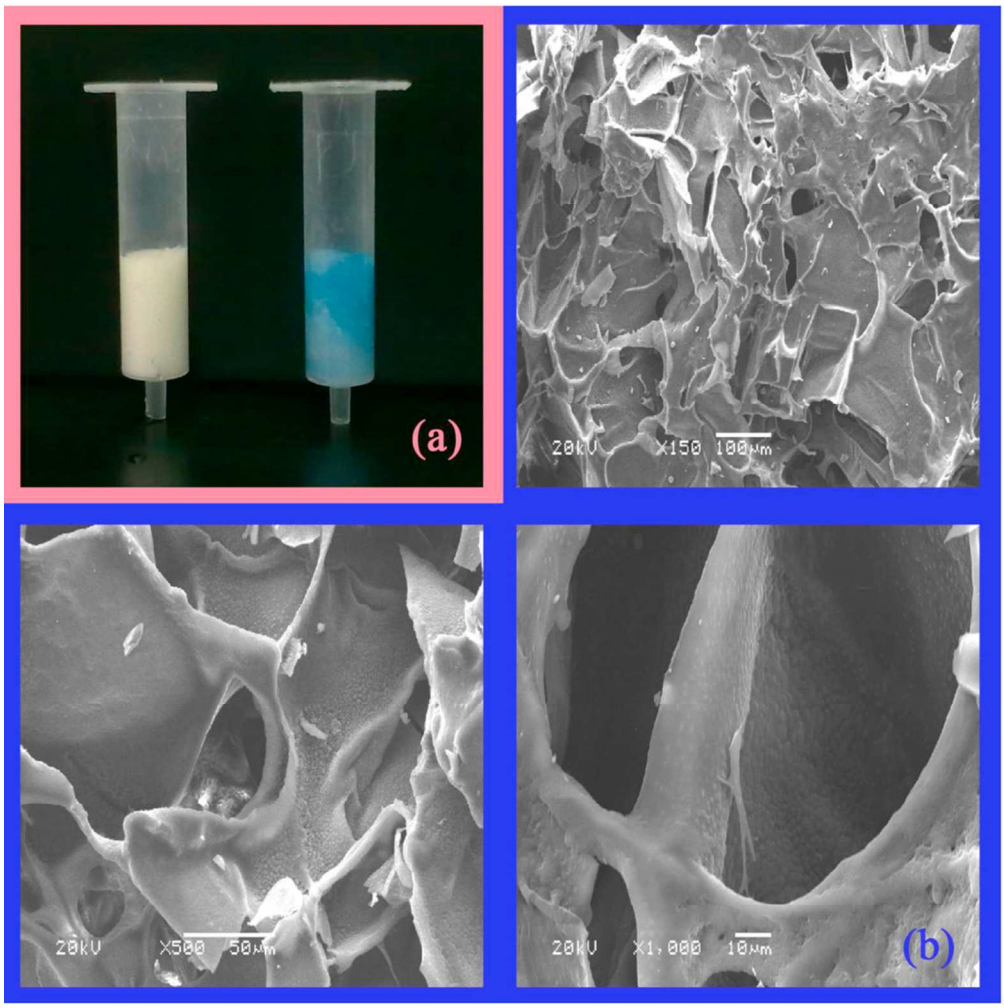

Figure 10. Optical photo (a) and SEM images (b) of the cryogels. White (Left): NanoProA/PHEMA; Blue (Right): CB-nanoProA/PHEMA [98].
101x101mm (300 x 300 DPI)

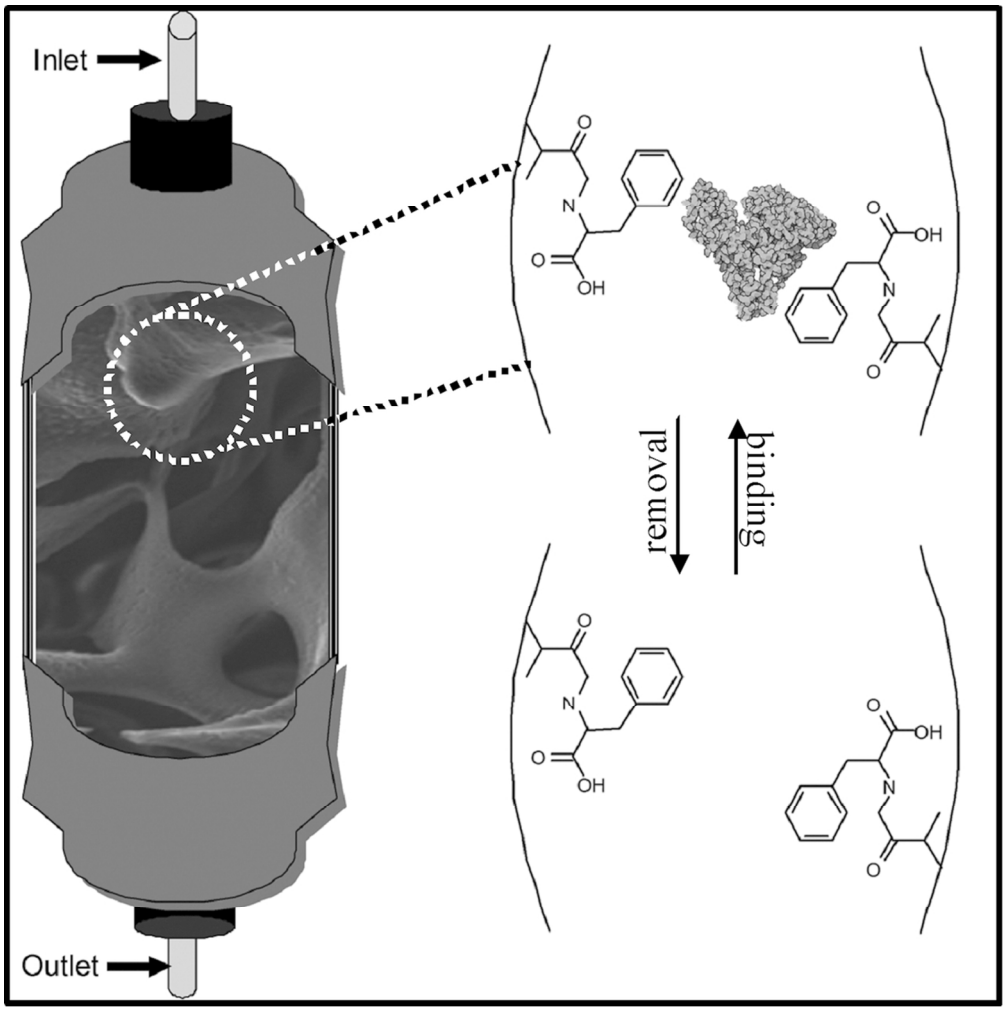

Figure 11. Schematic illustration of PHEMAPA-HSA cryogel [116].
109x109mm (300 x 300 DPI)

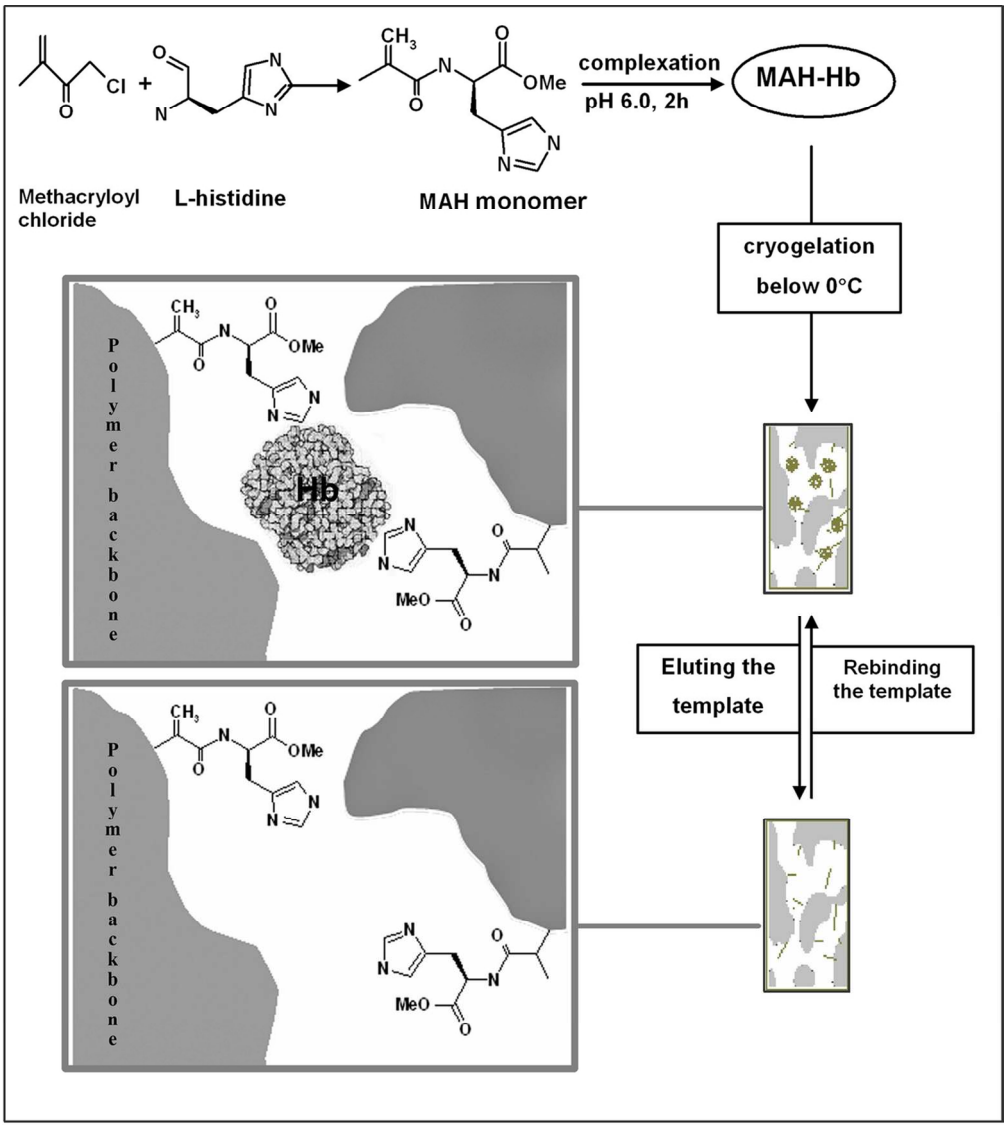

Figure 12. Schematic illustration of preparation of Hb-PHEMAH cryogel [118].
113x126mm (300 x 300 DPI)

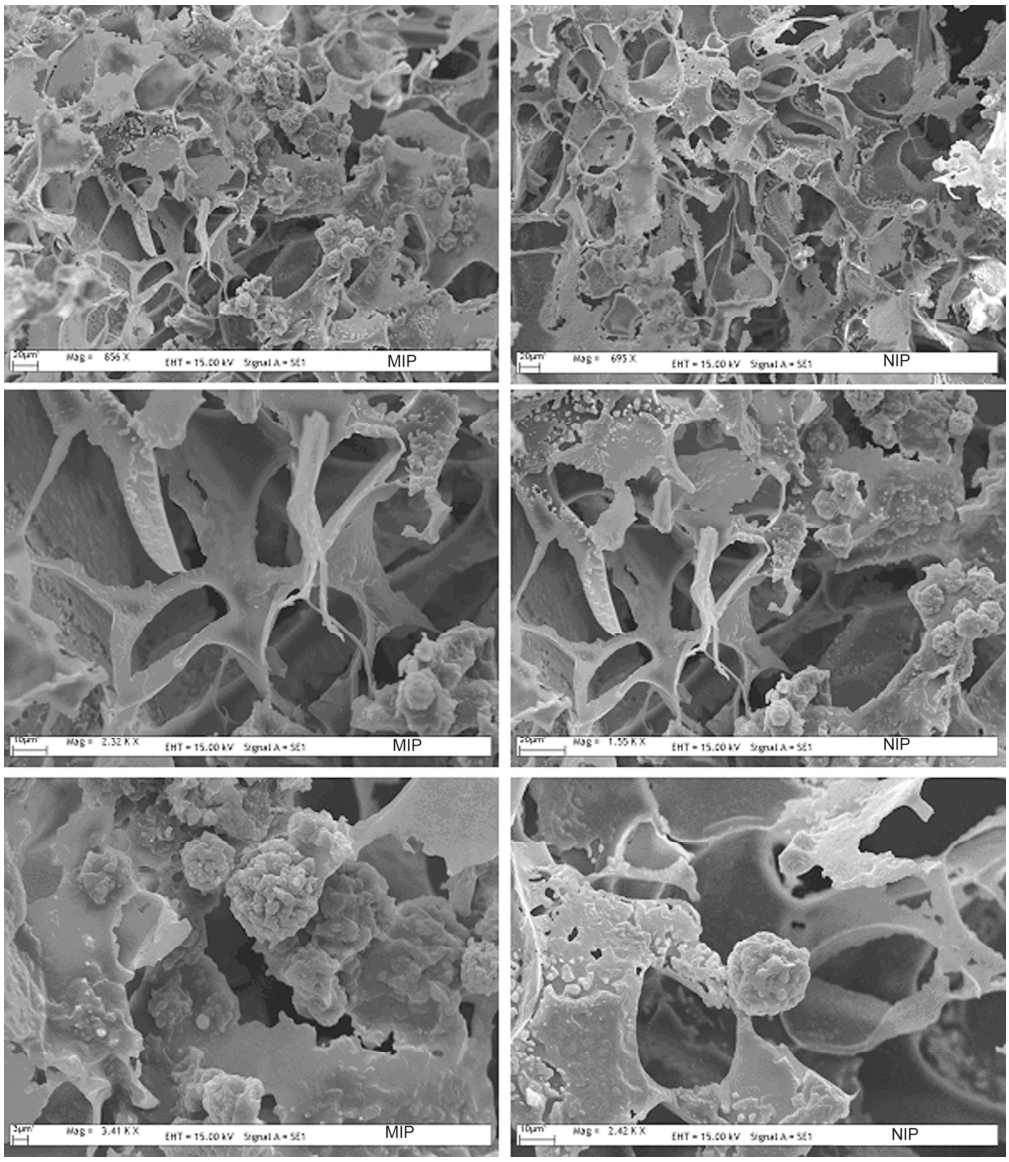

Figure 13. SEM photographs of HSA imprinted PGMA beads with 4000 times (1A) and 25000 times magnified (1B), MIP (1C and 1E) and NIP (1D and 1F) composite cryogels with different magnification levels [122].
146x168mm (300 x 300 DPI)