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Bioconjugates of Intelligent Polymers and Recognition Proteins for Use in Diagnostics and Affinity Separations

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Polymers that respond to small changes in environmental stimuli with large, sometimes discontinuous changes in their physical state or properties are often called "intelligent" or "smart" polymers. We have conjugated these polymers to different recognition proteins, including antibodies, protein A, streptavidin, and enzymes. These bioconjugates have been prepared by random polymer conjugation to lysine amino groups on the protein surface, and also by site-specific conjugation of the polymer to specific amino acid sites, such as cysteine sulfhydryl groups, that are genetically engineered into the known amino acid sequence of the protein. We have conjugated several different smart polymers to streptavidin, including temperature-, pH-, and light-sensitive polymers. The preparation of these conjugates and their many fascinating applications are reviewed here.

Smart Polymers and their Bioconjugates

Stimuli-responsive, "intelligent" polymers are polymers that respond with large property changes to small physical or chemical stimuli. They are also known as "smart", "stimuli-responsive", or "environmentally sensitive" polymers. These polymers can take many forms; they may be dissolved in aqueous solution, adsorbed or grafted on aqueous-solid interfaces, or cross-linked in the form of hydrogels (Fig. 1) *(1–3)*. Many different stimuli have been investigated, and they are listed in Table 1. Typically, when the polymer's critical response is stimulated, the smart polymer in solution will show a sudden onset of turbidity as it phase-separates; the surface-adsorbed or grafted smart polymer will collapse, converting the interface from hydrophilic to hydrophobic; and the smart polymer cross-linked in the form of a hydrogel will exhibit a sharp collapse and release much of its swelling solution. These phenomena are reversed when the stimulus is reversed, although the rate of reversion often is slower when the polymer has to redissolve or the gel has to reswell in aqueous medium.

Smart polymers may be physically mixed with or chemically conjugated to biomolecules to yield a large family of polymer-biomolecule systems that can respond to biological as well as to physical and chemical stimuli. Biomolecules that may be polymer-conjugated include proteins and oligopeptides, sugars and polysaccharides, single- and double-stranded oligonucleotides and DNA plasmids, simple lipids and phospholipids, and a wide spectrum of recognition ligands and synthetic drug molecules. In addition, polyethylene glycol may also be conjugated to the smart polymer backbone to provide it with "stealth" properties (Fig. 2). The conjugate of a synthetic polymer and a biomolecule produces a new, hybrid type of molecule that can synergistically combine the individual properties of the two components to yield new and unusual properties. One could say that these bioconjugates are "doubly smart". Among the most important polymer-biomolecule conjugates are polymer-drug conjugates *(4–7)* and polymer-protein conjugates *(8)*. There have been several successful applications in medicine and biotechnology for such smart polymer-biomolecule systems, and as such they represent an important extension of polymeric biomaterials beyond their well-known uses in implants and medical devices.

We have been combining smart polymers with a wide variety of biomolecules over the last 15 years [Refs. *(9–26)*; Monji et al., submitted for publication; and Shimoboji et al., unpublished work]. Our major activity in this area has been to conjugate temperature-sensitive polymers to proteins, both randomly [Refs. *(10–19)* and Monji et al., submitted for publication] and at specific sites [Refs. *(21–25)* and Hayashi et al., unpublished results], and this work will be the focus of this review. There are also many others who have randomly conjugated smart polymers to proteins, especially for affinity separations *(26–31)* and enzyme recovery *(32–38)*, but to our knowl-

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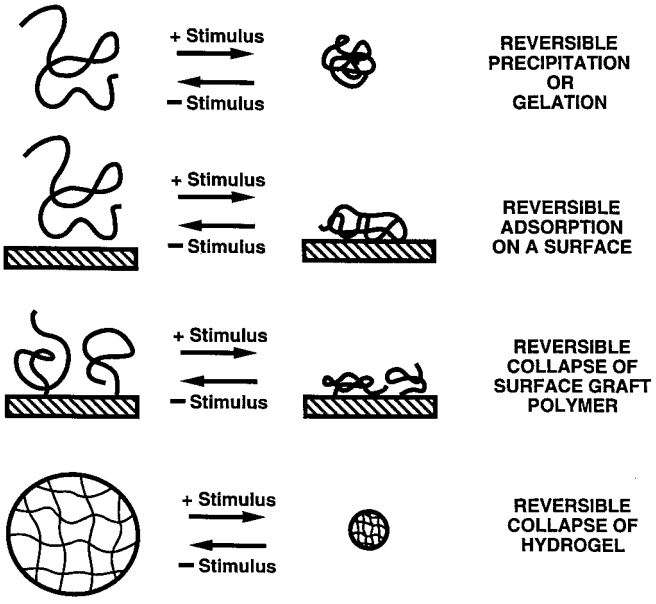

Fig. 1. Schematic of the different types of responses of intelligent polymer systems to environmental stimuli *(3)*.

Curved line is the polymer; *hatched rectangle* is a surface; *circle* is hydrogel.

edge, we are the only ones who have synthesized and investigated site-specific smart polymer bioconjugates.

There are many polymers that exhibit thermally-induced precipitation (Table 2), and the polymer we have studied most extensively is poly(*N*-isopropyl acrylamide), or PNIPAAm.[1] This polymer is soluble in water below 32 °C, and it precipitates sharply as the temperature is raised above 32 °C. The precipitation temperature is called the lower critical solution temperature (LCST). If NIPAAm monomer is copolymerized with more hydrophilic monomers such as acrylamide, the LCST increases and may even disappear. If NIPAAm monomer is copolymerized with more hydrophobic monomers, such as *n*-butyl acrylamide, the LCST decreases.

Synthesis of Polymer-Biomolecule Conjugates

RANDOM CONJUGATION

Proteins may be conjugated randomly to one end of a polymer or to pendant groups along the polymer backbone (Fig. 3). We have utilized chain-transfer free-radical polymerization to synthesize oligomers with one functional end group, which can then be derivatized to form a reactive group that can be conjugated to the protein. We have also copolymerized NIPAAm with reactive comonomers to yield a random copolymer with pendant reactive groups, which have then been conjugated to the protein *(23)*.

[1] Nonstandard abbreviations: PNIPAAm, poly(*N*-isopropyl acrylamide); LCST, lower critical solution temperature; SA, streptavidin; DMA, *N*,*N*-dimethylacrylamide; DMAA, copolymer of DMA and 4-phenylazophenyl acrylate; DMAAm, copolymer of DMA and *N*-4-phenylazophenyl acrylamide; and UV, ultraviolet.

Table 1. Environmental stimuli.

Physical	
	Temperature
	Ionic strength
	Solvents
	Electromagnetic radiation (UV, visible)
	Electric fields
	Mechanical stress, strain
	Sonic radiation
	Magnetic fields
Chemical	
	pH
	Specific ions
	Chemical agents
Biochemical	
	Enzyme substrates
	Affinity ligands
	Other biochemical agents

Usually the lysine amino groups are the most reactive sites for random polymer conjugation to proteins, and *N*-succinimide attachment chemistry is commonly used. Other possible sites include −COOH groups of aspartic or glutamic acids, −OH groups of serine or tyrosine, and −SH groups of cysteine residues. The most likely attachment site will be determined by the reactive group on the polymer and the reaction conditions, especially the pH. Because these conjugations are generally carried out in a nonspecific way, they can interfere sterically with the protein's active site and/or modify its microenvironment, and the bioactivity of the protein is often reduced [See Ref. *(18)* for an exception to this].

SITE-SPECIFIC CONJUGATION

We have also been able to conjugate the smart polymer to a specific site on some proteins (see Fig. 3) by inserting a specific reactive amino acid, such as a cysteine with its reactive −SH thiol group, at a selected site (Fig. 4). This is done by genetically engineering a site-specific mutation into the DNA sequence of the protein and then cloning the mutant in cell culture. Thus, this method is applicable only to proteins whose complete peptide sequence is known. The preparation of the reactive smart polymer is similar to the method described above, but now the reactive end or pendant groups and the reaction conditions are specifically designed to favor conjugation to −SH groups rather than to $-NH_2$ groups. Typical mercaptyl-reactive polymer groups include maleimide and vinyl sulfone groups.

For "site-specific" conjugation to $-NH_2$ groups, it should be noted that control of the reaction pH can provide more favorable conjugation to the terminal amine group vs pendant lysine amine groups. Furthermore, concerning the latter, some lysine amine groups will be more accessible, and therefore more reactive, than others.

The specific site for polymer conjugation can be located

far away from the active site, to avoid interference with the biological functioning of the protein, or nearby or even within the active site, to control the ligand-protein recognition process and the biological activity of the protein *(20, 21)*.

Properties and Applications of Random, Smart Polymer-Protein Bioconjugates

PHASE SEPARATIONS FOR RECOVERY

The thermally induced precipitation of a PNIPAAm-protein bioconjugate from a complex solution will simultaneously and selectively remove from solution only the protein that is conjugated to the PNIPAAm. We have used this phenomenon for the separation of an enzyme from its reaction solution to enable both recovery of the product from the supernatant and the recycle of the enzyme (Fig. 5) *(16–18)*.

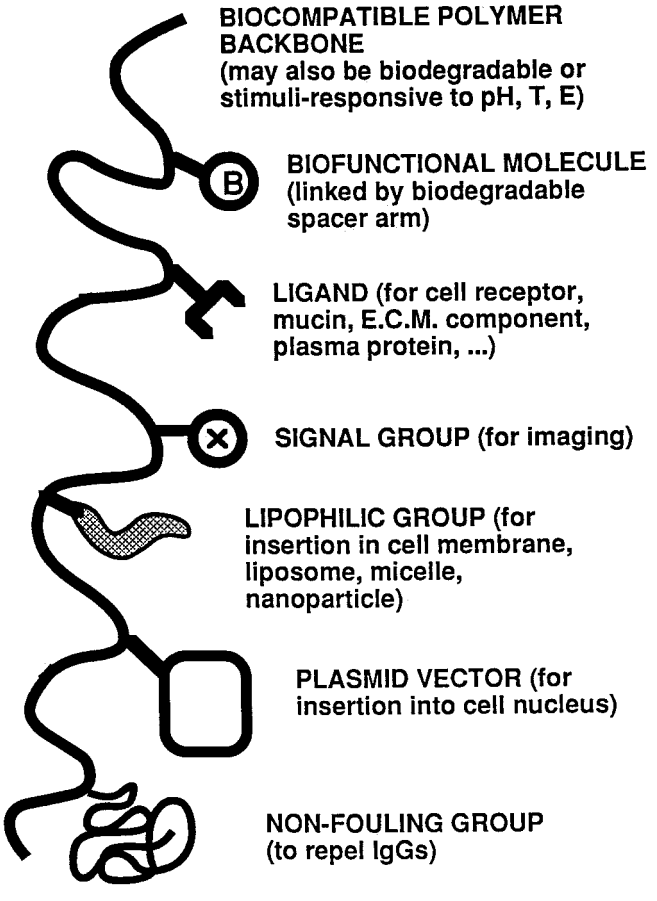

Fig. 2. Schematic of the variety of natural or synthetic biomolecules that may be conjugated to a smart polymer.

In some cases, only one molecule may be conjugated, such as a recognition protein, which may be linked to the protein at a reactive terminal group of the polymer, or it may be linked at a reactive pendant group along the polymer backbone. In other cases, more than one molecule may be conjugated along the polymer backbone, such as a targeting ligand along with many drug molecules *(3)*. *T*, temperature; *E*, electric field; *E.C.M.*, extracellular matrix.

Table 2. Some polymers and surfactants that show LCST behavior in aqueous solutions.

Ether groups
Poly(ethylene oxide)
Poly(EO/PO)[a] random copolymers
PEO-PPO-PEO triblock surfactants
Alkyl-PEO block surfactants
Poly(vinyl methyl ether)
Alcohol groups
Hydroxypropyl acrylate
Hydroxypropyl methylcellulose
Hydroxypropyl cellulose
Methylcellulose
Poly(vinyl alcohol) derivatives
Substituted amide groups
Poly(N-substituted acrylamides)
Poly(*N*-acryloyl pyrrolidine)
Poly(*N*-acryloyl piperidine)
Poly(acryl-L-amino acid amides)
Other
Poly(methacrylic acid)

[a] EO, ethylene oxide; PO, propylene oxide; PEO, poly(ethylene oxide); PPO, poly(propylene oxide).

If the conjugated protein forms a complex with another biomolecule, e.g., by affinity recognition, then the complex will also be selectively precipitated from solution (Fig. 6). We have used this phenomenon to selectively remove IgG from solution as a PNIPAAm-protein A·IgG complex, in a fashion similar to affinity chromatography, but in this case it is carried out by reversible phase separation from a solution, instead of binding and eluting from a packed column *(15)*.

This thermally induced affinity precipitation process may be extended to stimuli-induced phase separation of a biotinylated target molecule that is complexed to avidin or streptavidin (SA). In this case, a biotinylated target molecule is the first complexed with an excess of avidin or SA molecules in solution, such that at least one of the four biotin binding sites remains free (on average). An end-linked biotin-smart polymer conjugate is then permitted to bind to the free sites on the avidin or SA molecules. Following this, the bioconjugate-affinity complex can be phase-separated by raising the temperature above the LCST of the smart polymer (or by changing the pH if it is a pH-sensitive smart polymer), which selectively removes the biotinylated target molecule from solution *(26)*.

PHASE-SEPARATION IMMUNOASSAYS

We have extended the affinity phase-separation concept to the selective isolation and assay of an analyte from a complex mixture, such as a serum sample, by conjugating a first antibody to the polymer, complexing the analyte by affinity to the first antibody, and then introducing a

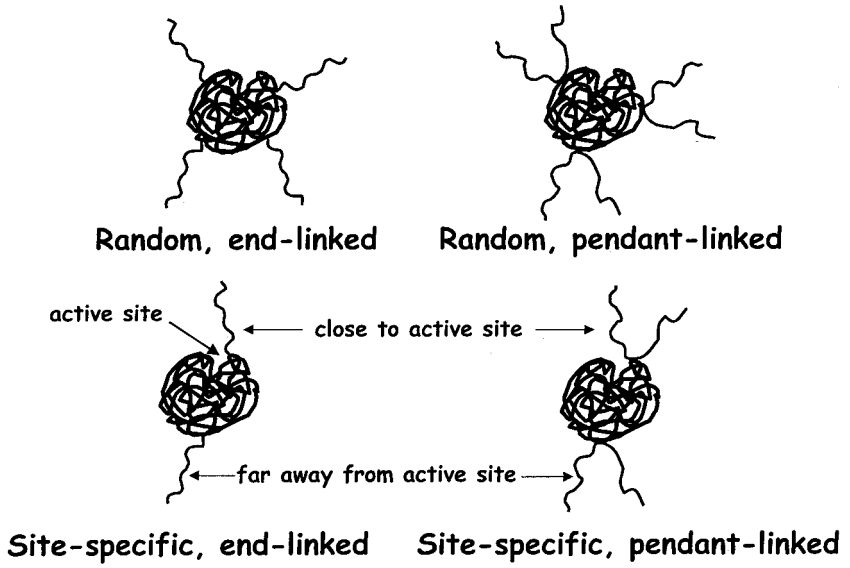

Fig. 3. Various types of random and site-specific smart polymer-protein conjugates.

In the latter case, conjugation near the active site of the protein is intended to provide stimulus control of the recognition process of the protein for its ligand, whereas conjugation far away from the active site should avoid any interference of the polymer with the protein's natural activity.

second, labeled antibody, which then binds to the analyte by affinity to a similar or a different site (epitope) on the analyte. This yields a temperature-sensitive polymer conjugated to an immune complex sandwich, which can then be removed selectively by thermally induced precipitation. This is an especially important separation step because an excess of the labeled, second antibody usually is added to the sample. Washing and resuspension in cold buffer permits easy assay of the analyte (Fig 7A) *(10–14)*. This immunoassay resembles ELISA done in solution. This concept has been extended to an assay of two different analytes in the same test sample. If NIPAAm is copolymerized with a more hydrophilic or a more hydrophobic co-monomer, then copolymers with higher and lower LCSTs can be obtained. If one of each of these two different LCST copolymers is conjugated to a different antibody, then two different analytes may be assayed in the same serum sample by raising the temperature of the system to sequentially phase-separate the two different polymer-conjugated immune complex sandwiches (Fig. 7B; Monji et al., submitted for publication). One could also carry out such a dual affinity separation or dual immunoassay using combinations of two different pH-sensitive smart polymers, or one temperature-sensitive and one pH-sensitive smart polymer. This use of a smart polymer-affinity recognition bioconjugate in an immunoassay may be extended to a DNA probe assay, where the analyte is a specific base sequence of an oligonucleotide in a library of oligonucleotides. Such an assay is illustrated schematically in Fig. 8 *(14)*.

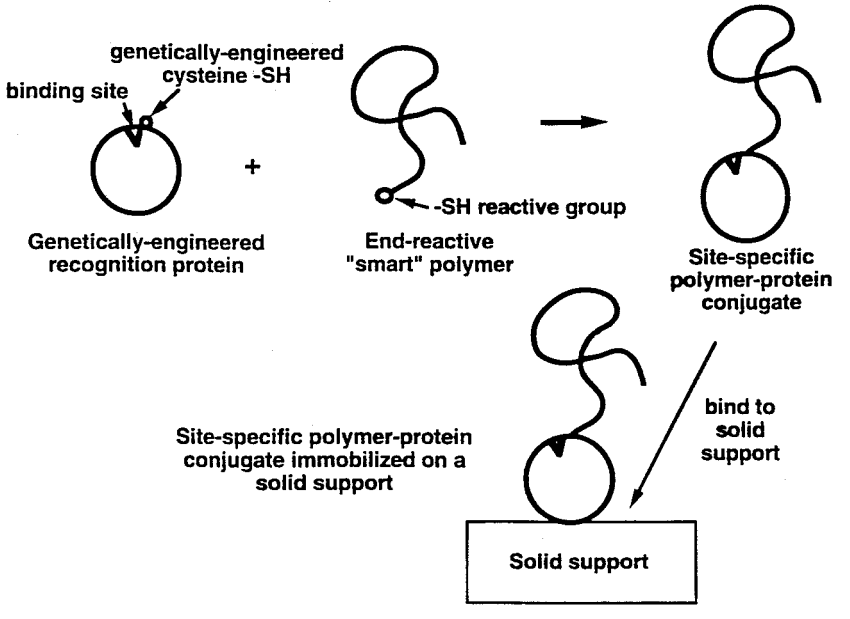

Fig. 4. Schematic of the process for preparing a site-specific conjugate of a smart polymer with a genetically engineered, mutant protein.

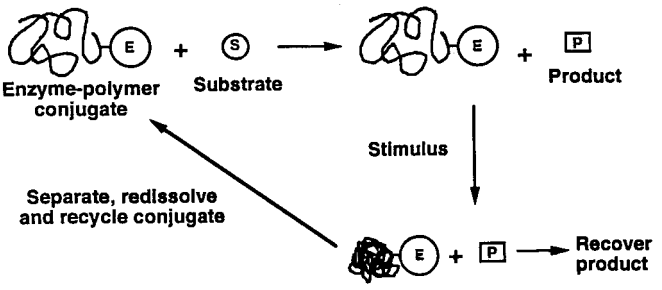

Fig. 5. Schematic of the stimuli-induced phase separation of a random conjugate of a smart polymer and an enzyme.

This general process should be useful for removing any specific molecule from a complex mixture for the purpose of recovery and possible recycle of that molecule *(3)*. *E*, enzyme; *S*, substrate; *P*, product.

Properties and Applications of Site-specific, Smart, Polymer-Protein Bioconjugates

SITE-SPECIFIC CYTOCHROME-B5 CONJUGATES LOCATED AWAY FROM THE ACTIVE SITE

We first created a mutant cytochrome-b5 with a cysteine located far from the active binding site, and conjugated PNIPAAm to the thiol group using maleimide chemistry *(20)*. We showed that the cytochrome-b5 was reversibly precipitated and redissolved by thermal cycling through the LCST, and also that the conjugation did not significantly reduce the activity of the protein. This is an extension of the randomly conjugated enzyme phase-separation process discussed above and shown in Fig. 5, where the site-specific conjugation is designed to assure a minimal loss in activity of the protein after conjugation with the smart polymer.

SITE-SPECIFIC CONJUGATES LOCATED NEAR THE ACTIVE SITE

Conjugation of a responsive polymer to a specific site near the ligand-binding pocket of a genetically engineered protein is a powerful new concept. The site-specific placement of a smart polymer near the active site of a protein can permit sensitive environmental control of the ligand-protein receptor recognition process, which controls all living systems. Small changes in environmental conditions can then cause large changes in the polymer conformation, leading to reversible "blocking" or "unblocking" of the protein's active site; such changes also can lead to triggered release of a bound ligand from the protein binding site (Fig. 9) [Ref. *(23)* and Hayashi et al., unpublished results].

SITE-SPECIFIC SA CONJUGATES THAT CONTROL BIOTIN-SA BINDING

We then selected SA to study. SA has become one of the most widely used proteins in affinity separations, laboratory assays, and clinical diagnostics because of its very high binding affinity for biotin. There are also several interesting in vivo applications under development for use of SA with biotinylated drugs or imaging agents. To attempt to control the biotin binding process, we decided to conjugate PNIPAAm near or within the active site of SA. We initially selected the N49 site, which is located on the loop above the biotin-binding pocket of SA, and genetically engineered a unique cysteine thiol group at that site, called the N49C mutant. (Native SA has no cysteine residues.) After that we conjugated a vinyl sulfone-terminated PNIPAAm to the thiol group at that site. We found that the thermally induced collapse of the polymer acted as a "molecular gate" to control the association of biotin with SA, which had been immobilized on a solid surface to avoid its precipitation from solution (as shown schematically in Fig. 9) *(21)*. Using another SA mutant, E116C, we found that the molecular weight of the polymer had an important influence on the gating process, which was only efficient above PNIPAAm molecular weights of ~7000, supporting the concept of steric interference of the biotin at the binding site [Ref. *(23)* and Hayashi et al., unpublished results]. We currently are extending these studies to the binding (or blocking) of biotinylated proteins.

TRIGGERED RELEASE OF BOUND BIOTIN FROM SITE-SPECIFIC SA CONJUGATES

Biotin was allowed to bind to the different PNIPAAm-SA conjugates at temperatures below the polymer's LCST. We then attempted to trigger the release of the bound biotin by thermally inducing the collapse of the polymer (as shown schematically in Fig. 9). We found that the collapse of the polymer did not "trigger" the release of the bound biotin in the N49C mutant, but it did release approximately one-half of the bound biotin molecules in the E116C mutant [Ref. *(23)* and Hayashi et al., unpublished results]. By cycling the temperature through the LCST several times for this latter mutant, we were able to release all of the bound biotin *(23)*.

Such triggered release of bound ligands could be used

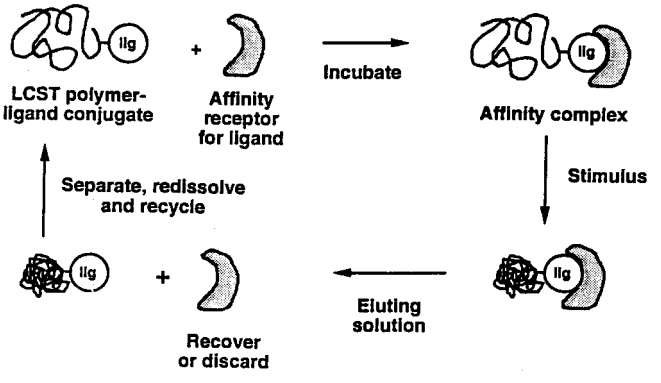

Fig. 6. Schematic of the stimuli-induced phase-separation of a random conjugate of a smart polymer and a receptor protein coupled to its recognition ligand.

This is essentially an affinity-chromatography process carried out in solution. It may be used to selectively separate a specific molecule for its recovery, assay, or removal (if it is a toxin or pollutant). Receptor proteins include antibodies, avidin and SA, protein A and protein G, cell membrane receptors (e.g., integrins), and many others *(1–3)*. The process may be used in "reverse", and a recognition ligand (e.g., biotin) may be attached to the smart polymer *(29)*. *lig*, ligand.

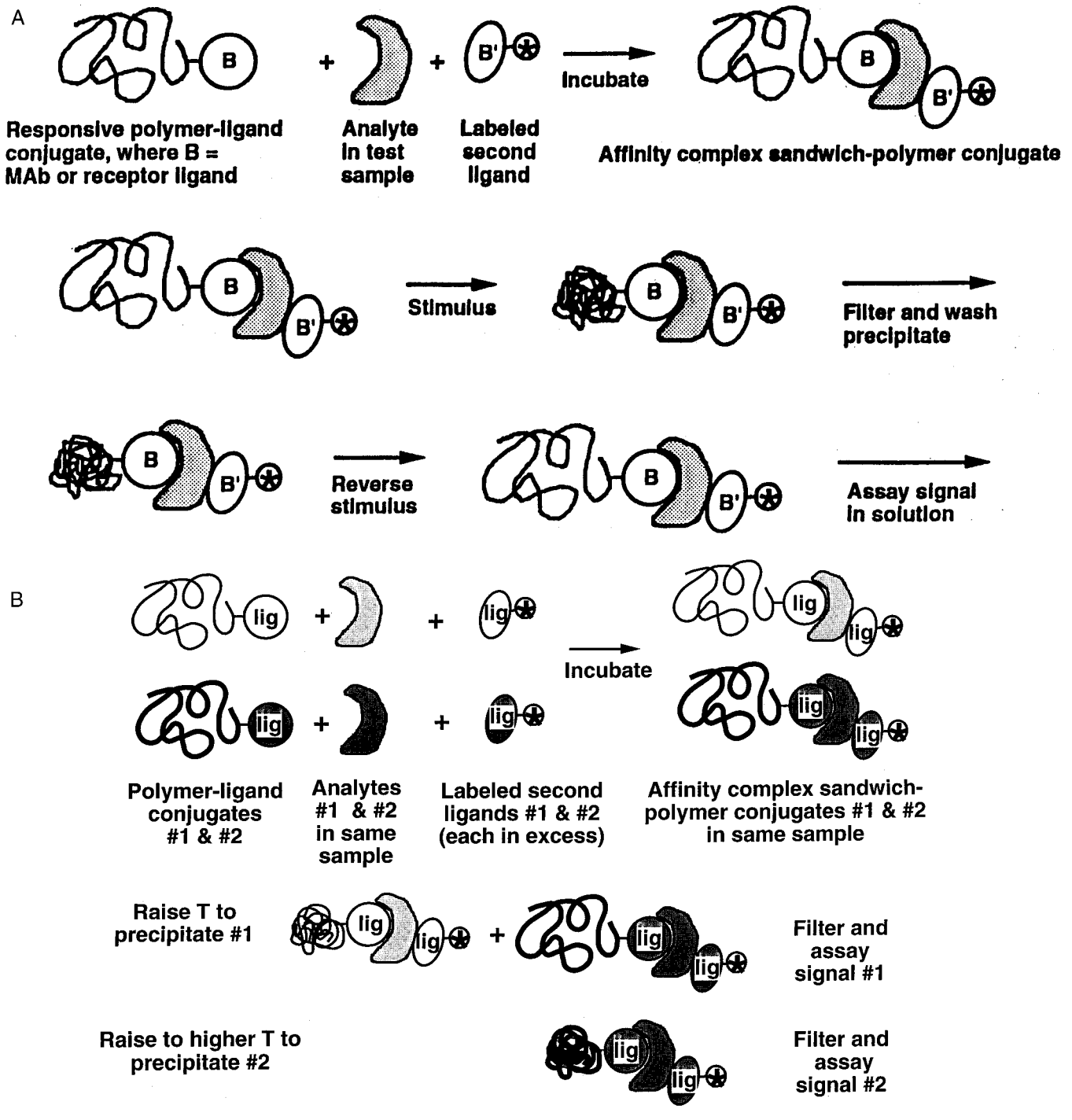

Fig. 7. Schematics of the stimuli-induced phase separation of a smart polymer-immune complex sandwich conjugate.

(*A*), this process is essentially an immunoassay, such as an ELISA, carried out in solution *(1–3)*. (*B*), this two-step double assay may use two temperature-sensitive polymers, two pH-sensitive polymers, or one of each. *lig*, ligand.

to release therapeutics, such as for topical drug delivery to the skin or mucosal surfaces of the body, and also for localized delivery of drugs within the body by stimulated release at pretargeted sites using noninvasive, focused stimuli, or delivery of stimuli from catheters. Triggered release could also be used to release and recover affinity-bound ligands from chromatographic and other supports in eluate-free conditions, including capture and release of specific cell populations to be used in stem cell and bone marrow transplantation. These processes could involve two different stimuli-responsive polymers with sensitivities to the same or different stimuli. For delicate target

ligands such as peptides and proteins, recovery could be affected without the need for time-consuming and harsh elution conditions. Triggered release could also be used to remove inhibitors, toxins, or fouling agents from the recognition sites of immobilized or free enzymes and affinity molecules, such as those used in biosensors, diagnostic assays, or affinity separations. This could be used to "regenerate" such recognition proteins for extended process use.

site-specific conjugation of a pH- and temperature-sensitive polymer to SA

We have observed similar results to those seen with the PNIPAAm-E116C SA conjugates when we prepared an E116C SA conjugate with a PNIPAAm-acrylic acid copolymer that exhibits combined temperature and pH sensitivity *(24)*. By incorporation of as little as 5.5 mol% of acrylic acid in a random copolymer with NIPAAm, we obtained a copolymer that is completely soluble at 37 °C and pH 7.4, and insoluble at 37 °C and pH 4.0. When this copolymer is conjugated to the E116C site of SA, biotin binding at 37 °C is significantly reduced at pH 4.0 compared with pH 7.4. This is most likely attributable to the more compact copolymer coil at pH 4.0 and 37 °C compared with that at pH 7.4 and 37 °C. After we allowed biotin to bind at 37 °C and pH 7.4, we were able to trigger the release of some of the bound biotin by changing the conditions to pH 4.0.

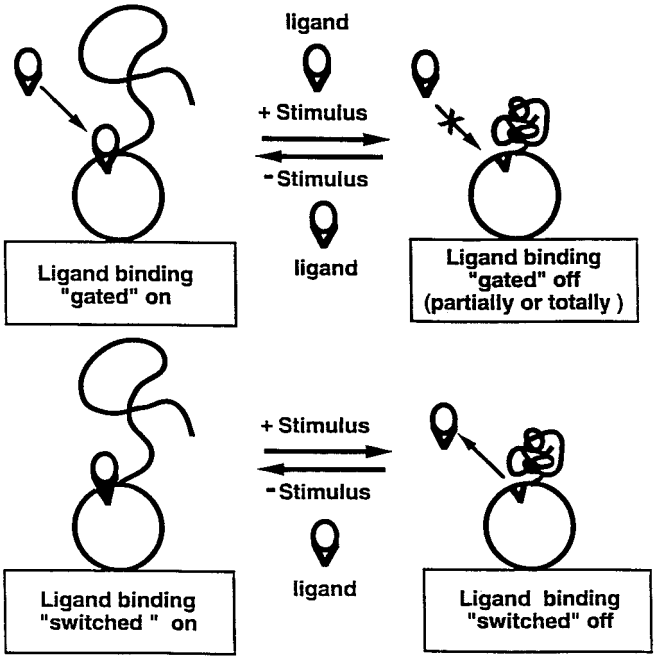

Fig. 8. Schematic of the "gating" and "switching" possibilities for a site-specific conjugate of a smart polymer and a mutant, recognition protein.

The switching action is referred to in the text as triggered release *(21–23)*.

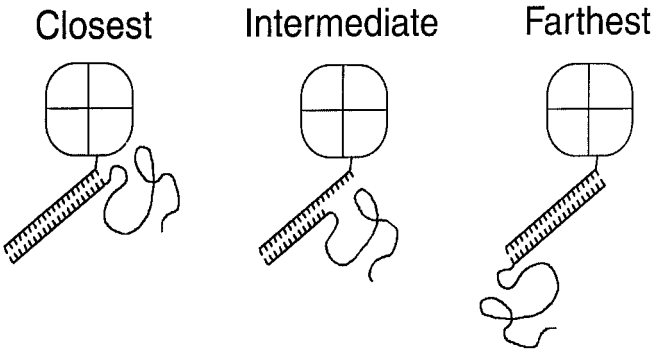

Fig. 9. Schematic of the use of a "molecular spacer arm" based on hybridization of oligonucleotide base pair sequences to control the distance of the smart polymer from the protein recognition site *(25)*.

site-specific conjugates of PNIPAAm-SA linked by hybridized DNA sequences to control the distance of the PNIPAAm from the SA binding site

We recently applied an interesting construct to control the distance of the PNIPAAm from the active site. For this purpose, we conjugated one sequence of complementary nucleotides to the E116C site near the binding pocket of SA, and a second sequence to the end of a PNIPAAm chain. By controlling the location and length of the complementary sequence, we could then use the self-assembly via hybridization of the two single chain DNA sequences to control the distance of the polymer from the SA-binding site. This concept is illustrated in Fig. 10 *(22, 25)*.

site-specific conjugates of temperature- and light-sensitive polymers to SA

To create photo-switchable enzymes, we have synthesized two different copolymers that exhibit combined temperature and photo sensitivity. The copolymers were prepared by copolymerizing *N*,*N*-dimethyl acrylamide (DMA) with two different light-sensitive co-monomers, 4-phenylazophenyl acrylate and *N*-4-phenylazophenyl acrylamide. The copolymers were identified as DMAA and DMAAm, respectively. DMAAm and DMAA showed ~8–10 °C differences in their LCSTs when irradiated with ultraviolet (UV) and visible light. Surprisingly, they showed the opposite photoresponses from each other using the same photo stimuli. Under the isothermal conditions at their photo-responsive temperatures, DMAAm precipitated under UV irradiation (350 nm), whereas DMAA dissolved under the same UV irradiation. This difference is related to the difference of the linkage between the azobenzene groups and the polymer backbone, which are amide and ester bonds. We derivatized the copolymers with vinyl sulfone end groups for specific conjugation to the E116C SA thiol group (Shimoboji et al., unpublished work).

One of these copolymer-E116C SA conjugates exhibited blocking of free biotin and triggered release of bound

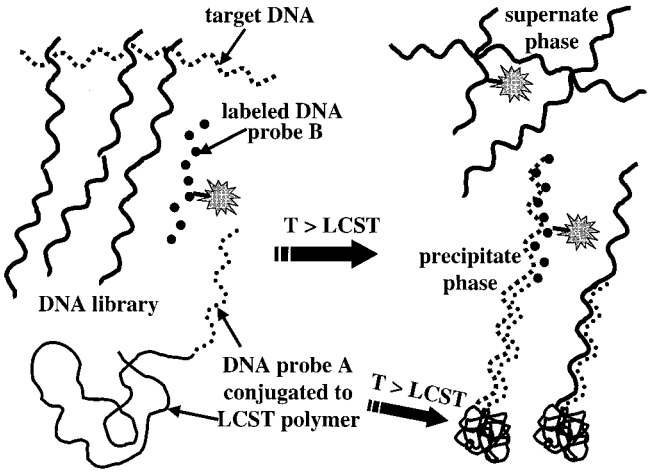

Fig. 10. Schematic of the use of smart polymer-oligonucleotide conjugates in a DNA probe assay.

In this assay, a stimulus induces the phase separation of the DNA complex formed from a bioconjugate of a smart polymer and a specific oligonucleotide sequence coupled by hybridization to a specific oligonucleotide analyte, which is also coupled to a second, labeled specific sequence of an oligonucleotide, to form a labeled, smart polymer-DNA complex conjugate. This process is similar to the immunoassay described in the legend of Fig. 7A. *T*, temperature.

biotin under visible irradiation, whereas the other demonstrated the same phenomena under UV irradiation. These opposite photo-responsive biotin-blocking or -releasing responses corresponded to the original photo-induced phase transition properties of the copolymers. The vinyl sulfone-terminated DMAAm and DMAA copolymers are also being used for site-specific conjugation to a mutant enzyme. That site-specific enzyme conjugate is currently in preparation for use as a photo-switchable enzyme. Such light-controlled binding and release of site-specific protein conjugates may be utilized as a molecular switch for various applications in biotechnology, medicine, and bioelectronics.

Conclusions

We have conjugated stimuli-responsive, smart polymers to random and specific sites on a variety of useful and important proteins, and in the latter case, we demonstrated the ability of the polymer to control the protein-ligand recognition and binding process. Table 3 summarizes the different possible molecular actions of these bioconjugates. Site-specific conjugation combines genetic engineering techniques that allow us to place the mutation residue in one (or more) selected location(s), with polymerization techniques that permit us to vary the molecular weight (and thus the polymer coil size) and the composition of the stimuli-responsive polymer (and thus the type and magnitude of the stimulus to be applied). Therefore, each polymer-protein conjugate can be molecularly engineered for each particular application. It should be emphasized that the site-specific placement of responsive polymers near the binding sites of recognition proteins can provide very sensitive and precise environmental control of the receptor protein-ligand recognition process, which is the process controlling all living systems. Furthermore, triggered release of a bound ligand may have many interesting applications in diagnostics, affinity separations, drug delivery, delivery of chemical or biochemical reagents in a process reaction, and signal generation (Table 4).

Table 3. Molecular actions of smart polymer-engineered protein conjugates.

- Control of enzyme-substrate reaction rates
- Control of affinity ligand binding rates and selectivities
- Eluate-free recovery of affinity ligands
- Triggered release of affinity-bound ligands
- Size-dependent enzyme-substrate reactions and affinity separations
- Capture and recovery of specific cells
- Response to and generation of optical or electrical signals
- Ability to switch protein recognition processes and products "on" and "off"

Table 4. Applications of smart polymer-engineered protein conjugates.

- Affinity separations of molecules and cells
- Clinical diagnostics and immunoassays
- Environmental sensors and biosensors
- Regeneration of "fouled" sensors
- Cell culture (fermentation) processes
- Triggered release (removal) of enzyme-bound inhibitors, products, toxins, and fouling agents
- Triggered release of drugs
- Drug "discovery"
- Information storage and retrieval

This review describes work by many collaborators, including: Patrick S. Stayton, Volga Bulmus, Guohua Chen, Jingping Chen, Chuck Cheung, Ashutosh Chilkoti, Zhongli Ding, Liangchang Dong, Robin Fong, Chantal A. Lackey, Cynthia J. Long, Morikazu Miura, John E. Morris, Niren Murthy, Yoshikuni Nabeshima, Tae Gwan Park, Ollie W. Press, Tsuyoshi Shimoboji, Sara Shoemaker, and Heung Joon Yang of the Departments of Bioengineering and Medicine, University of Washington, Seattle, WA; Nobuo Monji, Robert C. Nowinski, Carole Ann Cole, and John H. Priest of Genetics Systems, Inc., Seattle, WA; J. Milton Harris of Shearwater Polymers, Inc., Huntsville, AL; and Katsuhiko Nakamae, Takashi Nishino, and Takashi Miyata of the Department of Chemical Science and Engineering, University of Kobe, Kobe, Japan.

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