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(54) **Title**: PERMANENT AND REVERSIBLE ATTACHMENT OF MOLECULES TO SUBSTRATES BEARING THIOESTER BONDS

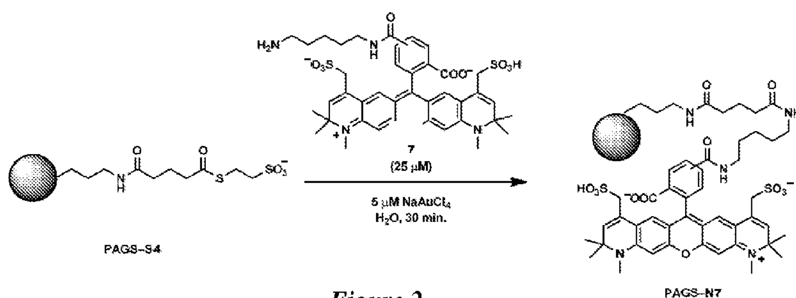

Figure 2

(57) **Abstract**: The invention provides methods for permanently or reversibly immobilizing molecules such as proteins, peptides, and polynucleotides on insoluble substrates such as silica, glass, and polymer beads functionalized with thioester groups. The methods employ Lewis acid-mediated thioester aminolysis and hiol-thioester exchange reactions.

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PERMANENT AND REVERSIBLE ATTACHMENT OF MOLECULES TO SUBSTRATES BEARING THIOESTER BONDS

5 **Cross-reference to Related Applications**

This application claims benefit of U.S. provisional application no. 61/393,107, filed October 14, 2010, which is hereby incorporated by reference.

Government Interest

This invention was made with Government support under National Institutes
10 of Health Award GM030367. The U.S. Government has certain rights in this invention.

Field of the Invention

The invention is in the field of organic chemistry and relates specifically to methods of attaching molecules to insoluble substrates.

15 **Background of the Invention**

The attachment of molecules to a solid substrate is a key step in many chemical and biological procedures. In solid-phase organic synthesis, loading substrates onto insoluble resins simplifies purification steps by eliminating the need for chromatographic separations. Among the best-known examples is the Merrifield
20 synthesis of polypeptides, where the *C*-terminus of a growing peptide chain is anchored to a polystyrene resin, usually via an ester bond, and the automated synthesis of oligonucleotides, where the 3′-terminal nucleoside is usually anchored via an ester or phosphate ester bond. Amino acid or nucleoside residues are installed sequentially, often in an automated process, and, at the end of each step, the resin-
25 bound product is separated by filtration from soluble reagents and byproducts. These methods rely on the availability of a robust but readily reversible binding of the oligomer to the solid substrate.

In affinity chromatography, biomolecules are isolated from crude mixtures by selectively and reversibly binding them to a solid substrate and releasing them to yield
30 a purified sample. An analogous method uses an immiscible liquid rather than a solid substrate. For example, a perfluorinated or “fluorous” solvent can be used in liquid-liquid extraction or countercurrent chromatography to extract molecules tagged with a

perfluorinated hydrocarbon residue selectively, leaving untagged materials and contaminants in the non-fluorous phase. The technique requires a reversible method of attaching the fluorous tag to the molecule of interest.

In materials science, molecules bound to solid phases are selected to tune the surface properties of materials, and analytical instruments employing surface effects, such as surface plasmon resonance, may employ monolayers of attached molecules as biosensors. These methods, too, rely on a specific method of binding the monolayer to the surface.

Numerous methods are available for linking molecules of interest to solid substrates like resins, beads, and surfaces. Chemical reactions can attach molecules to surfaces via the generation of covalent bonds, e.g., by reactions such as nucleophilic substitution, acylation with activated carboxylic acids, and thiol-disulfide interchange. (See S. Ostrove, S. Weiss, in *Methods in Enzymology, Vol. 182* (Ed.: M. P. Deutscher), Academic Press, Inc., New York, **1990**.) Alternatively, the surface and molecule can be labeled with complementary high-affinity binding partners and attached by mixing the labeled structures to form the bound complex. The small binding pair Ni^{2+}/oligohistidine ("His-tagging") is convenient for proteins, and biological binding partners, such as antibody-antigen pairs, biotin/(strept)avidin, protein A/IgG, and calmodulin binding peptide (CBP)/calmodulin, are frequently used for proteins and peptides.

Every binding system has its advantages and disadvantages, and no one system is regarded as universally optimal. (M. T. W. Hearn, D. Acosta, *J. Mol. Recognit.* **2001**, *14*, 323.) Methods that require the use of rigorously purified biomolecules, for example, tend to be expensive. High-affinity binding systems (e.g., biotin/avidin, $K_d = 10^{-15}$ M) can make it difficult to cleave the substrate from the surface without employing harsh conditions that limit the scope of compatible substrates. Non-specific methods of attachment, such as the glutaraldehyde cross-linking of proteins to aminated surfaces, are often unsatisfactory because the exact mechanism of binding is unclear, and the binding is not uniform. Covalent binding can also be difficult to reverse and can require modifications to the biomolecules of interest that interfere with the intended use of the materials. For example, some of the binding and linking moieties used to attach molecules to substrates are bulky and can interfere with the structure, activity, and solubility in the systems under study.

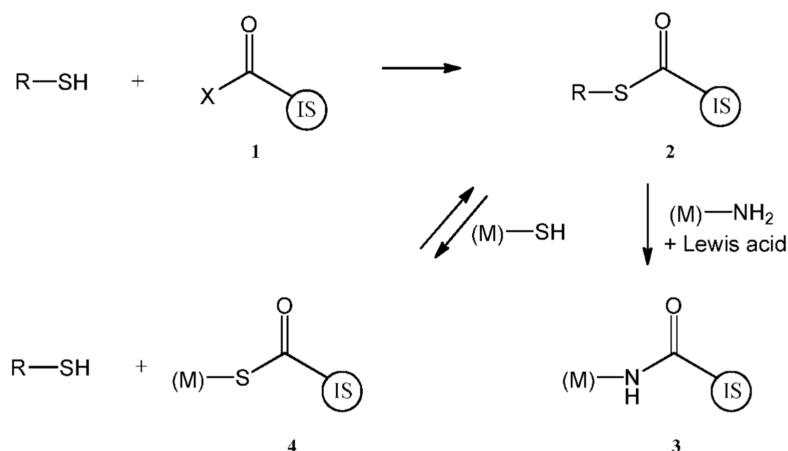
Glutaraldehyde can inactivate critical catalytic moieties and can introduce cross-links that prevent essential conformational changes. Covalent methods also suffer from a dearth of chemical reactions that proceed efficiently in water, the preferred solvent for studying biologically relevant interactions.

5 Accordingly, there remains a need for immobilization systems that function in water, have unambiguous chemical mechanisms of binding, offer high-affinity association, and allow cleavage steps that proceed under mild conditions.

Summary of the Invention

In aqueous solutions, thioesters react slowly with amines, alcohols, and water, 10 despite the large thermodynamic driving forces for these reactions. In contrast, the reaction between a thioester and a thiol to generate a new thioester and thiol (thiol–thioester exchange) is rapid in water at sufficiently alkaline pH. The present invention takes advantage of the reversibility of the thiol–thioester exchange reaction, the relatively slow rates for hydrolysis and aminolysis, and the availability of 15 selective, mild catalysis to provide useful methods for both reversible and irreversible attachment of molecules to substrates.

In one aspect, the invention provides a method for the selective covalent attachment of a target molecule ("M") to an insoluble substrate ("IS") and in certain embodiments also provides a method for the selective detachment of the target 20 molecule from the insoluble substrate. The durable (irreversible) covalent attachment employs metal-promoted aminolysis of thioester bonds, while the reversible attachment utilizes thiol–thioester exchange, as shown in Scheme 1:

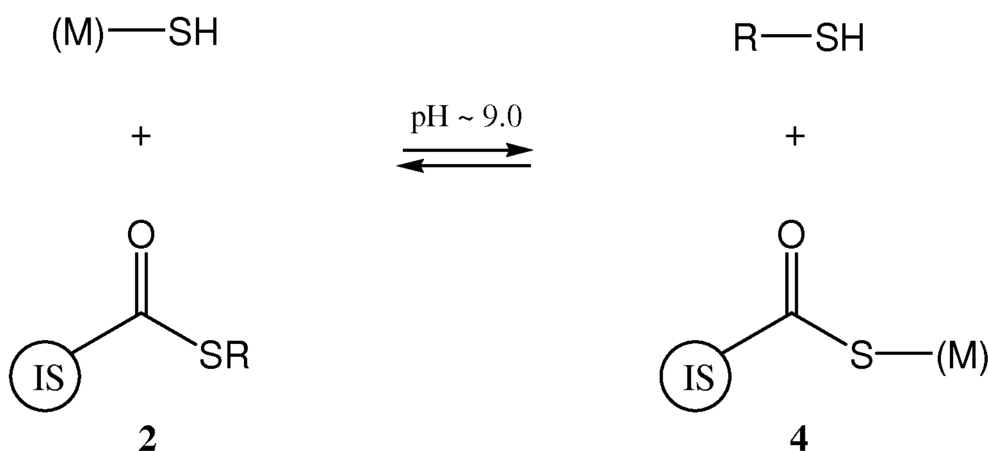


Scheme 1

In Scheme 1, X is a leaving group, such as a halide, alkylcarbonyloxy, or other leaving group characteristic of activated carboxylic acids, and R represents a small molecule residue such as an optionally substituted lower alkyl, heteroaryl, or aryl group. The structure **2** is a thioester-functionalized substrate that is stable to hydrolysis at neutral pH, while being a reactive substrate for metal-mediated aminolysis by the methods of the present invention. (M) represents the target molecule that is attached to (and optionally detached from) an insoluble substrate by the methods of the present invention. Permanent or durable attachment of the target molecule is realized by aminolysis of **2** with an amine derivative (M)-NH_2 — preferably in the presence of a suitable Lewis-acid promoter — while reversible attachment is realized by reaction with a thiol derivative (M)-SH — preferably in a mildly alkaline buffer.

Suitable promoters for the aminolysis reaction are thiophilic "soft" Lewis acid metal cations, including but not limited to Ag^{+}, Hg^{2+}, Pb^{2+}, Tl^{3+}, and Au^{3+}. Other promoters may be chosen, using criteria for "matching" hard and soft acids and bases that are known in the art (S. Woodward, *Tetrahedron*, 2002, **58**:1017-1050). High selectivity is possible because, in the absence of these metals, the thioester groups are stable to a variety of aqueous and non-aqueous environments, while at the same time, most other functional groups and covalent bonds are unaffected by the conditions for the thioester aminolysis.

High selectivity in the reversible attachment and detachment reactions is obtained through the use of the thiol–thioester exchange reaction depicted in Scheme 2:



Scheme 2

The thiol–thioester exchange reaction of Scheme 2 proceeds readily, under mild conditions, in an aqueous buffer at an effectively alkaline pH. As used herein, "effectively alkaline" refers to a pH value that generates a concentration of thiolate anion sufficient to promote the thiol–thioester exchange reaction, without generating sufficient hydroxide to cause excessive hydrolysis of the thioesters. Usually, the reaction is inconveniently slow if the pH is more than 3 units lower than the pK_a of the thiol (M)-SH. For example, if R-SH and (M)-SH are alkyl thiols having a pK_a of about 9.5, an effectively alkaline solution usually will have a pH of at least 6.5, preferably at least 7.5, and more preferably about 8.5. Most preferably, it will be buffered at a pH between 9.0 and 10.0. Thiols which have lower pK_a values, such as aryl and heteroaryl thiols, are expected to react effectively at correspondingly lower pH values.

High selectivity is possible because, in the absence of a Lewis-acid promoter, the thioester bond is kinetically stable to hydrolysis and aminolysis reactions in these aqueous buffers of mild pH, and because most other functional groups and covalent bonds are likewise unaffected.

Other features and advantages are described in the following detailed description, the drawings, and the claims.

Brief Description of the Drawings

Figure 1 shows representative syntheses of thioester-functionalized silica microspheres.

Figure 2 shows the covalent attachment of AlexaFluor™ 594 cadaverine dye to silica microspheres by catalyzed aminolysis of thioester groups.

Figure 3 is a SEM image of a sample of thioester-functionalized silica microspheres dried on a silicon wafer. The average diameter of the spheres is 454 ± 47 nm (95% confidence interval).

Figure 4 shows optical micrographs of the PAGS beads following immobilization of AlexaFluor 594 cadaverine dye. Fig. 4A, bright-field image; Fig. 4B, fluorescence image, $\lambda > 560$ nm.

Figure 5 shows a Coomassie Blue stained SDS-PAGE gel of purified 6xHis-EGFP (right); protein ladder on left.

Figure 6 shows optical micrographs of silica beads following immobilization of 6xHis-EGFP. Fig. 6A, bright-field image; Fig. 6B, fluorescence image, $\lambda > 515$ nm.

Figure 7 is an outline of processes suitable for the attachment and detachment
5 of a target molecule, via thioester bonds, to a representative insoluble substrate.

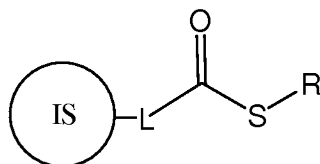
Figure 8 shows optical micrographs of silica beads following immobilization of AlexaFluor™ 488. Fig. 8A, bright-field image; Fig. 8B, fluorescence image, $\lambda >$ 515 nm.

Figure 9 shows the chemical steps used in the reversible attachment of
10 representative molecules to an insoluble substrate.

Detailed Description of the Invention

1. Overview.

The invention provides a method for the covalent attachment of a target molecule having at least one amino group to an insoluble substrate, by contacting the
15 target molecule with a thioester-functionalized insoluble substrate of formula



in the presence of a metal or suitable Lewis acid promoter (e.g., a soft Lewis acid metal promoter); wherein “IS” represents the insoluble substrate, L is a covalent bond or is a linker covalently or non-covalently attached to the insoluble substrate, and R-S
20 is a sulfenyl group, e.g., substituted or unsubstituted alkyl (such as C1 to C-8 alkyl), heteroaryl, or aryl group.

The term “alkyl,” as used herein, represents a straight, branched, or cyclic hydrocarbon group, which may be substituted or unsubstituted. Exemplary alkyl groups have from 1 to 10 carbons atoms, e.g., 1 to 8 carbon atoms.

25 The term “aryl,” as used herein, represents a mono-, bicyclic, or multicyclic carbocyclic ring system having one or two aromatic rings and is exemplified by phenyl, naphthyl, 1,2-dihydronaphthyl, 1,2,3,4-tetrahydronaphthyl, fluorenyl, indanyl, indenyl, and the like, and may be optionally substituted. Exemplary aryl groups include 6 to 10 carbon atoms.

The term “heteroaryl,” as used herein, represents a 5-, 6- or 7-membered ring, unless otherwise specified, containing one, two, three, or four heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur and being aromatic: i.e., they contain $4n+2$ pi electrons within the mono- or multicyclic ring system. In some embodiments, the heteroaryl is substituted. Exemplary heteroaryl groups include 2 to 9 carbon atoms. Heteroaromatic compounds from which R groups can be derived include furan, thiophene, pyrrole, pyridine, and indole.

The term “sulfenyl,” as used herein, presents a group R-S-, in which R is any group in which a carbon atom is bound to the sulfur atom. Exemplary R groups include unsubstituted or substituted alkyl, aryl, and heteroaryl groups.

Exemplary substituents for alkyl, aryl, and heteroaryl groups include acyl (e.g., C1-C6 alkyl carbonyl), carboxy, halogen, hydroxyl, oxo, sulfonyl, and sulfonic acid groups. Alkyl groups may also be substituted with aryl or heteroaryl groups; aryl groups may also be substituted with alkyl or heteroaryl groups; and heteroaryl groups may also be substituted with alkyl or aryl groups.

The promoter is preferably a salt of Ag^{+}, Hg^{2+}, Pb^{2+}, Tl^{3+} or Au^{3+}, and the moiety R-S is preferably derived from a thiol (R-SH) such as thiophenol, benzyl mercaptan, ethanethiol, 2-mercaptoethanol, 2-mercaptoacetic acid, 3-mercaptopropionic acid, 4-mercaptophenylsulfonic acid, and 2-mercaptoethanesulfonic acid.

The linker L may, in certain embodiments, be a direct covalent bond to the substrate but usually is one or more divalent linkers selected from the group consisting of C-1 to C-18 alkylene chains, polyethylene glycol chains having one to ten (CH_2CH_2O) residues, C-3 to C-6 dicarboxylic acid chains, C-2 to C-10 diamino alkylene chains of formula $H_2N(CH_2)_nNH_2$, amino acids of formula $H_2N(CH_2)_nCOOH$ where n is 1-10, and combinations thereof. The linker L is omitted for clarity in some of the illustrations herein. The linker may also be non-covalently attached to the insoluble substrate, e.g., by Ni^{2+}/oligohistidine (“His-tagging”), complementary oligonucleotides, antibody-antigen pairs, biotin/(strept)avidin, protein A/IgG, and calmodulin binding peptide (CBP)/calmodulin.

As used herein, the term “target molecule” refers to a molecule that has one or more thiol or amino groups, whether the group is present naturally or has been attached synthetically. In certain contexts, as a matter of convenience, the term

"target molecule" may refer to an unmodified molecule that requires introduction of one or more thiol or amino groups for the practice of the present invention. In preferred embodiments, the target molecule is a peptide or protein.

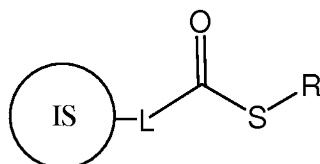
Suitable target molecules include dyes, pharmaceuticals, radiopharmaceuticals, imaging agents, contrast agents, proteins, peptides, polysaccharides, oligosaccharides, polynucleotides, oligonucleotides, catalysts, co-catalysts, and protein-binding ligands such as enzyme substrates and receptor ligands. Preferred target molecules are enzymes, enzyme substrates, enzyme inhibitors, co-factors, antibodies, antibody fragments, receptors, receptor ligands, affinity tags, oligonucleotide probes, oligonucleotide primers, and oligonucleotide coding sequences. In specific embodiments, the target molecule is a protein or peptide.

The term "insoluble substrate" as used herein refers to any organic or inorganic support, including but not limited to silica, alumina, glass, gold, paramagnetic and ferromagnetic beads, and polymers and co-polymers such as polystyrene, polyethylene glycol, polyacrylamide, cellulose, agarose, and dextran, whether cross-linked or not. Suitable supports are taught, for example, in *Organic Synthesis on Solid Phase: Supports, Linkers, Reactions*, by F. Z. Dörwald (Wiley-VCH, Weinheim, 2002). Furthermore, "insoluble substrate" refers to substrates that, while insoluble in at least one solvent, may be soluble in one or more alternative solvents, so that chemical reactions involving the substrate may be conducted in solution and isolation may be achieved by precipitation (D.E. Bergbreiter *et al.*, *Macromolecules*, 1998, **31**:6053–6062). The insoluble substrate need not be solid; for example, it may take the form of a gel. In certain embodiments of the invention, the term "insoluble substrate" refers to liquids, such as perfluorinated hydrocarbons or "fluorous tags," that are immiscible with at least one solvent but soluble in at least one other (A. Studer *et al.*, *Science*, 1997, **275**:823).

Similarly, the carboxyl group, activated derivative, or thioester in the insoluble substrate may be an inherent feature of the substrate (e.g., a polymer or copolymer of acrylic acid), or it may be artificially introduced for the purpose of practicing the present invention (e.g., via succinylation or carboxymethylation of amino or hydroxyl groups). As used herein, the term "insoluble substrate" refers to materials that have one or more carboxyl groups, activated derivative, or thioester, whether the pendant group is present naturally or has been introduced synthetically. In certain contexts, as

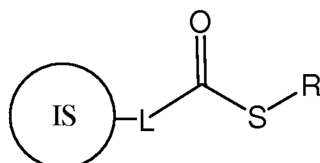
a matter of convenience, the terms "substrate" and "insoluble substrate" may refer to an unmodified molecule, polymer, or support that requires introduction of one or more carboxyl groups, activated derivatives, or thioester groups for the practice of the present invention.

5 The invention also provides kits for use in the attachment of target molecules to insoluble substrates, including one or more substrates of formula



as defined above, and one or more metals or suitable Lewis acid promoters (e.g., a soft Lewis acid metal promoter). Preferred promoters for these kits are salts of Ag^{+},
10 Hg^{2+}, Pb^{2+}, Tl^{3+}, or Au^{3+}.

The invention also provides a method for the covalent attachment of a target molecule having at least one thiol group to an insoluble substrate, by contacting the target molecule with a thioester-functionalized insoluble substrate of formula

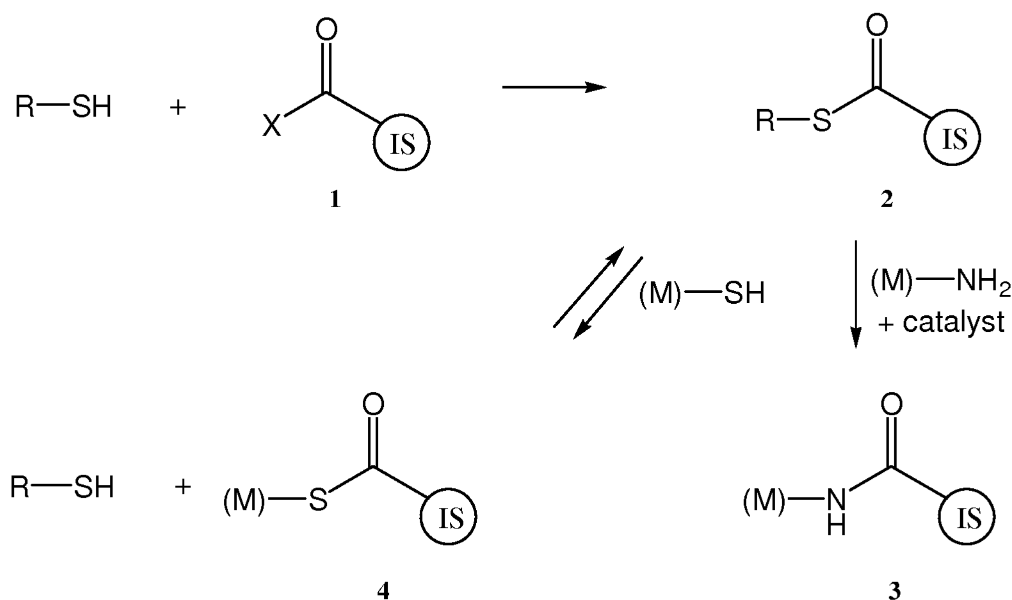


15 where IS, L, and R are as defined above, in an aqueous buffer of sufficiently alkaline pH.

The invention employs target molecules "M" that have at least one free thiol or amino group and substrates ("IS") that have one or more carboxylic acid groups, activated derivative thereof (e.g., acid halide, anhydride, or ester), or thioester groups.
20 Molecules and substrates that lack these features may be chemically modified, by methods well known in the art, to introduce these functional groups. For example, the thiol group in the molecule (M)-SH may be an inherent feature of the target molecule (e.g., where M is a cysteine-containing protein or peptide), or it may be introduced for the purpose of practicing the present invention (e.g., a recombinant protein may be
25 expressed with cysteine or poly-cysteine tags, or an ester or amide derivative of the molecule M may be formed with cysteine, glutathione, or mercaptoacetic acid). Other methods of thiol tagging are described by B. McCaughan *et al.*, *J. Chem. Crystallog.* 2010, **40**:417-422 and references therein. Similar considerations apply to the target

molecule, (M)-NH_2, where the amino group may be an inherent feature of the target molecule (e.g. a lysine residue) or may be introduced.

The methods for the covalent attachment of the target molecule (M) to the insoluble substrate (IS) include the formation of amide or thioester bonds, and the
5 methods for detachment include the cleavage of a thioester bond, as shown in Scheme 3:



Scheme 3

In Scheme 3, the insoluble substrate is denoted by a sphere labeled "IS"; this
10 representation alludes to an exemplary embodiment of the invention in which the insoluble substrate is a spherical particle of silica. This representation is used herein for illustrative purposes, as an aid to understanding the invention, but the invention is not limited to spherical particles or silica.

15 **2. Attachment of Target Molecules.**

The covalent attachment of molecules to insoluble substrates and surfaces is essential to a wide variety of applications. In synthetic chemistry, performing reactions on solid supports is used to simplify product isolation, by replacing tedious separations such as column chromatography with a simple filtration step or through
20 the use of flowing reagents in automated synthesis systems. In drug discovery, the attachment of molecules to surfaces can simplify how compounds are screened for activity. The anchoring of biomolecules to solid supports is particularly prevalent in biological research. Immobilized enzymes are used as readily recyclable catalysts and

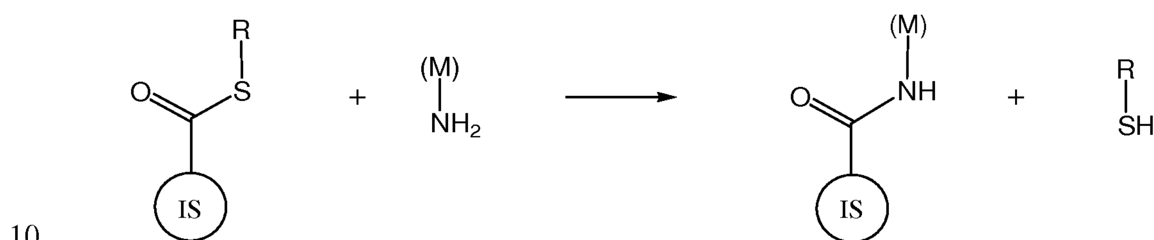
are the most commonly used biological components in biosensors, while surface-bound antibodies are important components of many immunoassays. Affinity chromatography often uses ligands bound covalently to resin supports for the purification of biomolecules such as proteins. Microarrays for the high-throughput study of interactions among biomolecules rely upon the immobilization of DNA, proteins, or carbohydrates to the surface of silicon chips or glass slides.

Prior art methods for immobilizing amines on solid substrates have attributes that limit their general use across the wide range of functional groups found in biology. Cross-linking reagents, for example, are frequently used to effect covalent linkage of amines to surfaces, but they are prone to cross-linking functional groups on the target molecule, which can disrupt its structure and function. Glutaraldehyde coupling, for example, while one of the most common methods of immobilizing proteins on surfaces (via imines formed from the side chains of lysine residues), often produces mixtures of products of ill-defined structure. Because proteins tend to have multiple amino and carboxyl groups, it is difficult to activate either group chemically in a way that leads to selective reaction with the desired substrate. Substrates with activated chemical groups are often prone to hydrolysis in the aqueous environments generally required for work with biomolecules; this hydrolysis reduces the number of target molecules that might otherwise bind to the substrate and the time that the activated substrates can be stored prior to use. Alkylation reactions (e.g., with halides and epoxides) are in most cases irreversible, as are reductive alkylations of aldehydes.

In J.C. Brown, H.R. Horton, *Fed. Proc. FASEB*, 1973, **32**:496; R.J. Brown *et al., Biochemistry*, 1979, **18**:4901-4906, a protein is anchored to an insoluble support via amide bond formation with a previously bound mercaptoacetate or mercaptopropionate linker. Detachment via thioester cleavage leaves the target molecule modified by the presence of the mercaptoacetyl or mercaptopropionyl amide. M.-L. Lesaicherre *et al., Bioorg. Med. Chem. Lett,*. 2002, **12**:2079 describes the use of a thioester-functionalized surface to ligate N-terminal cysteine containing peptides to glass slides via rapid intramolecular S-to-N acyl transfer (see, also, L.S. Wong *et al., Chem. Rev.*, 2009, 109:4025-4053). This attachment depends upon the reactivity of an N-terminal cysteine, the facility of an intramolecular S-to-N acyl transfer, and the driving force of amide bond formation, and as a result is generally limited to target molecules having N-terminal cysteine residues or other beta-amino

thiol moieties. The present invention provides a means for attachment of amines that are not beta-amino thiols. In certain embodiments of the invention, for either reversible or irreversible attachment, the target molecule is not capable of undergoing S-to-N acyl transfer; for example, the target molecule is not a beta-amino thiol.

5 In one embodiment of the present invention, a soft metal cation promoter is employed to activate thioester groups on an insoluble substrate (IS) selectively under mild conditions. This specific and selective catalysis allows the attachment of any target molecule (M) bearing an amino group to a thioester-functionalized substrate, as shown in Scheme 4:



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Scheme 4

Particularly preferred embodiments use Au^{3+} species, such as tetrachloroaurate salts, or Ag^{+} salts, as promoters to anchor amines to thioester-functionalized substrates. Other soft metal cations, which are also known to promote the hydrolysis

15 of thioesters, are suitable for rate enhancement of the aminolysis of the thioester bonds. (See Satchell and Secemski, *Tet. Lett.* 1969, **24**:1991-1994; Patel *et al.*, *J. Chem. Soc. Perkin Trans 2*, 1981, 1406-1410.) Hg^{2+}, Tl^{2+}, and Pb^{2+} salts are considered suitable, for example, but are less preferred where disposal of used reagents is concerned. The identity of the group R is not critical; in general, it may be

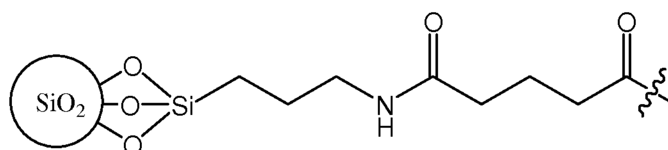
20 any alkyl, heteroaryl, or aryl group. Optional substituents on R may be present to confer water solubility or simplify product isolation. Substituents that reduce vapor pressure may also be desired, in view of the unpleasant odor of volatile mercaptans. Suitable examples of water-soluble, non-volatile mercaptans include 4-mercaptophenylsulfonic acid, N-acetylcysteine, $HSCH_2COOH$, $HSCH_2CH_2COOH$,

25 and $HSCH_2CH_2SO_3H$.

By way of example, a simple, inexpensive procedure is described herein for generating monodisperse, thioester-functionalized silica microspheres, and for immobilizing molecules bearing reactive amines to these microspheres. In the

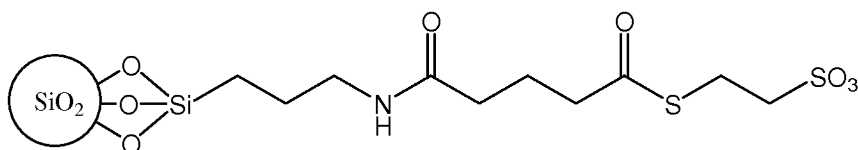
examples, silica microspheres having diameters on the order of 400–500 nm are prepared by the Stöber process (see Experimental section). The pendant thioester groups are stable to aminolysis and hydrolysis in buffered water at pH 7.5, yet they react readily with amines in the presence aqueous $NaAuCl_4$ to form surface-bound
5 amides, and, at an effectively alkaline pH, they undergo thiol–thioester exchange with added thiols to form new surface-bound thioesters. Because of the mild conditions of these reactions, the methods of the invention are suitable for use with biological molecules, as demonstrated by the immobilization of green fluorescent protein (GFP).

For convenience, terminology is employed which identifies the components of
10 the substrate-bound molecules. Thus, the acronym "PAGS" as used herein refers to silica microspheres having propylaminoglutaryl side chains:



Propylaminoglutaryl silica = "PAGS"

The atom bonded to the acyl group is identified by its elemental symbol (e.g., "S" for a thioester; "N" for an amide). Finally, the small molecule or target molecule that is
15 bound is identified by name or by a convenient identifier. For example, the following structure:



PAGS-S4

is referred to herein as PAGS–S4 ("4" representing the ethyl-2-sulfonate residue), and may also be referred to by more explicit recitation of the small molecule component,
20 e.g. as PAGS–S-$(CH_2)_2SO_3^-$ or PAGS–S-ethyl-2-sulfonate. With this nomenclature, the reader can immediately ascertain the identity of the substrate, the nature of the binding (amide or thioester), and the structure of the bound molecule.

The thioester-functionalized microspheres used in the examples herein were prepared from silica spheres produced by the method of Stöber (W. Stöber, A. Fink,
25 E. Bohn, *J. Colloid Interface Sci.* 1968, **26**:62; W. P. Hsu, R. C. Yu, E. Matijevic, *J.*

Colloid Interface Sci. 1993, **156**:56). With this technique, 3-aminopropyl-modified 0.5 μm silica microspheres (PAS) are readily prepared in gram quantities (see Experimental section). Treatment of the PAS spheres with an excess of glutaric anhydride in DMF produced carboxylate-terminated microspheres ("PAGS–OH"). Activation with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) hydrochloride and reaction with sodium 2-mercaptoethanesulfonate (M. Adamczyk, J. R. Fishpaugh, *Tet. Lett.* 1996, **37**:4305) produced the PAGS–S4 microspheres. Condensation of ethanethiol with PAGS–OH microspheres in DMF with diethyl phosphorocyanidate (DEPC) as the activating agent (Y. Yokoyama et al., *Chem. Pharm. Bull.* 1977, **25**:2423) similarly provided *S*-ethylthiocarboxylate-terminated microspheres (PAGS–S-Et; or "PAGS–S5"). These representative reactions for the preparation of thioester-functionalized microspheres are summarized in Figure 1.

Silica microspheres suspended in water scatter UV light, but, when they are suspended in chloroform, the indices of refraction of the solvent and solid are sufficiently matched to permit meaningful UV spectra to be obtained. A suspension of the hydrophobic PAGS–S5 beads in chloroform, for example, absorbs UV light with λ_{max} = 238 nm, an absorption band typical of thioesters; the band is absent from the spectrum of PAGS–OH beads suspended in chloroform. PAGS–S4 beads do not form a stable suspension in chloroform but readily form suspensions in water, consistent with the presence of charged functional groups on the surface of the beads.

The attachment of amine-containing molecules to insoluble substrates by the method of the invention was demonstrated (Figure 2) by use of a fluorescent amine dye (AlexaFluor™ 594, **7**) as a model molecule, the binding of which could be monitored by fluorescence microscopy. Thus, PAGS–S4 beads were suspended in a 25 μM solution of **7** in the presence of 5 μM $NaAuCl_4$ for 30 minutes. After being washed and isolated, the resulting PAGS–N7 beads fluoresced above 560 nm, as expected of a rhodamine dye (Figure 4). This fluorescence persisted after the beads were washed several times with water and brine, showing that the attachment was robust.

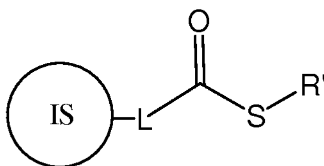
When a 25 μM solution of **7** was stirred with the PAGS–S4 microspheres for 1 day at room temperature in unbuffered water, no dye was observed to attach, and when the PAGS–S4 microspheres were suspended in 1 M NaOH for three minutes and washed, subsequent attempts to attach the dye failed. These experiments confirm

that both the thioester surface and the metal ion promoter are necessary for the immobilization of simple amines.

The attachment of larger biomolecules by the method of the invention was demonstrated with green fluorescent protein (GFP) as a model protein. A plasmid that encodes for enhanced green fluorescent protein with a 6xHis tag (6xHis-EGFP) was transformed into *E. coli*, expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG), and the protein purified on Ni-NTA agarose beads. Analysis by SDS-PAGE (Figure 5) confirmed the homogeneity of the protein.

PAGS–S4 thioester microspheres were suspended in a 50 μM solution of 6xHis-EGFP and 80 μM $NaAuCl_4$ in PBS for 25 minutes. After washing and isolation, the beads fluoresced above 515 nm in the region expected of GFP (Figure 6). As was the case with the AlexaFluor™ dye, the attachment was robust, and control experiments omitting the metal promoter did not generate fluorescent microspheres.

In addition to the representative examples above, there are many known and commercially-available functionalized insoluble substrates, such as aminopropyl silicas, porous glass beads, and glass surfaces; carboxylated polystyrene, dextran, agarose, and cellulose products; polyacrylate and polymethacrylate polymers and co-polymers; and C_8 and larger perfluorinated carboxylic acids. By the methods of the present invention, any solid, liquid, or gel-phase substrate known to be useful for covalent attachment of target molecules of interest may be converted into thioester-functionalized substrates of general formula



wherein “IS” is an insoluble substrate, L is as described herein, and R’ represents R, as described herein, or a target molecule of interest (M).

The linker L may be any linker known in the art to be suitable for attachment of molecules to insoluble substrates, as taught for example in *Linker strategies in solid-phase organic synthesis*, by Peter J. H. Scott (Wiley, Chichester, 2009). Suitable linkers include, but are not limited to, one or more of: C-1 to C-18 alkylene chains, polyethylene glycol chains having one to ten (CH_2CH_2O) residues, C-3 to C-6 dicarboxylic acid chains, C-2 to C-10 dialkylamine chains, amino acids of formula

$H_2N(CH_2)_nCOOH$ where n is 1-10, and combinations and permutations thereof. The linker may also be non-covalently attached to the insoluble substrate, as described herein.

The target molecule, M, may be any molecule suitable for attachment to an insoluble substrate, including but not limited to dyes, pharmaceuticals, radiopharmaceuticals, imaging and contrast agents, proteins, peptides, protein-binding ligands, polysaccharides, oligosaccharides, polynucleotides, and oligonucleotides. More specifically, M may be an enzyme, enzyme substrate, enzyme inhibitor, co-factor, antibody, antibody fragment, receptor, receptor ligand, or an affinity tag. M may also be an oligonucleotide probe, primer, or coding sequence, or a catalyst, co-catalyst, or a ligand (such as a phosphine or diphosphine) for a catalyst.

The invention provides a simple procedure for making thioester-functionalized silica spheres in gram quantities from inexpensive, commercially-available reagents. The amide bond formation occurs in phosphate buffered or unbuffered water at room temperature in the presence of a mild promoter, conditions well-suited for the immobilization of sensitive biomolecules.

In addition to the selectivity and mildness of the metal-promoted reactions with thioester-functionalized substrates, the present invention has the advantage that thioester substrates do not hydrolyze at an appreciable rate in the absence of a promoter or a significant concentration of hydroxide ion. Relative to the prior art alternatives (e.g., NHS and HOBT esters, mixed anhydrides, and acid chlorides), the thioester-functionalized substrates of the present invention have a much longer shelf life when stored in the presence of water. The thioester-functionalized silica microspheres described herein can be stored in pH 7.4 PBS for months. Accordingly, they may be manufactured in bulk, stored in water, and marketed commercially as ready-to-use reactive substrates for the immobilization and tagging of proteins, peptides, and other target molecules. The invention also provides kits comprising one or more thioester-functionalized substrates in combination with one or more Lewis acid promoters, e.g., metal cations.

Several examples of the methods and materials of the invention involve the attachment of amine-functionalized fluorescent dyes to the insoluble substrate. These molecules were selected as model amines because they allowed the progress of the reactions to be monitored by fluorescence microscopy. Non-specific adsorption of

organic molecules to the solid support was avoided by using microspheres of silica, rather than an organic polymer such as polystyrene. These particular embodiments were chosen for illustrative purposes, and do not constitute or represent limitations on the scope of the claims.

3. Detachment of Molecules

In another aspect, the present invention provides a method for the reversible attachment of molecules to an insoluble substrate by thiol–thioester exchange, under mild, aqueous conditions that are compatible with biological molecules. The thioester attachment is a robust covalent bond that does not significantly dissociate, hydrolyze, or aminolyze on short time scales at physiological pH. The molecules attached by thioester linkages can be detached from the substrate via: (i) thiol–thioester exchange, by addition of another thiol; (ii) hydrolysis, by raising the pH or adding a metal to promote hydrolysis; (iii) thioester-exchange-assisted aminolysis, by addition of cysteamine or cysteine, or (iv) aminolysis, by a direct or metal-assisted reaction with ammonia or an organic amine, as described above. Options (i) through (iii) are illustrated in Fig. 8.

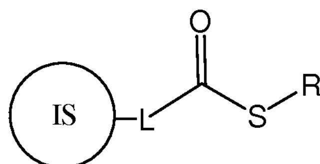
Thus, in combination with the methods of thioester attachment described herein, the present invention provides a method for isolating target molecules from a mixture comprising non-target molecules, which comprises (i) attachment of the target molecules to an insoluble substrate via thioester bonds, (ii) separation of the insoluble substrate from non-target molecules, and (iii) detachment of the target molecules from the insoluble substrate.

The use of thiol–thioester exchange as a binding event for bioconjugation has several attractive properties, including robust attachment via a covalent bond, the use of heterologous functional groups (ensuring that like species do not bind to each other), and mild “biomolecule-friendly” conditions for both attachment and detachment. The method has applications in numerous fields, such as affinity chromatography, protein purification, and reversible self-assembly.

Another attractive property of the thioester linkage is its suitability for multivalent attachment. A target molecule having two or more thiol groups may be bound to a substrate of the present invention by two or more thioester linkages. As the number of thiol groups rises, and the number of thioester bonds to the substrate

increases, the equilibrium constant for formation of the bound species rises considerably, resulting in a tremendous increase in the affinity of the substrate for the target molecule. This effect may be utilized in affinity chromatography, and for the isolation of proteins carrying poly-cysteine tags. The process is analogous to the isolation of poly-histidine tagged proteins on a substrate carrying nickel or cobalt ions. Poly-cysteine tagged proteins suitable for use in the present invention may be prepared by analogous methods.

The reversible thiol–thioester exchange reaction allows elution of the bound poly-cysteine tagged species from the substrate by displacement with a high concentration of a low-molecular-weight mercaptan. Accordingly, one embodiment of the present invention provides a method for separating a target molecule having *n* thiol groups from a mixture comprising molecules having fewer than *n* thiol groups. The method comprises contacting the mixture with a thioester-functionalized insoluble substrate of formula



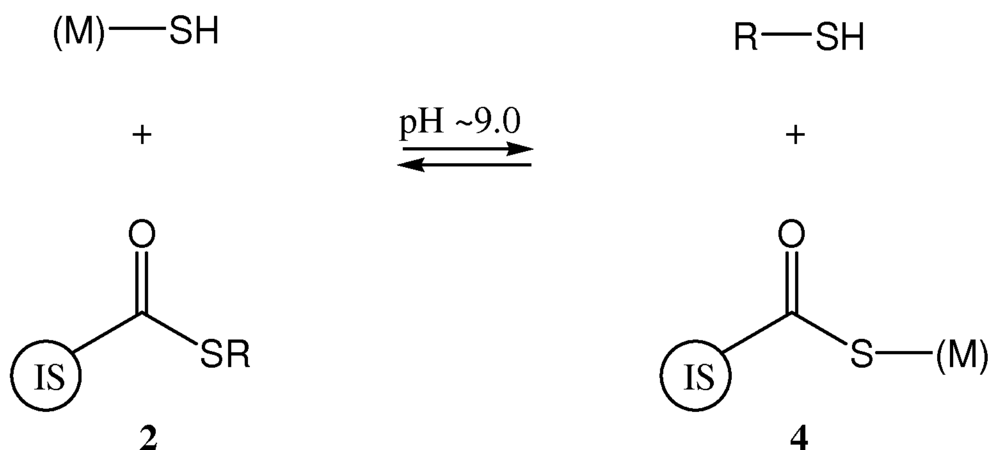
in a buffered solution having an effectively alkaline pH, as described above, followed by the elution any unbound molecules. The bound molecules are then eluted with a buffer solution containing a soluble displacing mercaptan. The latter elution is preferably carried out with a gradient of an increasing concentration of the displacing mercaptan. The gradient may be a step or continuous gradient, or any combination thereof.

The soluble displacing mercaptan may be any sulfide or mercaptan that is readily separable from the target molecule, for example by means of dialysis, size exclusion chromatography, gel filtration, or protein precipitation. Volatile mercaptans may be separated by evaporation or lyophilization. Suitable examples include, but are not limited to, sodium sulfide, ethyl mercaptan, 2-mercaptoethanol, 2-mercaptoethylamine, 2-mercaptoacetic acid, 3-mercaptopropionic acid, 2-mercaptoethanesulfonic acid, cysteine, and the like. In applications where unpleasant odors are undesirable, the use of non-volatile mercaptans will be preferred.

Target molecules having a plurality of thioester bonds to the substrate, which are very tightly bound, may optionally be cleaved from the substrate irreversibly by hydrolysis or aminolysis of the multiple thioester bonds. Use of a β-aminomercaptan or a soft metal cation promoter, as described above, is preferable where mild conditions are desired.

In principle, the generation of the thioester-functionalized surfaces for use in the present invention, may be carried out by any of a wide variety of synthetic methods known to be useful for the preparation of thioesters. (See, e.g., S. Fujiwara, N. Kambe, in *Chalcogenocarboxylic Acid Derivatives, Vol. 251*, Springer-Verlag, Berlin, **2005**, p. 87.) A preferred method is the reaction between a thiol and a thermodynamically activated derivative of a carboxylic acid, such as an acid chloride, anhydride, or mixed anhydride. (A. W. Ralston, E. W. Segebrecht, S. T. Bauer, *J. Org. Chem.* 1939, **4**:502; H. M. Meshram et al., *Synlett* 1998, 877; A. A. Schleppnik, F. B. Zienty, *J. Org. Chem.* 1964, **29**:1910.) Although the usual carbodiimide coupling reagents (e.g. DCC) are not particularly effective, dichlorophosphate esters (A. Arrieta *et al.*, *Synth. Commun.* 1983, **13**:471) and halopyridinium salts (Y. Watanabe, S. I. Shoda, T. Mukaiyama, *Chem. Lett.* 1976, 741) are efficient coupling agents for the preparation of thioesters from carboxylic acids.

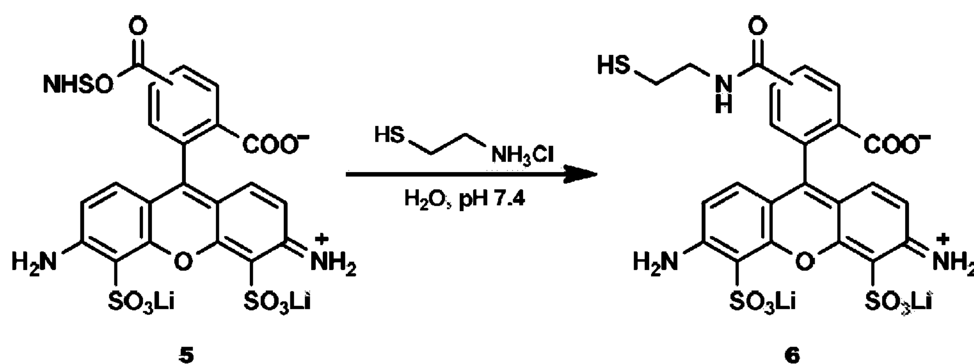
The above methods are generally suitable for preparing thioester-functionalized insoluble substrates of formula **2**, where R is a "disposable" small molecule residue as described above. Where the molecule of interest (M) is a biomolecule, however, milder and more selective methods may be preferred. Accordingly, in preferred embodiments of the present invention, the structure **2** is employed as an activated substrate for attachment of the molecule of interest, via a thiol–thioester exchange reaction as shown in Scheme 5. The species (M)-SH may represent the target molecule, if thiol groups are natively present; alternatively it may represent a thiol-tagged derivative of the target molecule (M). The exchange reaction is preferably carried out in a buffer containing a reagent that reduces disulfides, referred to herein as a "thioester exchange buffer". One suitable thioester exchange buffer contains 100 mM carbonate and 2 mM tris(2-carboxyethyl)phosphine at pH 9.0.



Scheme 5

For illustrative purposes, the examples presented herein employ fluorescent dyes as model target molecules (M). The dyes have good solubility in water, large extinction coefficients in the visible spectrum, and high fluorescence quantum yields. These properties make possible the convenient qualitative monitoring of the binding by fluorescence microscopy. The high fluorescence quantum yield of the dyes allows detection of the binding event despite the small quantity of thiol attached to the beads. The nomenclature used to describe the bound complexes is the same as used in above in connection with the attachment studies.

The thiol-tagged dye **6** is an example of a fluorescent target molecule (M)-SH. The NHS ester of the commercially available dye AlexaFluor™ 488 (**5**) was treated with one equivalent of cysteamine hydrochloride to yield the thiol-tagged dye **6** (Scheme 6):



Scheme 6

To prevent oxidation of the thiols, the water-soluble reducing agent tris-(2-carboxyethyl) phosphine (TCEP) was present in the thioester exchange buffer used to run the binding reactions (J. Houk, G.M. Whitesides, *J. Am. Chem. Soc.* 1987,

109:6825.) TCEP reduces any disulfides that might form as a result of air oxidation of the thiols.

A sample of PAGS–S4 microspheres was suspended in a buffered (pH 9) solution that contained the thiol-tagged dye **6** and 2 mM TCEP, and binding of the dye to form PAGS–S6 microspheres was monitored by fluorescence microscopy. The PAGS–S4 microspheres did not fluoresce, but as shown in Figure 8, after stirring for 1 hour at pH 9, intensely fluorescent PAGS–S6 microspheres were obtained. Carboxylate silica spheres (PAGS–OH) exposed to thiolated dye **6** in the same buffer did not fluoresce, consistent with thiol–thioester exchange as the mechanism of binding.

Exposure of the PAGS–S6 spheres to aqueous buffer of pH 11 for 90 minutes cleaved the dye from the microspheres, rendering them non-fluorescent. Stirring fluorescent PAGS–S6 spheres at pH 9 for 90 minutes in 30 mM 2-mercaptoethane sulfonate, or in 300 mM cysteamine, also rendered the spheres non-fluorescent. Fluorescence consistent with the dye **5** was observed in the supernatant solution. In control experiments, in the absence of thiols, the PAGS–S6 microspheres retained their fluorescence when suspended in the same pH 9 buffer. When the spheres cleaved with 2-mercaptoethanesulfonate were exposed to a 200 mM solution of **6**, the dye **6** re-attached, and the microspheres regained fluorescence. These results demonstrate the reversibility of the thioester binding system. The synthesis of the support and the various means of attachment and detachment of the target molecules are summarized in Figure 9.

The dye-terminated polypeptides **9**, **10**, and **11** were each attached to the silica microspheres by the methods described above, in order to demonstrate polyvalent attachment:

H_2N-GGCGG-CO-(fluorescein) (**9**) (SEQ ID NO: 1)

H_2N-GGCCGG-CO-(rhodamine) (**10**) (SEQ ID NO: 2)

H_2N-GGCCCCCCGG-CO-(fluorescein) (**11**) (SEQ ID NO: 3)

A 10 mM solution of 2-mercaptoethanesulfonate in pH 9 buffer was able to eliminate most of the fluorescence from the PAGS–S9 (monovalent tag) and PAGS–S10 (divalent tag) microspheres, but the hexavalent-tagged PAGS–S11 microspheres were resistant to detachment of the dye. This demonstrates that a plurality of thiols on

the target molecule leads to a more durable, multivalent attachment to the insoluble support.

The methods of the present invention provide for reversible attachment and detachment of target molecules to insoluble supports, under conditions suitable for
5 most biochemical and biological applications. Because the thiol–thioester exchange reaction selectively binds thiols in the presence of other nucleophiles, the method of the invention is orthogonal to most other methods that use binding pairs (e.g., biotin-avidin), and the method is expected to work in conjunction with most systems without interference.

10 Representative experimental procedures are described below, in order to illustrate the invention in greater detail. These serve merely as examples, and do not represent limitations on the scope of the invention or the attached claims.

EXAMPLES

Reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Alfa
15 Aesar (Ward Hill, MA), or TCI-America (Portland, OR). AlexaFluor™ dyes were purchased from Molecular Probes (Eugene, OR). Glutaric anhydride from Aldrich and Alfa Aesar was found to contain small grains of insoluble black grit; it was dissolved in dry (low-water, not rigorously anhydrous) *N*,*N*-dimethylformamide (DMF) and filtered prior to use to prevent particulate contamination of the
20 microspheres. All other commercial reagents were used without further purification.

Fluorescein-terminated peptides H_2N-GGCGG-CO-(fluorescein) (SEQ ID NO: 1) and H_2N-GGCCCCCCGG-CO-(fluorescein) (SEQ ID NO: 3) were obtained from the solid phase peptide synthesis facility at Harvard Medical School's Department of Pharmacology (Boston, MA). The rhodamine-terminated peptide
25 H_2N-GGCCGG-CO-(rhodamine) (SEQ ID NO: 2) was obtained from Biomer Technology (Pleasanton, CA).

Phosphate buffered saline (PBS) solution was pH 7.4 with 10 mM Na_xH_yPO4, 2.7 mM KCl, and 138 mM NaCl. "Thioester exchange buffer" contained 100 mM carbonate ion and 2 mM tris(2-carboxyethyl)phosphine (TCEP) hydrochloride titrated
30 to pH 9.0. Following each chemical reaction, the solid microspheres were rinsed repeatedly to remove excess reagents and other impurities. One "rinse cycle" consisted of suspending the beads in the stated volume of solvent, shaking for 60 s, centrifuging the sample, and discarding the supernatant.

Preparation of Amine-functionalized Silica Microspheres (PAS). At room temperature, a 50 ml heavy-walled glass tube was filled with 1.5 ml 28% aqueous ammonium hydroxide, 9.0 ml 7 N ammonia in methanol, 17 ml isopropyl alcohol, 4 ml tetrabutylorthosilicate, and a Teflon-coated stir bar. The tube was sealed with a Teflon screw cap and heated at 40 °C with stirring for 8.5 h. Over the course of this time period, the clear mixture became white and opaque.

The tube was cooled with tap water to minimize the loss of ammonia upon opening of the sealed vessel. Next, 2.0 ml of 3-aminopropyltrimethoxysilane (**2**) was added, and the mixture stirred for an additional 14 h at 40 °C while sealed. The tube was opened and heated at 55 °C for 30 min to expel most of the ammonia from the solution. The sample was centrifuged, the supernatant discarded, and the solid washed with 40 ml volumes of ethanol (2x), milli-Q water (2x), and DMF (2x).

Preparation of Propylaminoglutaric Acid-functionalized Silica Microspheres (PAGS–OH). The aminopropyl PAS beads were suspended in 30 ml DMF and 3.7 g of glutaric anhydride was added as a solid. This mixture was stirred at room temperature for 2 h. The beads were sonicated for 20 min, then centrifuged and decanted. The beads were re-suspended in 50 ml DMF and 3.0 g of glutaric anhydride was added. The mixture was stirred for 2 h at 40 °C, then for an additional 13 h at room temperature. The beads were centrifuged and decanted, then washed with 40 ml volumes of solvent (four times with milli-Q water, then twice with ethanol).

Preparation of PAGS–S4 Thioester Beads. Carboxylic acid-terminated PAGS–OH spheres were suspended in 30 ml of deionized water. EDC hydrochloride (1.5 g) and sodium 2-mercaptoethanesulfonate (1.5 g) were added, and the mixture stirred at room temperature for 40 h (M. Adamczyk, J. R. Fishpaugh, *Tet. Lett.* 1996, **37**:4305.) The beads were washed with five 40 ml portions of milli-Q water, then stored at 4 °C prior to use.

Preparation of PAGS–S5 Thioester Beads. Carboxylic acid-terminated PAGS–OH spheres (prepared from roughly 1.6 mmol of tetraorthosilicate) suspended in PBS were centrifuged, and the supernatant was decanted. The sample was rinsed twice with DMF by suspending, centrifuging, and decanting, to remove residual water, and then suspended in 20 ml of DMF in a 50 ml round-bottomed flask. The flask was sealed with a rubber septum, and in sequence, 2.2 ml of ethanethiol, 2.4 ml of diethyl

phosphorocyanidate (DEPC), and 1.9 ml of triethylamine were injected by syringe. The mixture was stirred at room temperature for 6 h with no significant change in color. The mixture was transferred to a 50 ml centrifuge tube and centrifuged, and the solvent was decanted.

5 The beads were rinsed, in sequence, once with 40 ml ethanol, once with 40 ml PBS, twice with 40 ml ethanol, and twice with 40 ml ethanol. The beads suspended homogeneously in ethanol, but aggregated and foamed in PBS.

Anchoring of AlexaFluor 594 Cadaverine Dye (7). Thioester-functionalized PAGS–S4 spheres were suspended in 400 μl of deionized water. A 500 μl aliquot of a 10 50 μM solution of the amine dye **7** was added, followed by 100 μl of 50 μM $NaAuCl_4$. The suspension was agitated for 30 min at room temperature, centrifuged, and decanted. The particles were washed twice with 1 ml milli-Q water, twice with brine, and once more with milli-Q water prior to analysis by fluorescence microscopy.

Cloning of EGFP. A plasmid encoding EGFP (enhanced green fluorescent 15 protein) with a 6xHis tag at the *C*-terminus was prepared. The coding sequence for EGFP was amplified using PCR (Taq polymerase) from the vector pEGFP (Clontech) and a forward primer that encodes an *Nco I* restriction site (CCGGCCATGGTGAGCAAGGGCGAGGA) (SEQ ID NO: 4) and a reverse primer that encodes a *Hind III* restriction site 20 (CCCAAGCTTGGGCTTGTACAGCTCGTCCATGCC) (SEQ ID NO: 5). PCR (25 cycles; 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min) used 0.5 μl of each primer (200 mM), 1 μl pEGFP (0.5 ng/μl), 10 μl dNTPs (2 mM), 2 μl Taq polymerase, 50 μl Failsafe Buffer E (Epicentre), and 36 μl H_2O. PCR products were purified using a Strataprep™ PCR Purification Kit (Stratagene), digested using *Nco I* and *Hind III* 25 (New England Biolabs), and purified again. The digested PCR product was ligated into pET28a+ (Novagen) using T4 DNA ligase (New England Biolabs), and the crude ligation mixture was transformed into chemically competent *E. coli* NM554 growing on LB/agar plates containing kanamycin (50 μg/ml). Five colonies were selected after overnight incubation (37 °C) and grown in liquid culture (LB media, 50 μg/ml 30 kanamycin) for 24 h. Plasmid DNA from each culture was purified using a Miniprep Plasmid Purification Kit (Qiagen). Each plasmid was digested using *Nco I* and *Hind III*, and the EGFP fragment was observed using agarose gel electrophoresis. Plasmid

DNA that contained the EGFP fragment (referred to as pET28a-EGFP) was sequenced with an ABI DNA Analyzer (Applied Biosystems).

Bacterial Transformation and Protein Purification. Chemically competent *E. coli* BL21(DE3) were transformed with pET28a-EGFP, and colonies were selected on LB/agar plates containing kanamycin (50 μg/ml) after incubation overnight (37 °C). For protein expression, cells were grown to an $O.D._{600}$ of 0.6 in LB media containing 50 μg/ml kanamycin, and isopropyl-β-D-thiogalactoside (1 mM final concentration) was added to induce the expression of EGFP. Bacteria were harvested after 4 h of incubation at 37 °C.

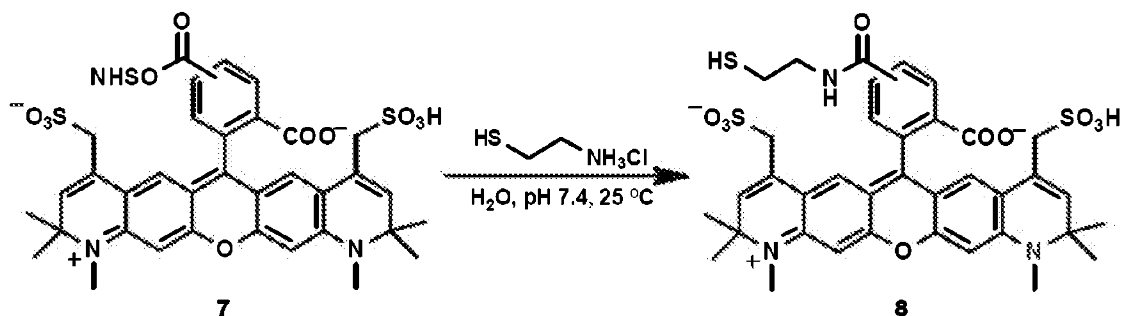
The bacteria were centrifuged and subjected to three liquid nitrogen freeze/thaw cycles, suspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 0.005% Tween™ 20, pH 8.0) and sonicated six times (10 s duration with a 5 s pause). The crude lysate was centrifuged at 10,000 x g for 30 minutes at 4 °C. The protein was purified from the supernatant using Ni-NTA resin (Qiagen). The resin was washed with wash buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.005% Tween™ 20), and the protein was separated from the resin using elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 250 mM imidazole, 0.005% Tween™ 20). The purity of the protein was confirmed by SDS-PAGE.

Anchoring of 6xHis-EGFP. Thioester-functionalized PAGS–S4 spheres were suspended in 50 μl of a 46 μM solution of 6xHis-EGFP in PBS, pH 7.4. A 5 μl aliquot of 50 μM $NaAuCl_4$ was added, and the suspension was agitated for 25 min at room temperature, centrifuged, and decanted. The beads were washed twice with 1 ml PBS prior to analysis by fluorescence microscopy.

Thiol "Tagging" of Substrates:

Compound **6**. The NHS-ester-functionalized AlexaFluor 488 dye **5** (1 mg, 1.6 μmol) (Molecular Probes, Eugene OR) was dissolved in 1.6 ml of PBS to yield a 1 mM solution. To a 1.4 ml aliquot of this solution, cystamine hydrochloride (0.14 mg, 1.2 μmol) was added, and the mixture was stirred for 1 hour. Without characterization, this mixture was treated as a 1 mM solution of compound **6**, thiol-labeled AlexaFluor 488.

Compound **8**.



The NHS-ester-functionalized AlexaFluor 594 dye **7** (1 mg, 1.2 μmol) (Molecular Probes, Eugene OR) was dissolved in 1.2 ml of PBS to yield a 1 mM solution. To a 1.0 ml aliquot of this solution, cystamine hydrochloride (0.10 mg, 0.9 μmol) was added, and the mixture was stirred for 1 hour. Without characterization, this mixture was treated as a 1 mM solution of compound **8**, thiol-labeled AlexaFluor 594.

Thiol–thioester exchange of ***6*** *with PAGS–S4 microspheres.* A 100 μl portion of PAGS–S4 beads were suspended in 800 μl of pH 9 exchange buffer. A 200 μl aliquot of a 1 mM solution of **6** was added, and the mixture was agitated for 60 minutes at room temperature. The sample was centrifuged, the supernatant extracted, and the beads washed four times with 1 ml PBS, pH 7.4. The beads fluoresced when irradiated with blue light on an optical microscope.

Control: Reaction of ***6*** *with PAGS–OH microspheres.* A 100 μl portion of carboxylic acid terminated silica microspheres (PAGS–OH) was suspended in 800 μl of pH 9 exchange buffer. A 200 μl aliquot of a 1 mM solution of **6** was added, and the mixture was agitated for 60 minutes at room temperature. The sample was centrifuged, the supernatant extracted, and the beads washed four times with 1 ml PBS, pH 7.4. The beads did not fluoresce when irradiated with blue light on an optical microscope.

Cleavage Experiments: Four 50 μl aliquots of a suspension of the fluorescent dye-linked spheres PAGS–S6 were placed into four microcentrifuge vials, each vial containing one of the following solutions: i) pH 9 exchange buffer, ii) 300 mM cysteamine in pH 9 exchange buffer, iii) 30 mM sodium 2-mercaptoethanesulfonate in pH 9 exchange buffer, and iv) pH 11 buffer. After 90 minutes of incubation at 23 °C, the beads from the first vial (pH 9 exchange buffer) remained brightly fluorescent. There was minimal residual fluorescence from the sample in the third vial, and no residual fluorescence from the beads in the second and fourth vials.

Reversibility of attachment of Alexafluor-488. PAGS–S4 (100 μl of stock suspension) was added to a 2 ml Eppendorf™ vial. After centrifugation and removal of the supernatant liquid, a pH 9 buffered solution of thiol-modified AlexaFluor-488 (**6**) was introduced, and the suspension was agitated for 60 minutes at room
5 temperature. The beads were separated by centrifugation and washed three times with 1 ml pH 7.4 PBS buffer. Fluorescence microscopy verified that the microspheres were fluorescent. The microspheres were suspended in 300 μl of 100 mM 2-mercaptoethanesulfonate and 700 μl of pH 9 (exchange) buffer, agitated for 60 minutes, isolated by centrifugation, and washed three times with pH 7.4 PBS buffer.
10 Fluorescence microscopy showed greatly diminished fluorescence. The cleavage solution was analyzed in a fluorimeter, where strong emission at 488 nm indicated the presence of unbound AlexaFluor-488 dye (**6**). The isolated microspheres were re-dispersed in the original pH 9-buffered solution of thiol-modified AlexaFluor-488 solution for 60 minutes, then isolated and washed as before. Fluorescence
15 microscopy verified that the microspheres were again fluorescent.

Other embodiments

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the
20 invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

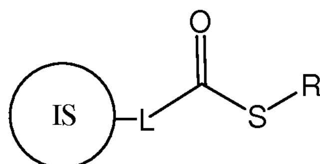
All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent
25 or patent application was specifically and individually indicated to be incorporated by reference in its entirety. Where a term in the present application is found to be defined differently in a document incorporated herein by reference, the definition provided herein is to serve as the definition for the term.

Other embodiments are in the claims.

30 What is claimed is:

CLAIMS

1. A method for the covalent attachment of a target molecule having at least one amino group to an insoluble substrate, comprising contacting said target molecule with a thioester-functionalized insoluble substrate of formula



in the presence of a metal or Lewis acid promoter;
wherein "IS" represents said insoluble substrate, L is a covalent bond or is a linker covalently or non-covalently attached to the insoluble substrate, and R-S is a sulfenyl group.

2. The method of claim 1, wherein the promoter is selected from the group consisting of salts of Ag^{+}, Hg^{2+}, Pb^{2+}, Tl^{3+}, and Au^{3+}.

3. The method of any preceding claim, wherein the moiety R-S is derived from a thiol R-SH, selected from the group consisting of thiophenol, benzyl mercaptan, ethanethiol, 2-mercaptoethanol, 2-mercaptoacetic acid, 3-mercaptopropionic acid, 4-mercaptophenylsulfonic acid, 2-mercaptoethanesulfonic acid, and N-acetylcysteine.

4. The method of any preceding claim, wherein L comprises one or more divalent linkers selected from the group consisting of C-1 to C-18 alkylene chains, polyethylene glycol chains having one to ten (CH_2CH_2O) residues, C-3 to C-6 dicarboxylic acid chains, diamino alkylene chains of formula $H_2N(CH_2)_nNH_2$ where n is 2-10, amino acids of formula $H_2N(CH_2)_nCOOH$ where n is 1-10, and combinations thereof.

5. The method of any preceding claim, wherein the target molecule is selected from the group consisting of dyes, pharmaceuticals, radiopharmaceuticals,

imaging agents, contrast agents, proteins, peptides, polysaccharides, oligosaccharides, polynucleotides, oligonucleotides, catalysts, co-catalysts, catalyst ligands, and protein-binding ligands.

6. The method of any preceding claim, wherein the target molecule is selected from the group consisting of enzymes, enzyme substrates, enzyme inhibitors, co-factors, antibodies, antibody fragments, receptors, receptor ligands, affinity tags, oligonucleotide probes, oligonucleotide primers, and oligonucleotide coding sequences.

7. The method of any preceding claim, wherein the target molecule is a protein or peptide.

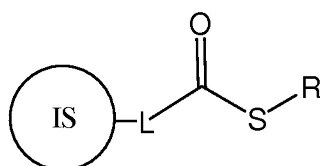
8. The method of any preceding claim, wherein R is a substituted or unsubstituted alkyl, heteroaryl, or aryl group.

9. The method of any preceding claim, wherein R is a C-1 to C-8 alkyl group.

10. The method of any preceding claim, wherein L is a covalent bond or is a linker covalently attached to the insoluble substrate.

11. The method of any preceding claim, wherein the Lewis acid promoter is a soft Lewis acid metal promoter.

12. A kit for attachment of target molecules to insoluble substrates, comprising one or more thioester-functionalized insoluble substrates of formula



and one or more metals or Lewis acid promoters, wherein "IS" represents an insoluble substrate, L is a covalent bond or is a linker covalently or non-covalently attached to the insoluble substrate, and R-S is a sulfenyl group.

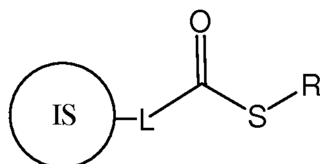
13. The method of claim 12, wherein R is a substituted or unsubstituted alkyl, heteroaryl, or aryl group.

14. The method of claim 12 or 13, wherein R is a C1-C8 alkyl group.

15. The method of any of claims 12-14, wherein L is a covalent bond or is a linker covalently attached to the insoluble substrate.

16. The method of any of claims 12-15, wherein the Lewis acid promoter is a soft Lewis acid metal promoter.

17. A method for the covalent attachment of a target molecule having at least one thiol group to an insoluble substrate, comprising contacting said target molecule with a thioester-functionalized insoluble substrate of formula



in a buffered solution of effectively alkaline pH;
wherein "IS" represents said insoluble substrate, L is a covalent bond or is a linker covalently or non-covalently attached to the insoluble substrate, and R-S is a sulfenyl group.

18 The method of claim 17, wherein the buffer solution has a pH of at least 8.5.

19. The method of claim 17 or 18, wherein the buffer solution has a pH between 9.0 and 10.0.

20. The method of any of claims 17-19, wherein the moiety R-S is derived from a thiol R-SH, selected from the group consisting of thiophenol, benzyl mercaptan, ethanethiol, 2-mercaptoethanol, 2-mercaptoacetic acid, 3-mercaptopropionic acid, 4-mercaptophenylsulfonic acid, 2-mercaptoethanesulfonic acid, and N-acetylcysteine.

21. The method of any of claims 17-20, wherein L comprises one or more divalent linkers selected from the group consisting of C-1 to C-18 alkylene chains, polyethylene glycol chains having one to ten (CH_2CH_2O) residues, C-3 to C-6 dicarboxylic acid chains, diamino alkylene chains of formula $H_2N(CH_2)_nNH_2$ where n is 2-10, amino acids of formula $H_2N(CH_2)_nCOOH$ where n is 1-10, and combinations thereof.

22. The method of any of claims 17-21, wherein the target molecule is selected from the group consisting of dyes, pharmaceuticals, radiopharmaceuticals, imaging agents, contrast agents, proteins, peptides, polysaccharides, oligosaccharides, polynucleotides, oligonucleotides, catalysts, co-catalysts, and ligands for catalytic processes.

23. The method of any of claims 17-22, wherein the target molecule is selected from the group consisting of enzymes, enzyme substrates, enzyme inhibitors, co-factors, antibodies, antibody fragments, receptors, receptor ligands, affinity tags, oligonucleotide probes, oligonucleotide primers, and oligonucleotide coding sequences.

24. The method of any of claims 17-23, wherein the target molecule is a protein or peptide.

25. The method of any of claims 17-24, wherein the target molecule has two or more thiol groups.

26. The method of any of claims 17-25, further comprising the steps of
(i) separation of the insoluble substrate from non-target molecules, and
(ii) detachment of the target molecules from the insoluble substrate.

27. The method of claim 26, wherein step (ii) comprises contacting the insoluble substrate with a mercaptan, with a Lewis acid, with ammonia or an organic

amine in the presence of a metal or Lewis acid, or with an aqueous solution of sufficiently high pH to promote hydrolysis.

28. The method of any of claims 17-27, wherein R is a substituted or unsubstituted alkyl, heteroaryl, or aryl group.

29. The method of any of claims 17-28, wherein R is a C1-C8 alkyl group.

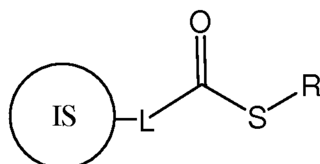
30. The method of any of claims 17-29, wherein L is a covalent bond or is a linker covalently attached to the insoluble substrate.

31. The method of any of claims 26-30, wherein the Lewis acid is a soft Lewis acid metal promoter.

32. A method for detaching target molecules attached via thioester bonds to an insoluble substrate, comprising contacting the insoluble substrate with an amine in the presence of a metal or Lewis acid promoter.

33. The method of claim 32, wherein the Lewis acid promoter is a soft Lewis acid metal promoter.

34. A method for separating a target molecule having *n* thiol groups from a mixture comprising molecules having fewer than *n* thiol groups, comprising the steps of (i) contacting the mixture with a thioester-functionalized insoluble substrate of formula



in a buffer solution having an effectively alkaline pH;
(ii) eluting any unbound molecules; and
(iii) detaching and eluting the bound molecules with a buffer solution containing a soluble, low-molecular-weight mercaptan;

wherein “IS” represents said insoluble substrate, L is a covalent bond or is a linker covalently or non-covalently attached to the insoluble substrate, and R is a sulfenyl group.

35. The method of claim 34, wherein step (iii) is a gradient elution with a buffer having an increasing concentration of the soluble, low-molecular-weight mercaptan.

36. The method of any of claims 34-35, wherein R is a substituted or unsubstituted alkyl, heteroaryl, or aryl group.

37. The method of any of claims 34-36, wherein R is a C1-C8 alkyl group.

38. The method of any of claims 34-37, wherein L is a covalent bond or is a linker covalently attached to the insoluble substrate.

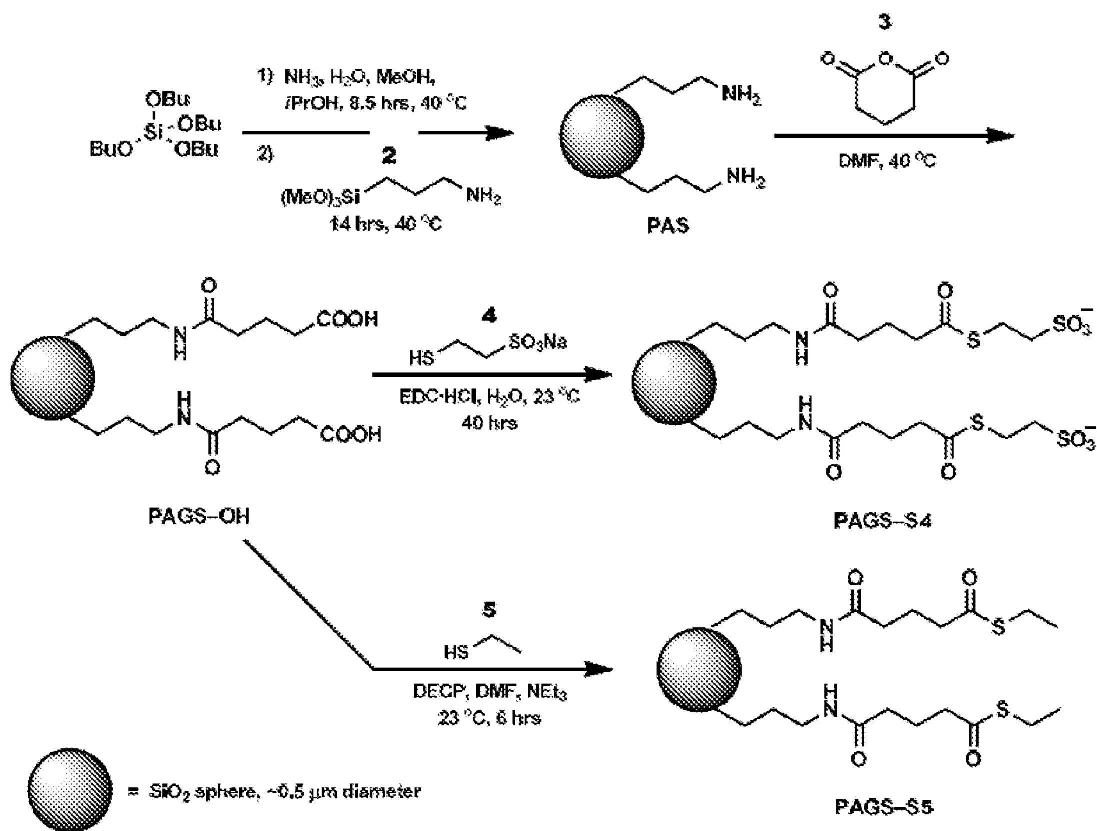


Figure 1

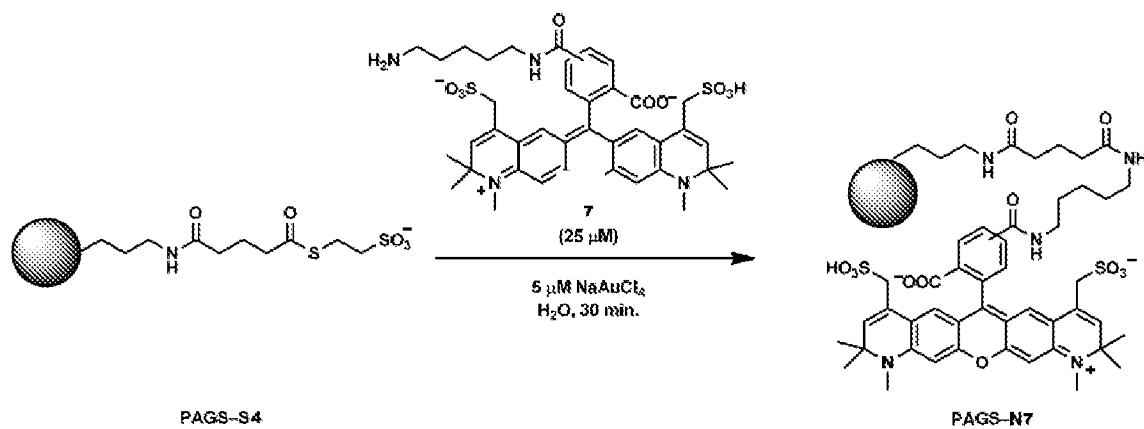


Figure 2

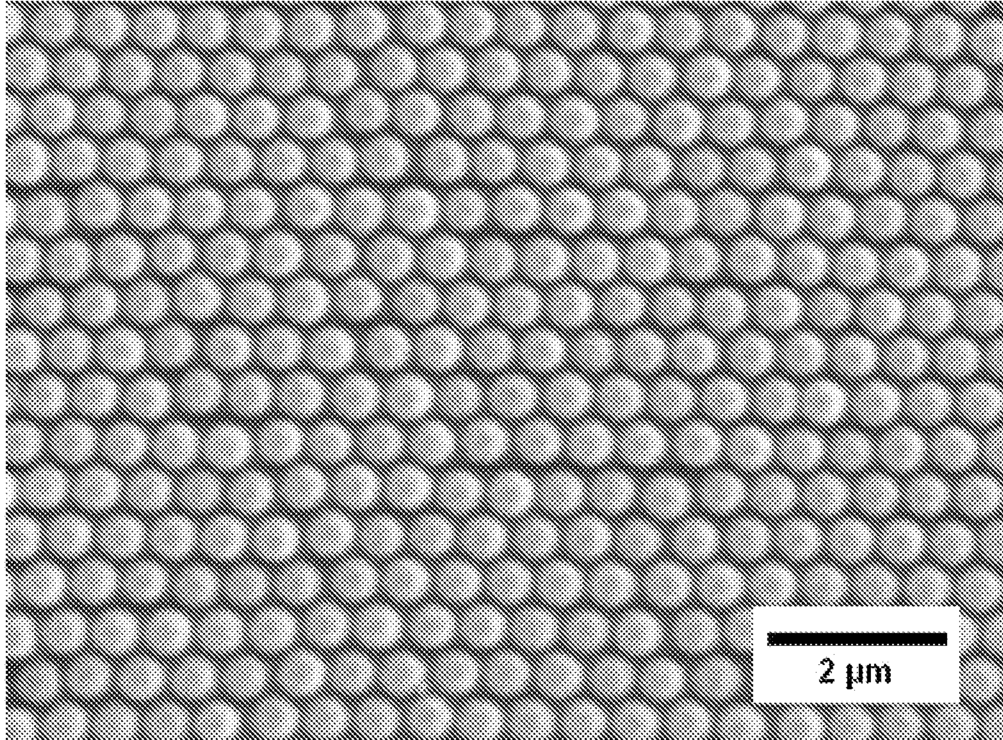


Figure 3

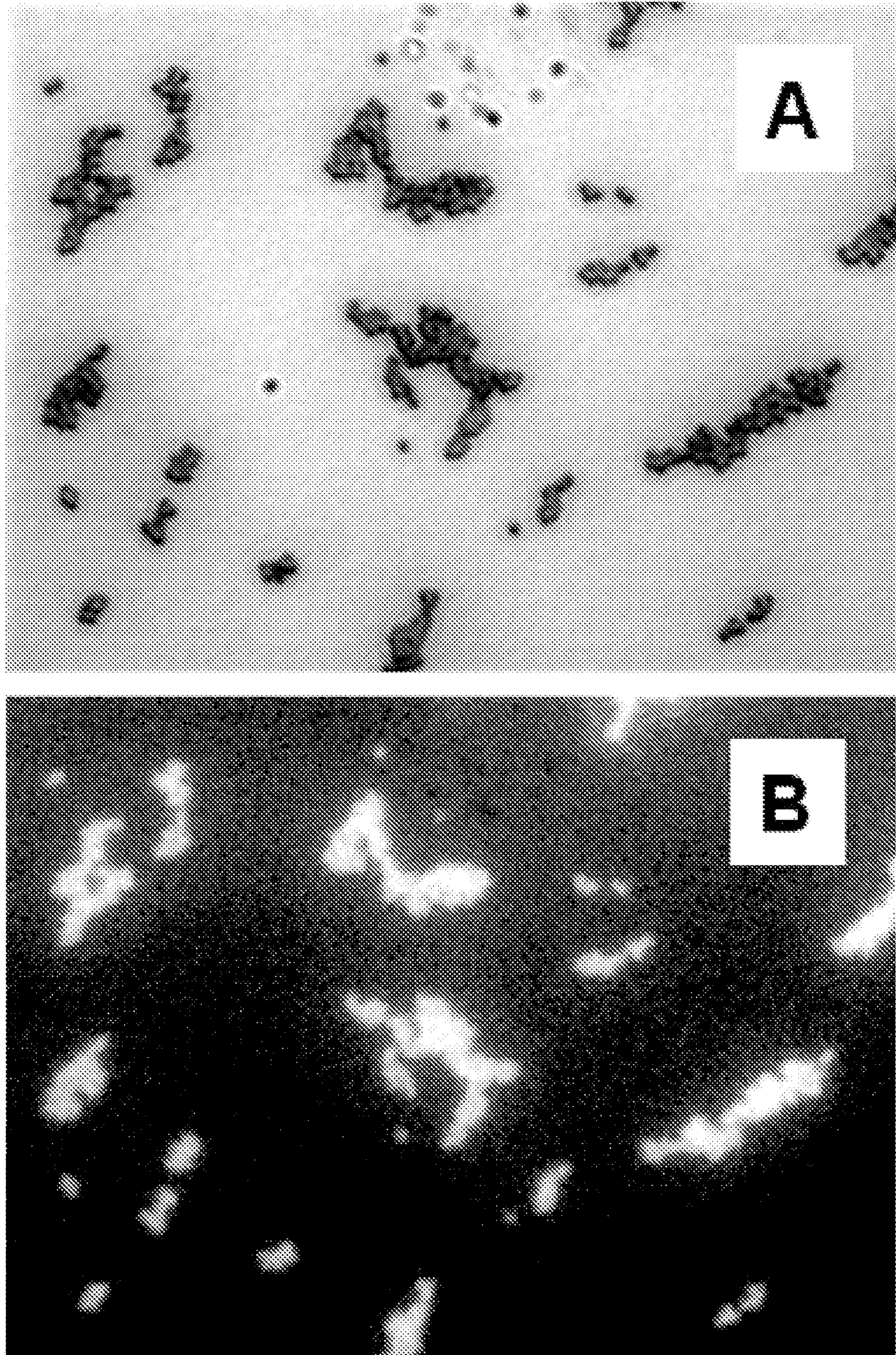


Figure 4

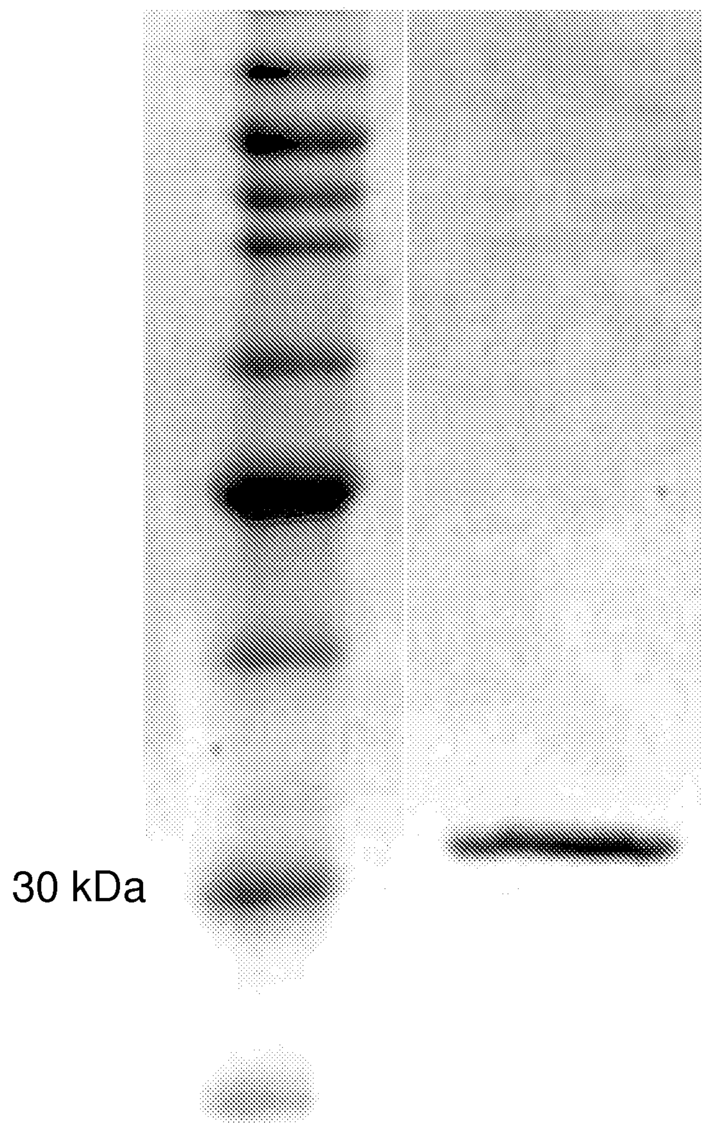


Figure 5

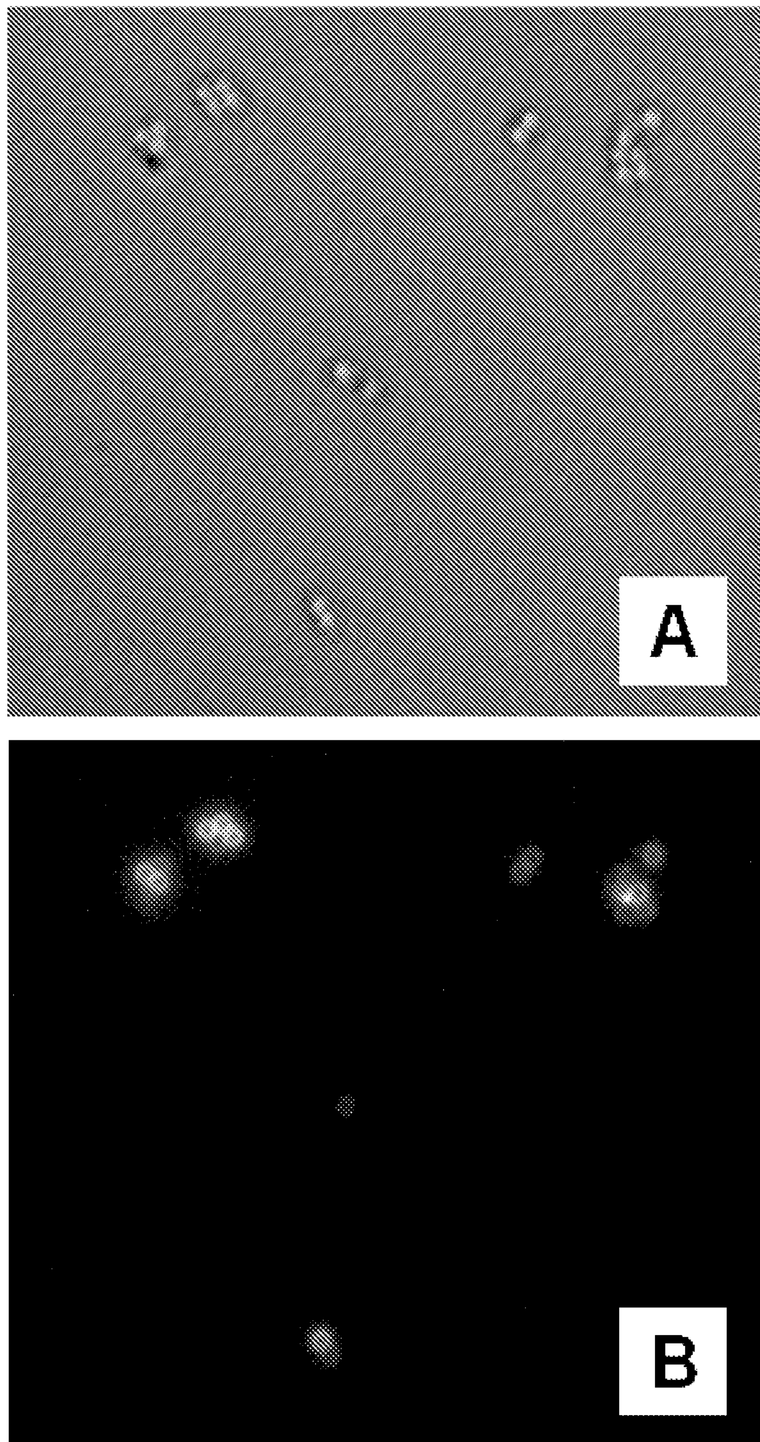


Figure 6

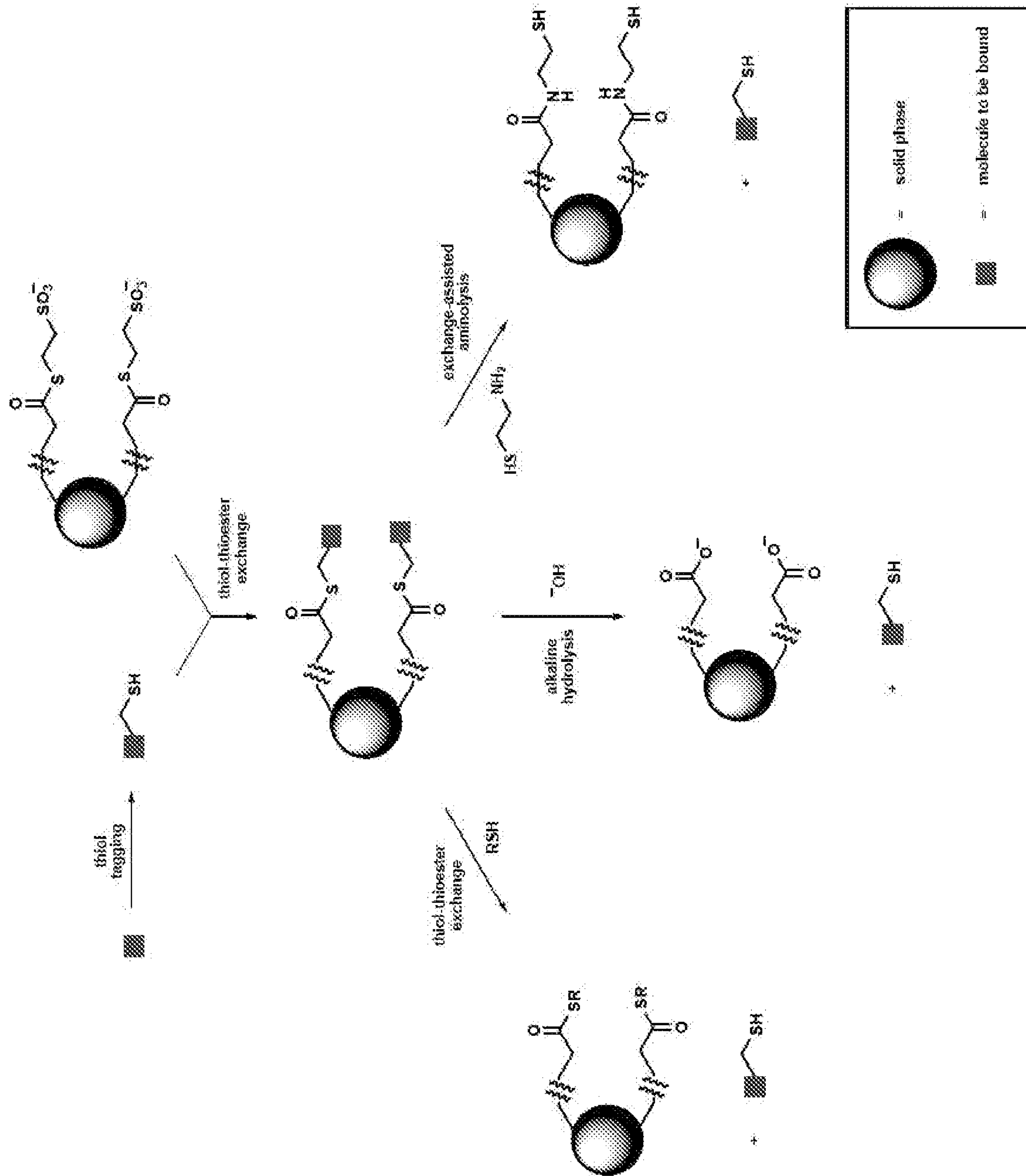
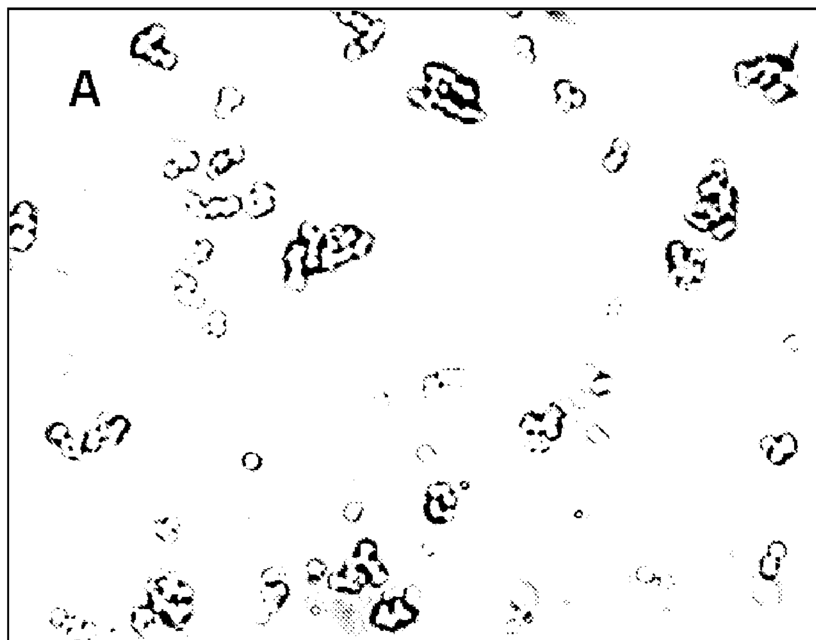


Figure 7



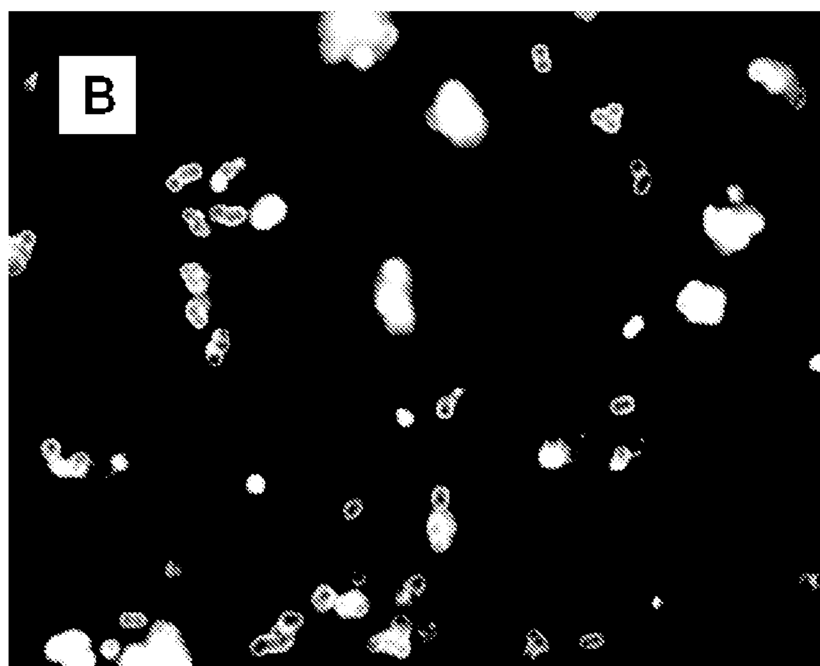


Figure 8

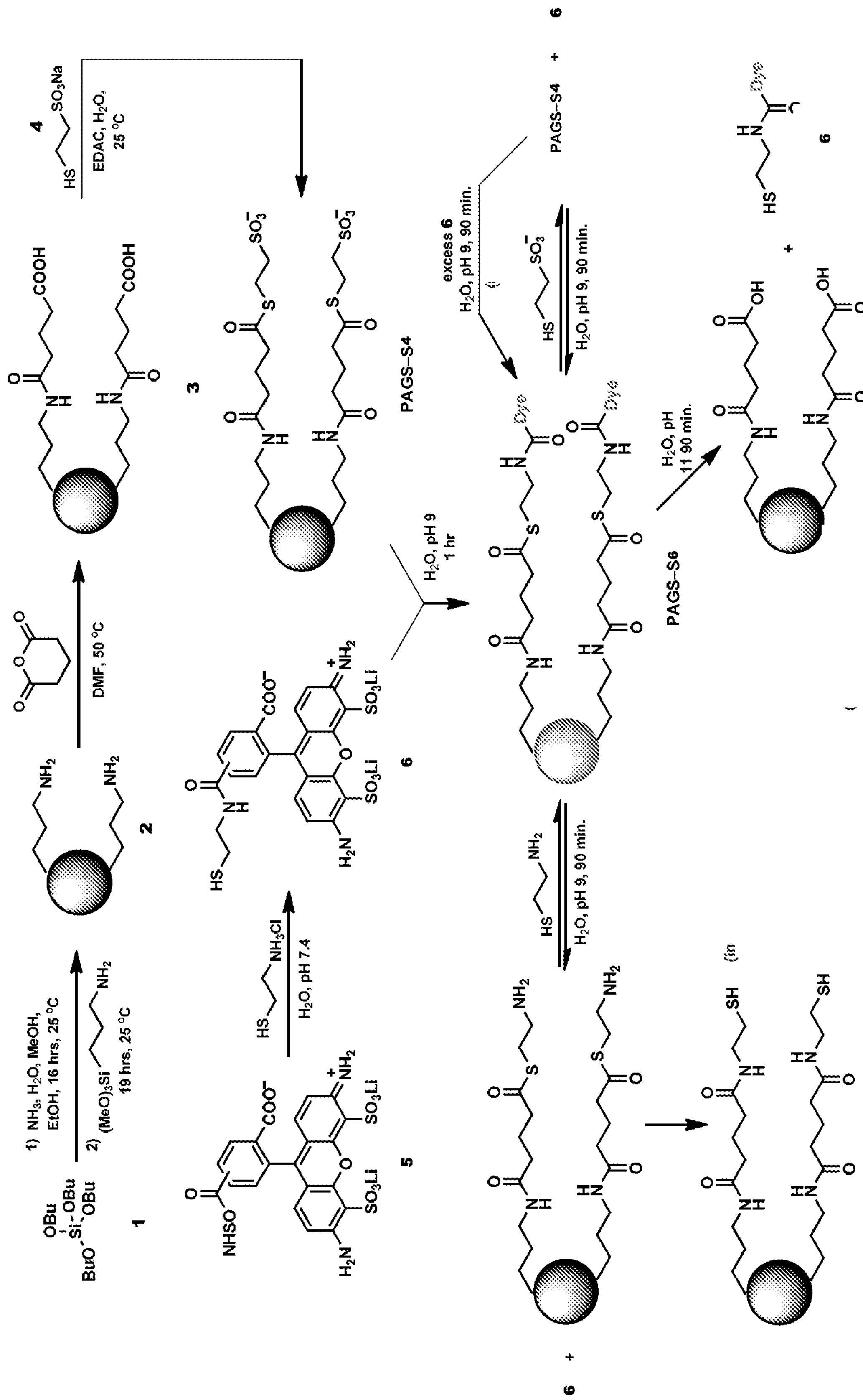


Figure 9

INTERNATIONAL SEARCH REPORT	International application No. PCT/US 11/56165

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 17/00 (2012.01)
USPC - 530/408, 345
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8)- C07K 17/00 (2012.01);
USPC- 530/408, 345

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Patents and NPL (classification, keyword; search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWest (US Pat, PgPub, EPO, JPO), GoogleScholar (PL, NPL), FreePatentsOnline (US Pat, PgPub, EPO, JPO, WIPO, NPL); search terms: covalent, attach, tether, bind, bond, thioester, thiolester, functional, group, substrate, promote, metal, Lewis, salt, ion, Ag, Hg, Pb, Tl, Au, thiophenol, benzyl mercaptan, ethanethiol, mercaptoethanol...

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2007/0082019 A1 (HUANG et al.) 12 April 2007 (12.04.2007), Fig. 2; arpara [0015]-[0018], [0025], [0080], [0084], [0093]-[0096], [0113], [0120], [0136], [0143], [0145], [0151], [0204], [0231], [0245], [0306]	1, 3, 12-14, 17-19 -------- 2, 33-36
X -- Y	US 2006/0024808 A1 (DARZINS et al.) 02 February 2006 (02.02.2006), para [0007], [0008], [0026], [0028], [0151], [0154], [0226], [0233]	32 -------- 2, 33-36
Y	US 2009/0104435 A1 (HUTCHISON et al.) 23 April 2009 (23.04.2009), para [0046]-[0070]	1-3, 12-14, 17-19, 32-36
Y	US 2005/0042623 A1 (AULT-RICHE et al.) 24 February 2005 (24.02.2005), para [0012]-[0032], [0250], [0389], [0393], [0394], [1037]	1-3, 12-14, 17-19, 32-36

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 January 2012 (17.01.2012)	31 JAN 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/56165

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-11, 15, 16, 20-31, 37, and 38
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.