Title: POLYMER SUPPORTED REAGENTS FOR NATURAL PRODUCTS PURIFICATION

Abstract: There is provided a method for the purification of bio-molecules after chemical modification using one or more insoluble polymer supported reagents. More particularly, the present invention provides a method for the fast purification of labeled bio-molecules using insoluble polymer supported quenching reagents. There are also provided novel polymer supported reagents of the formula (I): Rs-Sp-Re, wherein Rs is an insoluble polymer, Re is one or more reagent residues and Sp is one or more chemically robust spacer that join Rs and Re; and a process for their preparation.
Polymer supported reagents for natural products purification

Labeling of bio-molecules with a series of tags, such as biotinylating and/or fluorescent tags is a frequently used method in biochemical and biophysical laboratories. These compounds are useful for different applications, such as for example for assays or binding studies.

Since the purity of the desired product is important for the quality and the reliability of the subsequent applications, a series of purification steps, such as gel-filtration, ion exchange chromatography or reverse-phase RP-HPLC, must be done after the labeling of the bio-molecule. Nevertheless, during these steps a pure product with a high recovery cannot often be achieved.

In the field of the combinatorial chemistry the introduction of polymer-supported scavengers opened the possibility of a simple and efficient method to facilitate the purification of a desired product from un-reacted reagents and/or byproducts. Once reacted with the solid support, it can be removed from the reaction mixture, e.g. by filtration or extraction. A series of applications of scavengers bound on a solid support and their application in the practice of organic synthesis, combinatorial synthesis and automated organic synthesis have been developed. Examples of the solid support are polystyrene resins, Merrifield resins, polyamine resins, poly(styrene-divinylbenzene) / poly(ethylene glycol) grafted copolymer. These resins exhibit a significant stability towards organic solvents and temperature and pressure. Some examples are described in WO-A-9742230 and in the following references: Thompson L. A. Curr. Opin. Chemical Biol., 2000; 4: 324-337; Wentworth Jr., P. Trends Biotechnol., 1999, 17: 448-452.

Protein purification by covalent chromatography is also known; the desired product is extracted from a crude reaction mixture by its selective reaction with a polymeric support, followed by filtration and rinsing. In a second step the protein can be recovered by cleavage from the resin. The polymeric support in the covalent chromatography must react with the desired product in a reaction mixture in the presence of other (undesired) compounds and the bound product has to be cleavable and retrieved from the polymer.

The present invention provides a method for the purification of bio-molecules after their chemical modification using one or more insoluble polymer supported reagents. Said polymer supported reagents are put in contact with the mixture containing the desired chemically modified bio-molecule in order to react covalently with excess of reagents...
and/or unwanted byproducts. Bio-molecules are substances originated by living organisms. They can be proteins, peptides, DNA, RNA, lipids, small molecules, all originated by living organisms. The polymer supported reagent reacts with byproducts or reactants, leaving the desired pure product in solution.

The use of polymer supported reagents (also named scavenger resins) provides a quick and simple way of enhancing the purity of chemically modified bio-molecules. The purification is carried out by incubating the polymer supported reagents with the mixture containing the desired bio-molecule and undesired byproducts and educts and removing the solid support. The solid support after incubation can be filtered from the solution or separated from the solution by aspiration.

These scavenger resins can be used packed in columns or in a batch system. The incubation and the separation of the reaction mixture from the insoluble polymeric support can be repeated one or more times. Preferably, the method of purification of this invention is performed under non-denaturing conditions. The bio-molecule maintains its three dimensional structure during the treatment with the scavenger resin. Therefore it does not have to be re-natured in a second step. The method of the present invention can be applied as an alternative and/or in combination with other purification steps, like ion exchange chromatography, affinity chromatography and gel filtration. Therefore, the invention also comprises the combination with other purification material such as ion-exchange resin; gel-filtration resin; NH₂-reactive resins, (such as Actigel B, N-hydroxy succinimide esters activated columns, CNBr activated columns, Epoxy-activated columns, Carbonyl diimidazole activated columns, but not limited to these resins); SH-reactive resins (such as Iodoacetyl, Bromoacetyl, maleimide, pyridyl disulfide columns, but not limited to it) chelating resins, desalting columns, reverse-phase adsorbants. The invention comprises the incubation of the reaction mixture with the scavenger resins combined with no, one or more of the other purification materials in one or in more subsequent steps.

A further embodiment of this invention includes the automatic performance of the process and the setup for a parallel purification array. Preferably, high concentrations of the reacting groups are present on the solid support, so an addition of a small amount of polymer is required.
In particular, the present invention is relating to a method for the fast purification of labeled bio-molecules using an insoluble polymer supported quenching reagent. The labeling of the bio-molecules can be, for example, biotinylation or the introduction of fluorescent tags.

Therefore, in a preferred embodiment, the present invention provides a method of removing biotinylating and/or fluorescent agent from a reaction mixture containing labeled bio-molecules, such as for example proteins, protein mixtures and/or peptides, which are either obtained by cellular expression or by chemical or enzymatic cleavage from proteins.

As stated above, this invention is addressed to the improvement of the purity of a modified bio-molecule by trapping unwanted byproducts and/or un-reacted starting material. Bio-molecules can be proteins, peptides obtained by enzymatic and/or chemical cleavage from proteins and/or produced by cellular systems. These proteins and peptides can contain post-translational modifications, such as phosphorylations, glycosylations, myristoylations and palmitoylations. The bio-molecules can also be DNA, RNA, oligonucleotides, lipids, nucleotides, nucleosides, phospholipids, carbohydrates, small molecules (like NAD, NADH, ATP, ADP, AMP, GTP, GDP, GMP, but not limited to it) originated from living organisms.

The chemical modification of these bio-molecules can be the tagging with biotinylating or fluorescent agents. The biotinylating and fluorescent agents contain three parts: biotin and/or the fluorescent tag, no, one or more linkers and a chemical group, which permits the binding to the bio-molecule. The linker is expected to be chemically robust at the conditions the binding and quenching step is carried out. It is generally composed from CH₂-CH₂; CH₂-CH; CH-CH₂; CH=CH; CH=N; CH₂-N; CH=O; CH₂-O; CH-S; CH₂-S; CD₂-CD₂; CD₂-CD; CD-CD₂; CD=CD; CD=N; CD₂-N; CD-O; CD₂-O; CD-S; CD₂-S C(=O)-N; N-C(=O); C(=O)-O; O-C(=O); O-C(=O)-N; N-C(=O)-O; N-C(=O)-N; O-C(=O)-O and combinations thereof. (D is equal deuterium). The group reacting with the bio-molecule can be iodoalkyl, bromoalkyl, maleimido, dithiopyridine, disulphides, isothiocyanat, succinimidyl esters, sulphonesuccinimidyl esters, aldehydes, ketones, dichlorotriazines, diazoles carboxylic acids, sulphonyl chlorides, acyl azides, acyl nitriles, acid chlorides, amino groups, hydrazines, hydrazides, hydroxylamines, alcohol, carbodiimides and combinations thereof.
The fluorescent tags include all molecules, which are able to emit photons after having been excited by light or heat. Examples are fluorescein, eosin, dinitrophenyl, naphthalene and substituted naphtalenes (such as dansyl), coumarin, [Ru(bipy)_3]^{2+}, BODIPY® fluorophores, rhodamine, Texas Red™, Indocarbocyanine (Cy3) or indodicarbocyanine (Cy5), Alexa Fluor®, Nile Red, allophyocyanine, Oregon Green®, indotricarbocyanine (CY7), Europium trisbipyridine cryptate, N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl].

The method of the present invention is particularly attractive for the nanotechnological approach, owing to two characteristics. It is a simple one step approach, in which the desired product does not have to bind or interact with a solid support. Second, high concentrations of the quenching groups on the solid support allow the addition of a small amount of quenching polymer.

The insoluble polymer supported reagents to be used in the present purification method are consisting of a polymer bearing at least one reagent residue. Preferably, the reagent residue is selected from the group consisting of thiol (-SH), hydroxyl (-OH), carboxyl, formyl, keto (COOH, CH=O, C=O), guanidino, amino groups and derivative thereof (NR_1R_2 wherein R_1 and R_2 independently represent hydrogen atom, C_1-C_{12} alkyl or aryl group, NR_1R_2, OH), azlactone. Preferably, at least 0.01 mmoles of reagent residues, more preferably at least 0.05 mmoles, are covalently bonded or immobilized on 1 gram of resin. In general, there are two synthetic strategies by which the preferred high loading of quenching functionality on polymeric supports is achieved. In the first strategy, a polymer with existing functionalities of greater than 0.01 mmol per gram of polymer is chemically modified to give a novel polymer-supported quenching reagent which has greater than 0.01 mmol of quenching functionalities per gram of polymer. In the second strategy, polyfunctional or dendritic molecules bearing connecting functional groups and two or more quenching functional groups are attached to polymers with less than 2 mmol of attachment sites per gram of polymer. In this manner, the number of quenching sites is amplified compared to the number of attachment sites. One required characteristic of the polymers to be used according to the present invention is the insolvability. The term insolubility means that the polymer does not dissolve in the solvent, wherein the purification reaction is carried out, such as water, but also water/organic solvent mixtures and organic solvents. For example, the polymeric support can
be built up by cross-linked polystyrenes, polyacrylamides and polyacrylates, poly(styrene-divinylbenzene), poly(styrene-divinylbenzene/poly-(ethylene glycol) copolymer (known also as Tentagel® resin). Of course, also other polymeric supports known in the art can be conveniently used in the present invention, such as for example polyamide, polyamide/polyethylene glycol copolymer, polysulfone, latex, polyester, paper, polypropylene, polyethylene, nitrocellulose. Tentagel is a trade mark of Rapp Polymere GmbH. Tentagel resins are grafted copolymers consisting of a low cross linked polystyrene matrix on which polyethylene glycol (PEG or POE) is grafted.

Another classes of polymeric supports are polysaccharide resins like Sepharose, agarose (non cross-linked and cross-linked), cellulose, polysaccharides copolymerized with acrylamide (for example N,N'-methylene-bis(acrylamide), N-acryloyl-2-amino-2-hydroxymethyl-1,3-propane diol, but not limited to these two compounds). Even though these last compounds do not show such a high stability as those above, they keep being the mostly used resins in the purification procedures for proteins.

The reagent residue may be linked to the polymeric supports by means of a spacer. The spacer is chemically resistant at the conditions in which the purification or quenching step is carried out, and is generally of the formula -CH₂-CH₂--; -CH₂-CH--; -CH-CH--; -CH=CH--; =CH-N--; -CH₂-N--; -CH-O--; -CH₂-O--; -CH-S--; -CH₂-S--; C(=O)-N--; -N-C(=O)-O--; -C(=O)-O--; -O-C(=O)-; -O-C(=O)-N--; -N-C(=O)-O--; -N-C(=O)-N--; -O-C(=O)-O or combinations thereof, and the like. A spacer can be linked to one or more reagent residues, and can also be linked to another spacer, of the same formula or different. The insoluble polymer supported reagents to be used in the method of this invention may be prepared by converting a polymeric starting material into a polymer supported reagent in one to four synthetic steps, rinsing thoroughly with one or more solvents after each synthetic step, for example as described in WO-A-9742230. The preparation of the polymer supported reagents starts from known polymers. Polymer supported reagents are made in one to four synthetic steps from readily available starting materials, such as for example, insoluble polymers or derivatives thereof which contain convenient linker functionality, and one or more polyfunctional reagents which bear a compatible connecting functionality and one or more functionalities used in the purification or quenching reaction process.
The preferred polymeric starting materials are well-known to those skilled in the art of solid-phase peptide or solid-phase organic synthesis. The present invention also provides polymer supported reagent of the formula I:

\[
\text{Rs-Sp-Re} \quad \text{I}
\]

wherein Rs is insoluble polymer selected from polysaccharide resins, cellulose, polysaccharides copolymerized with acrylamide; Re is one or more reagent residues that are capable of selective covalent reaction with unwanted byproducts, or excess reagents; and Sp is one or more chemically robust spacer that join Rs and Re. Preferably, the reagent residue Re is selected from the group consisting of thiol, hydroxyl, carboxyl, formyl, keto, guanidino, amino groups and derivative thereof as defined above, azlactone and the spacer Sp is as defined above. It is a further object of the invention a process of preparing a compound of the formula I as defined above, which comprises conversion of a polymeric starting material Rs as above defined into a compound of Formula I in one to four synthetic steps, rinsing thoroughly with one or more solvents after each synthetic step.

The starting materials are well-known to or those skilled in the art of purification of biomolecules. They are commercially available or are known in the scientific literature.

A method which affords novel polymer supported reagents are described in the following schemes, wherein Rs and Sp are as defined above:

\[
\text{Rs-Br + NH}_3 \text{ or H}_2\text{N-Sp-NH}_2 \rightarrow \text{Rs-NH}_2 \text{ or Rs-NH-Sp-NH}_2
\]

\[
\text{Rs-Br + HS-Sp-SH} \rightarrow \text{Rs-S-Sp-SH}
\]

Specific methods which afford selected examples of most preferred polymer-supported quenching reagents are illustrated in Examples 1 and 2. The following examples illustrate the invention without limiting it:

**Description of Actigel B resin:**

<table>
<thead>
<tr>
<th>Specifications</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose bead conc.</td>
<td>6%</td>
</tr>
<tr>
<td>Molecular exclusion limit:</td>
<td>4 million Daltons</td>
</tr>
<tr>
<td>Bead size:</td>
<td>60-160 μm</td>
</tr>
<tr>
<td>Spacer:</td>
<td>7 atoms, hydrophilic</td>
</tr>
</tbody>
</table>
Example 1
Preparation of the Actigel B-NH₂ resin

Matrix-O-spacer-O-CH₂-CH(OH)-CHBr + H₂N-CH₂-CH₂-NH₂ -->

Matrix-O-spacer-O-CH₂-CH(OH)-CH₂-NH-CH₂-CH₂-NH₂

Actigel B resin (1 ml, 150 mmole/l) was washed for 3 times with 0.1 M K₂CO₃ and incubated with 333 µl of ethylenediamine (300 mg, 5 mmole) in 4.5 ml of 0.1 M K₂CO₃ overnight at 4°C.

The resin was washed with 0.1 M K₂CO₃ (3 x 5 ml), PBS (saline phosphate buffer, 3 x 5 ml) and finally with PBS/20% ethanol (3 x 5 ml). For storage the resin was kept in a solution of PBS/ethanol (4:1, v:v).

Example 2
Preparation of the Actigel B-SH resin

Matrix-O-spacer-O-CH₂-CH(OH)-CHBr + HS-CH₂-CH₂-CH₂-SH -->

Matrix-O-spacer-O-CH₂-CH(OH)-CH₂-S-CH₂-CH₂-CH₂-SH

Actigel B resin (1 ml, 150 mmole/l) was washed for 3 times with 0.1 M K₂CO₃ and incubated with 500 µl propanedithiol (540 mg, 5 mmole) in 4.5 ml 0.1 M K₂CO₃ overnight at 4°C.

The resin was washed with 0.1 M K₂CO₃ (3 x 5 ml), PBS (3 x 5 ml) and for storage with PBS/20% ethanol (3 x 5 ml).

Example 3
Validation of the Actigel B-NH₂ resin

60 µg of fluorescein-5(6)-carboxamido-caproic acid-N-hydroxy succinimide ester (0.10 mmole) were dissolved in 6 µl DMF, diluted with 100 µl PBS and incubated with 2, 10 and 20 µl Actigel B-NH₂ resin for 90 minutes to overnight at 4 °C. Finally the solid support is filtered and washed with PBS. The amount of the not bound fluorescein can be established by fluorescence spectroscopy.

Example 4
Validation of the Actigel B-SH resin
70 μg of fluorescein maleimide (0.16 μmoles) were dissolved in 7 μl DMF, diluted with 100 μl PBS, pH=7.2, and incubated with 5, 20 and 50 μl Actigel B-SH resin for 90 minutes to overnight at 4 °C. Finally the solid support was filtered and washed with PBS. The amount of the not bound fluorescein can be established by fluorescence spectroscopy.

Example 5
Usage of the resins for purification:
40 μg of a mixture of casein proteins, dissolved in 40 μl NH₄HCO₃ 50mM, pH=8, were incubated with 20 μl of a solution containing 20 μg of EZ-Link PEO Biotin (Pierce) in NH₄HCO₃ 50mM, pH=8, for 2 hours at 37°C. After that, 10 μl Actigel B-SH resin were added to the reaction mixture and the incubation carried out for another 30-60 minutes. The resin was removed by filtration or spinning down. The resin was washed twice with 10 μl NH₄HCO₃ 50mM, pH=8 and the washing solution was added to the protein solution. In order to validate this approach and to check the efficiency of the quenching, the supernatant solution was injected in a HP1090 separation system using a RP-C4 column (Vydac 4.6x250 mm, 300Å pore size, 7.5 μ). The efficiency was monitored by comparing the absorption of the traces at wavelengths of 220 and 280 nm. A reduction of 90% of EZ Link PEO-Biotin could be observed, whereas the recovery of the protein was >90%.
Claims

1. A method for the purification of bio-molecules after chemical modification using one or more insoluble polymer supported reagents.

2. A method according to claim 1 for the fast purification of labeled bio-molecules using an insoluble polymer supported quenching reagent.

3. A method according to claim 2 in which the bio-molecule was labeled by biotinylating or by introducing fluorescent tags.

4. A method according to claim 3 of removing biotinylating and/or fluorescent agent from a reaction mixture containing proteins, protein mixtures and/or peptides, which are either obtained by cellular expression or by chemical or enzymatic cleavage from proteins.

5. A method according to claim 1 in which polymer supported reagents are put in contact with the mixture containing the desired chemically modified bio-molecule in order to react covalently with excess of reagents and/or unwanted byproducts.

6. A method according to claim 1 in which the bio-molecules are proteins, peptides, DNA, RNA, lipids or small molecules, all originated by living organisms.

7. A method according to claim 1 which is carried out by incubating the polymer supported reagents with the mixture containing the desired bio-molecule and undesired byproducts and educts and removing the solid support by filtration or aspiration.

8. A method according to claim 1 in which the polymer supported reagents are packed in columns or in a batch system.

9. A method according to claim 1 or 2 that is carried out repeating one or more times the incubation and the separation of the reaction mixture from the insoluble polymeric support.

10. A method according to claim 1 or 2 that is carried out under non-denaturing conditions.

11. A method according to claim 1 or 2 that is carried out as an alternative and/or in combination with other purification steps.
12. A method according to claim 1 or 2 in which the polymer supported reagents are characterized from high concentrations of the reacting groups present on the solid support.

13. A method according to claim 1 or 2 which is carried out in the nanotechnological approach.

14. A method according to claim 1 or 2 in which the polymer supported reagents are consisting of a polymer bearing at least one reagent residue, said reagent residue being selected from the group consisting of thiol, hydroxyl, carboxyl, formyl, keto, guanidino, amino groups and derivative thereof and azlactone, and with at least 0.01 mmoles of said reagent residues covalently bonded or immobilized on 1 gram of resin.

15. A method according to claim 1 or 2 in which the polymer supported reagents are built up by cross-linked polystyrenes, polyacrylamides and polyacrylates, poly(styrene-divinylbenzene), poly(styrene-divinylbenzene/poly- (ethyleneglycol) copolymer, polysaccharide resins like Sepharose, agarose (non cross-linked and cross-linked), cellulose, polysaccharides copolymerized with acrylamide.

16. A method according to claim 1 or 2 in which the polymer supported reagents are characterized by a spacer linking the reagent residues to the polymeric supports, said spacer being chemically resistant at the conditions in which the purification or quenching step is carried out.

17. A method according to claim 16 in which the spacer is of the formula

\[ -CH_2-CH_2-; -CH_2-CH-; -CH=CH_2; -CH=CH-; =CH-N=; -CH_2-N; \]
\[ -CH-O-; -CH_2-O-; -CH-S-; -CH_2-S-; -C(=O)-N-; -N-C(=O)-; -C(=O)-O-; \]
\[ -O-C(=O)-; -O-C(=O)-N-; -N-C(=O)-O-; -N-C(=O)-N-; -O-C(=O)-O \]

or combinations thereof, and the like.

18. A method according to claim 16 or 17, in which the spacer is linked to one or more reagent residues, and/or is linked to another spacer, of the same formula or different.

19. A polymer supported reagent of the formula I:

\[ \text{Rs-Sp-Re} \quad I \]

wherein Rs is an insoluble polymer selected from polysaccharide resins,
cellulose, polysaccharides copolymerized with acrylamide; Re is one or more reagent residues that are capable of selective covalent reaction with unwanted byproducts, or excess reagents; and Sp is one or more chemically robust spacer that join Rs and Re.

20. A polymer supported reagent according to claim 19 wherein Re is selected from the group consisting of thiol, hydroxyl, carboxyl, formyl, keto, guanidino, amino groups and derivative thereof and azlactone as defined in claim 14, and the spacer is as defined in claim 17.

21. A process of preparing a compound of the formula I as defined in claim 19, which comprises conversion of a polymeric starting material Rs as defined in claim 19 into a compound of the formula I in one to four synthetic steps, rinsing thoroughly with one or more solvents after each synthetic step.