Bioseparation **7:** 177–184, 1999.

Smart hydrogels for bioseparation

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Received 11 May 1998; accepted 14 August 1998

Key words: bioseparation, hydrogels, pH-sensitive hydrogels, size exclusion, smart hydrogels, temperature-sensitive

Abstract

Smart hydrogels are hydrogels which alter their dimension (i.e., either swell or shrink) dramatically upon a small change in an environmental condition, such as temperature, pH, ionic strength, salt type, solvent, etc. Due to large changes in the swelling ratio, the smart hydrogels have been used widely in the separation of various molecules including proteins. Bioseparation using smart hydrogels is convenient, cost effective, and operable in mild conditions. The use of mild conditions during separation is critical for proteins which can be easily denatured or degraded. Smart hydrogels currently used in bioseparation and their limitations as well as improvements to be made are described here.

Introduction

Separation is an important part of the biochemical processes for obtaining bioactive compounds in pure and/or concentrated form. In the past, distillation, liquid-liquid extraction, crystallization, and filtration dominated conventional separation processes. For separation of biological molecules (i.e., bioseparation), however, the conventional separation methods are not adequate. They are expensive, difficult, and less appropriate for handling low molecular weight biomolecules (e.g., hormones, vitamins, and amino acid), natural polymers (proteins, polysaccharides, and nucleic acids), viruses, and cells due to their low stability and complex physicochemical properties. One area of the main focuses of current biotechnology is the production of therapeutic proteins (Evens and Witcher, 1993). Global sales of genetically engineered protein drugs were $10.7 billion in 1995 and the sales of macromolecular drug are projected to reach $20 billion in 2000 (Patton, 1997). As more advances are made in biotechnology, and thus in the mass production of therapeutic proteins, new and more cost-effective separation methods are necessary.

Hydrogel is a three-dimensional network of hydrophilic polymers that can hold a large amount of water while maintaining the solid state. A three-dimensional network is usually formed by chemical or physical crosslinking of hydrophilic polymer chains (Kamath and Park, 1993; Park et al., 1993). The key feature of hydrogels is the swelling/deswelling property in aqueous solution. Those hydrogels which swell or deswell (i.e., shrink) upon changes in the environmental condition are known as smart hydrogels. The property which makes the smart hydrogels unique is that the swelling (or shrinking) occurs by very small changes in the environmental condition. For example, a temperature change in a few degrees may result in swelling or shrinking of hydrogels. The change in hydrogel volume can reach more than a few hundreds. This unique property of smart hydrogels has been exploited in bioseparation. The environmental factors which affect the swelling (or shrinking) of hydrogels are listed in Table 1. The most commonly used environmental stimuli are temperature and pH, since the two variable are relatively easy to change. Other stimuli are electricity, ions, solvents, light, and pressure. For biological processes, smart hydrogels which respond to specific ligand molecules are also highly useful. The main advantage of using smart hydrogels in bioseparation is that the separation process can be operated without large changes in environmental factors. Thus, mild conditions for biomolecules can be maintained during the entire processes.

Table 1. Environmental factors which cause sharp response of hydrogels

Factor	Applications	References
Temperature	Drug delivery	(Bae et al., 1991; Chun and Kim, 1996; Dinarvand and D'Emanuele, 1995; Dong and Hoffman, 1990)
	Separation	(Cussler et al., 1984; Freitas and Cussler, 1987)
	Bioreaction	(Park and Hoffman, 1993)
	Shape memory	(Hu et al., 1995)
	Artificial muscle	(Kishi et al., 1993)
	Enzyme immobilization	(Shiroya et al., 1995)
pH	Drug delivery	(Bala and Vasudevan, 1982; Brazel and Peppas, 1996; Dong and Hoffman, 1991)
Electric field	Drug delivery	(Kwon et al., 1991; Sawahata et al., 1990)
	Artificial muscle	(Kajiwara and Ross-Murphy, 1992; Osada et al., 1992)
Ions		(Park and Hoffman, 1993; Starodoubtsev et al., 1995)
Solvents		(Tanaka, 1981)
Light		(Mamada et al., 1990; Suzuki et al., 1996a; Suzuki and Tanaka, 1990; Zhang et al., 1995)
Pressure		(Lee et al., 1990; Zhong et al., 1996)
Specific molecule	Drug delivery	(Kokufata et al., 1991; Obaidat and Park, 1997; Suzuki et al., 1996b)

Separation using hydrogels

Separation using temperature-sensitive hydrogels

Probably the most useful environmental factor controlling the swelling behavior of smart hydrogels is temperature. Most hydrogels swell more as temperature increases. Many hydrogels, however, exhibit the opposite behavior. Some hydrogels shrink as temperature is increased above a certain value known as the lower critical solution temperature (LCST). Upon a very small change in temperature around the LCST, hydrogels can either swell or shrink drastically. The temperature-sensitive hydrogels can be allowed to swell in a solution containing molecules to be concentrated. Hydrogels swell by absorbing water, and while water is absorbed, molecules smaller than the pore size of hydrogels can be absorbed into the hydrogel. Molecules larger than the pore size of hydrogels are excluded in the process. After equilibrium swelling is reached, the hydrogels are physically removed from the solution and transferred into another solution. The deswelling (or shrinking) of fully swollen hydrogels can be achieved by a small change in temperature. The shrinking process releases small molecules which were previously absorbed into the hydrogels. The shrinking process is also called "collapse" due to a sudden decrease in the hydrogel volume. The collapsed hydrogels are ready to be used again. This cyclic process is schematically described in Figure 1. As water is absorbed into the swelling hydrogel, the volume of aqueous solution in the container is reduced, and this results in concentration of large molecules remaining in solution. The small molecules absorbed into the hydrogel can be removed from the hydrogel by shrinking the swollen hydrogel through altering the environmental temperature. Since the only change in this process is small change in temperature, the bioseparation can be achieved relatively easily compared with conventional separation approaches. For temperature-sensitive hydrogels containing ionizable groups (e.g., carboxyl or amine groups), pH can also be used to control the swelling property.

Currently, soy protein isolates are commonly obtained by the acid precipitation process which cause the loss of albumins, which are the most valuable solutes in the feed and account for approximately 10% of the total soluble proteins. The albumins do not precipitate at pH 4.5 where the other proteins precipitate as a "curd" (protein-rich precipitate) which is separated from the "whey" (acidic liquor) afterwards, washed, and redispersed at a pH of about 7. The dispersed protein is subjected to spray drying to yield the soy protein isolate. This conventional process produces about 33 kg of isolate from 100 kg of defatted soybean flakes. The unrecovered soluble albumin in the whey is the main loss along with the carbohydrates and other salts. Cussler and coworkers

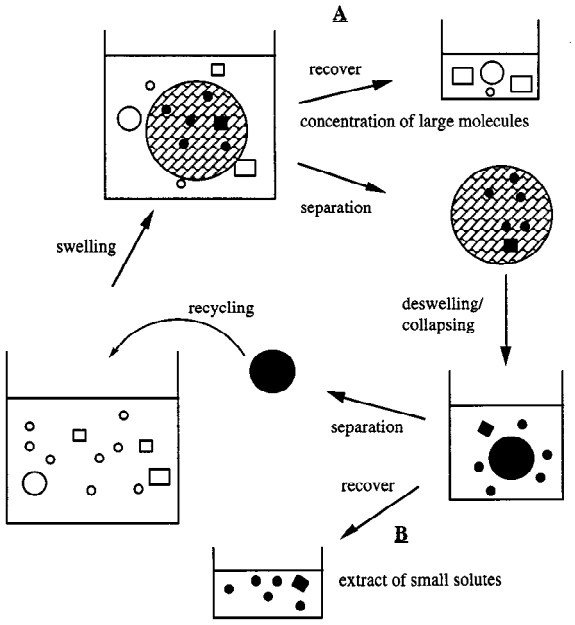

Figure 1. An example of hydrogel-based bioseparation. In process A, large molecules can be selectively excluded from the stimuli-sensitive hydrogels. In an extraction process B, small solutes entrapped inside the hydrogels can be extracted by deswelling of the hydrogels. (From Sassi et al., 1996a)

have applied the size selective extraction technology to soy protein isolation process (Trank et al., 1989; Wang et al., 1993). Their process utilizes gel particles made of temperature-sensitive poly(N-isopropyl acrylamide) (PNIPAAm).

In the first stage, collapsed PNIPAAm hydrogel particles (i.e., those shrunken at temperatures above the LCST) are placed into the defatted protein solution at 5 °C. Approximately 40% of the water is absorbed by the gel as the gel particles swell. The swollen gel particles are removed by centrifugation, and the retentate is diluted with water to the original volume. The water removal process is repeated using additional collapsed gel particles. In the final stage the retentate is not diluted, but is concentrated for spray drying. This alternative process produces 45 kg of protein isolate containing albumins excluded in the acid precipitation process from 100 kg of defatted soybean flakes. Furthermore, it provides more native proteins which may have better flavor and excludes undesirable components such as phytins which are toxic in large amounts.

Temperature-sensitive hydrogel membranes can also be used for sequential separation of molecules of different sizes from a mixture (Feil et al., 1991). A crosslinked membrane of poly (N-isopropylacrylamide-co-butylmethacrylate) (95:5 mol%) was used to sequentially separate uranine (Mol. Wt. of 376), small dextran (Mol. Wt. of 4400 and large dextran (Mol. Wt. of 150 000). The mesh size of the hydrogel membrane was controlled by changing the temperature. As temperature is lowered, the mesh size of the membrane increased and larger molecules can diffuse through the membrane. The separation by this method resulted in high purity and recovery of the separated compounds. One limitation of this approach was that it took a long time (100∼460 h). It is not only similar to conventional methods such as dialysis and membrane filtration, but also difficult to operate in large scale.

As shown by the above examples, size selective bioseparation can concentrate the solution at mild conditions without any harmful effect on biomolecules such as proteins. In addition to proteins, enzymes, small solutes, polymer latex particles (Cussler et al., 1984), and even virus (Roepke et al., 1987) can be separated by the size selective separation process. Temperature-sensitive hydrogels do not require any severe changes in other environmental conditions, such as ionic strength, pH, pressure, or shear conditions of the medium during the process (Park and Orozco-Avila, 1992). Since most thermo-responsive hydrogels have LCST under 50 °C, they require low energy to operate (Galaev and Mattiasson, 1993).

Separation using antibody-attached hydrogels

Antibody-attached hydrogels can be used as intelligent bioconjugates. Crosslinked polyacrylamide microparticles with antibodies covalently bound only at their surfaces were used as a solid support in solid-phase immunoassay (Tarcha et al., 1989). These antibody-containing hydrogel microparticles, in the range of 106-150 μm, provided a relatively high surface area per unit volume and suspendability. In addition, they had a concentrating effect on the analyte in the bulk solution as a result of high swelling property of polyacrylamide hydrogel. Reduction in volume results in decrease in the diffusion path length of antigen (e.g., β-human chorionic gonadotropin, HCG) contributing to a faster overall rate of antigen capture than that of conventional polystyrene spheres. More affinity functionalized hydrogels are expected to be developed in the near future for bioseparation.

Separation using temperature-responsive liquid chromatography

A new concept in chromatography was proposed that utilized a temperature- responsive surface with a constant aqueous mobile phase (Kanazawa et al., 1997; Kanazawa et al., 1996). A temperature-responsive semitelechelic copolymer, poly(N-isopropylacrylamide-co-butyl methacrylate), was grafted to the surface of (aminopropyl)silica through the reaction of activated ester-amine coupling. Separation of steroids and proteins (insulin chains A and B, and β-endorphin fragment 1-27) was investigated using the polymer-modified silica as a packing material. As the hydrophobicity of the polymer-modified silica increased the capacity factors and retention times for steroids increased. Retention times of insulin A and B, and β-endorphin fragment 1-27 were less than 15 minutes, while those of steroids were less than 30 minutes at 25 °C. The hydrophobicity of the column was achieved by temperature increase. Only a small change of column temperature, in the range of 5 °C to 35 °C, was the major controlling factor in this new liquid chromatography. There was no need to change the aqueous mobile phase. Temperature-responsive chromatography is able to separate solutes without the use of organic solvents. This technology may provide cost effective separation of proteins and peptides maintaining biological activity in preparatory liquid chromatography.

Separation using thermoreversible matrices for electrophoresis

Agarose and polyacrylamide are the most common separation media for gel electrophoresis of DNA and proteins. The study of DNA and proteins would be extremely difficult without electrophoresis. In a recent issue of the journal "BioTechniques" (Vol. 24, No. 3, 1988), more than three quarters of all the papers published in the issue used electrophoresis. Both agarose and polyacrylamide are selectively used according to the size of DNA or proteins and experimental condition. Cross-linked polyacrylamide hydrogel is used for fine resolution of DNA fragments smaller than 2000 base pairs while agarose gels are effectively used for separating large DNA fragments ranging from thousands to millions of base pairs. Even though the preparation of polyacrylamide gels is straightforward, the potential of exposing the user

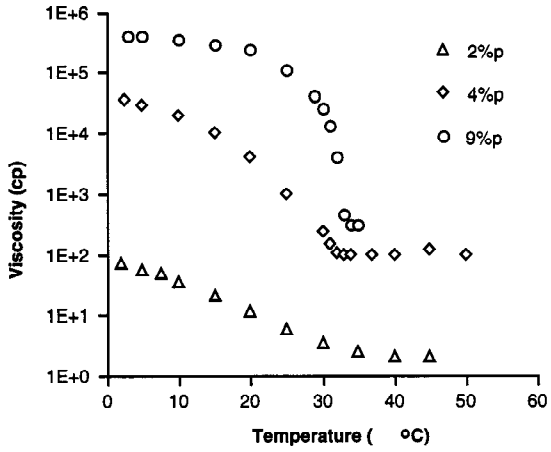

Figure 2. Viscosity behavior of aqueous suspension of temperature-sensitive PNIAAm microspheres as a function of temperature and polymer concentration (%p). Increasing temperature causes decrease of viscosity in suspension and the viscosity transition occurs dramatically in a higher polymer concentration (9%p). (From Sassi et al., 1996b)

to neurotoxic acrylamide monomer is the major drawback in practical applications of polyacrylamide gels. Sassi and coworkers reported new hydrogels which are thermoreversible and useful for separation of double stranded DNA fragments (<2000 base pairs) in capillary, tube, and slab electrophoresis (Sassi et al., 1996b). They have investigated systems of LCST polymers where the phase transition behavior was used to drive a viscosity transition (Figure 2). Since each microsphere is swollen at temperatures below the transition temperature (about 32 °C), the volume fraction of swollen microspheres is increased. At a higher polymer concentration this makes a suspension extremely viscous (over 100,000 centipoise). On the other hand a suspension is highly fluid at temperatures above the transition temperature due to collapsed microspheres. They investigated two classes of formulations, gel microsphere suspensions and solutions of uncrosslinked polymers. In their work, separation of DNA fragments at single-base resolution was achieved for DNA fragments with up to 150 base pairs in the capillary format.

In capillary electrophoresis, one of the problems is the difficulty of loading the polymeric media into capillaries (Gelfi et al., 1995; Grossman, 1994). If there are reversible viscosity transitions without the bulk phase separation or aggregation in suspensions or solutions, such viscosity responsiveness can be used effectively in capillary electrophoresis. In addition, the temperature responsiveness enables active control of

sieving properties during a run. Temperature-sensitive polymers, such as poly(N,N′-dimethylacrylamide) or poly(N,N′-diethylacrylamide), can be used for this application. Usually high resolution is obtained using solutions of polymers which are highly entangled at high concentrations. These highly entangled polymer solutions are often highly viscous, and thus, high pressures are required to load viscous polymer solutions in narrow-bore capillaries. Temperature-sensitive polymers, however, eliminate this particular problem, since viscosity can be lowered by increasing the temperature.

After the separation by electrophoresis, the separated proteins can be easily recovered by using temperature-sensitive hydrogels (Yoshioka et al., 1994). Crosslinked PNIPAAm gel was used for electrophoretic separation like normal polyacrylamide gel. After the electrophoresis, separated bands that contained desired substances were excised, and finely crushed. Finally these gels underwent shrinking and swelling alternately three times at 37 °C and 4 °C respectively. By shrinking the gel at 37 °C, horse heart myoglobin (MW, 18,800) and bovine hemoglobin (MW, 64,500) were effectively recovered at almost 100% yield. This recovery technique is advantageous for the high recovery yield in addition to the mild operating condition that prevents proteins from the denaturation. Electrophoresis using stimuli-sensitive hydrogels may be applicable to other proteins and DNAs.

Improvements to be made

For the maximum utilization in bioseparation, a number of properties of smart hydrogels need to be improved (Galaev and Mattiasson, 1993; Huang et al., 1989).

Faster swelling and shrinking rates

The duration of a bioseparation process depends on the kinetics of swelling and shrinking of smart hydrogels. Because the higher swelling (or shrinking) rate results in faster separation process, many attempts have been made to increase the swelling (or shrinking) rate as much as possible. The swelling kinetics of a dried gel depends on the diffusion of water or relaxation of the polymer chains (Hirose et al., 1987; Tanaka and Fillmore, 1979). For hydrogels of which swelling depends on diffusion of water, the time (t_v) for volume change is described by the following equation:

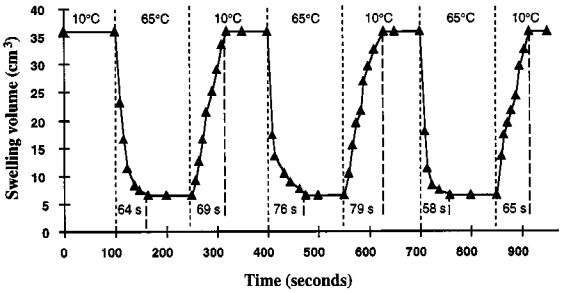

Figure 3. Fast swelling and deswelling kinetics of poly(N-isopropylacrylamide-co-acrylamide) hydrogels. The hydrogels have numerous pores all connected each other to form open channels. The size of the dried hydrogel was 1.9 cm × 2.3 cm. (From Chen, 1997)

$$t_v = \frac{l_c^2}{D_c}, \tag{1}$$

where l_c and D_c are characteristic length of the gel and the diffusion coefficient of water in the gel, respectively. Since D_c is usually in the order of 10^{-7} cm^2 s^{-1}, the time needed for swelling is 10^7 s for spherical gel particles of 1 cm radius but is only 10^{-1} s for those with 1 μ radius. For this reason, hydrogel particles with very small diameters are commonly used. The small gel particles, however, make it more difficult to handle.

To make dried gel particles swell faster than determined by the diffusion of water through the polymer matrix, hydrogels with porous structures have been prepared. Microporous and macroporous thermoresponsive hydrogels have been synthesized by various methods (Huang et al., 1987; Kabra et al., 1992; Wu et al., 1992; Yan and Hoffman, 1995). While micro- and macro-porous hydrogels swell faster than conventional hydrogels by an order of magnitude, the swelling can be still too slow for many applications. Recently, superporous hydrogels were synthesized to increase the swelling even faster (Chen, 1997; Park et al., 1997; Park and Park, 1994a; Park and Park, 1994b). The most important aspect in the preparation of superporous hydrogels is that all the pores are interconnected to form open channels. It is this open channel system that makes the dried hydrogel swell quickly in a matter of a minute regardless of the size of the dried hydrogels. As shown in Figure 3, temperature-sensitive superporous hydrogels made of poly(N-isopropylacrylamide-co-acrylamide) swelled in a matter of a minute even though the size of

the dried hydrogel was 1.9 cm × 2.3 cm (Chen, 1997). This type of fast swelling hydrogels are expected to reduce the time for the overall bioseparation process.

Improved size selectivity

For the best results in the size selective separation, hydrogels need to have selectivity, i.e., hydrogels should be able to selectively absorb molecules based on their size. If there are no specific interactions between hydrogels and molecules to be separated, gel selectivity can be obtained simply by changing the mesh size of hydrogels. This can be easily achieved by adjusting crosslinking density and monomer concentration in feed solution (Freitas and Cussler, 1987; Peppas and Lustig, 1986). The partition coefficient of a protein in a charged, temperature-sensitive hydrogel is influenced by various properties such as pH, temperature and ionic strength, and as well as material properties such as gel composition, charge density, and crosslinking density. Even at 0.1 M ionic strength, changing pH had a strong influence on the partition coefficient of cytochrome into poly(N-isopropylacrylamide-co-acrylate) hydrogels (Sassi et al., 1996a). The optimization of the size selectivity depends on the nature of smart hydrogels. The approach used for the optimization of temperature-sensitive hydrogels may be very much different from that of hydrogels sensitive to other factors.

Mechanical strength of hydrogels

The mechanical strength of hydrogels is important for easier handling in recycling processes. The hydrogel materials must have enough rigidity to retain their mechanical integrity and to minimize the attritional losses during the operation. Badiger et al. reported the use of a lightly crosslinked, sulfonated poly(styrene-divinyl benzene) hydrogel for the concentration of macromolecular solutions (such as casein, dextran, bovine serum albumin, egg albumin, poly(ethylene glycol), and insulin) (Badiger et al., 1992). Due to the low crosslinking density, the hydrogels had a much higher swelling capacity than conventional ion-exchange resins used in the water treatment. The use of these highly swellable hydrogels (which are ion-exchange resins) is named “swellex process”. Due to the rather high mechanical property of the hydrogels used, the swellex process could be performed in a column operation. The semicontinuous column operation is easy to scale-up and more applicable in a certain condition. For most of hydrogels, however, the mechanical strength is still low. One way of increasing the mechanical strength is to make hydrogel composites (Chen, 1997; Park et al., submitted).

Reduction of protein adsorption onto the hydrogel surface

One of the reasons for the poor separation efficiency of proteins by hydrogels is the adsorption of proteins onto the surface of hydrogels. If the separation efficiency is not well correlated with the mesh size of the hydrogels, it is most likely due to the adsorption of solute (especially proteins) at the gel surface. The protein adsorption may cause the gel shrinking (Jin et al., 1995). During hydrogel ultrafiltration of recombinant alkaline phosphatase, separation efficiency and enzyme recovery was low mainly due to enzyme entrapment between gel particles and attachment of enzymes to the gel surface (Park et al., 1997). Bovine serum albumin is also known to adsorb onto poly(N-n-propylacrylamide) surface. The protein adsorption onto hydrogel surfaces can be reduced by grafting hydrophilic, flexible polymer chains on the gel surface by copolymerization (Sun et al., 1991; Sun et al., 1992).

Prevention of thermal deactivation of enzyme

Separation efficiency could be decreased by exposure of enzyme at the collapse temperature during separation cycles (Park et al., 1997). Swelling temperature also had a large effect on separation efficiency. Even though enzyme recovery varied only slightly (92–95%), separation efficiency increased from 57.6% to 84.0% as the temperature decreased from 30 °C to 20 °C in concentrating alkaline serine protease using PNIPAAm gel (Han et al., 1995). For more practical bioseparation of thermo-labile materials, temperature-sensitive hydrogels with lower LCST would be much more useful.

Smart hydrogels have a lot of potential in many practical applications. Bioseparation is one of the areas where separation has been improved by use of temperature-sensitive hydrogels. As new polymers with unique properties are synthesized, bioseparation will also be advanced. Soon, we will see that the application of smart hydrogels in bioseparation is limited only by one’s imagination.

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