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Convenient supported recyclable material based on dihydrolipoyl-residue for the reduction of disulfide derivatives

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ABSTRACT

A quantitative method for the reduction of disulfides, which uses a totally recyclable solid phase supported reducing agent, is reported. D,L-α-Lipoic acid was quantitatively condensed on a highly stable 100% PEG Aminomethyl-ChemMatrix® resin that can swell in aqueous media as well as in organic solvents. Lipoic residue, subsequently reduced to its dihydrolipoyl form, was utilized as a reducing agent for highly valuable disulfide compounds.

1. Introduction

The thiol-disulfide redox system is a key regulatory process in biological systems[1] and plays an important role in organosulfur chemistry.[2] This system is largely exploited in nanotechnology[3] for drug and gene delivery.[4–7] For example, formulation of stable nanometric monomolecular DNA complexes was obtained via thiol detergent dimerization into disulfide lipid on the DNA matrix. Conversely, the reduction of disulfide into thiol in intracellular medium (which is a reducing medium) was exploited for facilitating the gene release into the cytoplasm from the cell-internalized DNA nanoparticles. This redox system was also extensively used for the surface modifications of liposomes, vesicles and nanoparticles used as delivery vehicles with inert PEG groups ('stealth' effect) and with specific ligands for achieving targeted delivery.

Reduction of disulfides into free thiols can be achieved using a large variety of reagents, including among others organic thiols or phosphines. In many cases, the experimental conditions are not always compatible with the stability of the compounds to be reduced, and/or purifications are needed for further investigations.[2] The reduction of disulfides by action of thiols (such as mercaptoethanol or thioglycolic acid) is more particularly adapted for highly valuable products.[2,8] However, for performing this thiol-disulfide exchange reaction, these reagents must be used in a large excess because of the equilibrium constant (close to one) for the interchange reaction between disulfides and thiols.[8] To bypass this stoichiometric concern, dithiothreitol (DTT) among the thiols is of special interest.[9] This derivative exhibits a high reducing potential and is able to maintain thiol under its reduced form thanks to the low redox potential of the very stable six-membered dithiolane, which is formed upon DTT oxidation. It should be mentioned that, whatever the method utilized for performing disulfide reduction, removal of the remaining reducing agent as well as its oxidized state can then be cumbersome. Besides, it often results in partial recovery due to the re-oxidation that may occur during the whole process. To overcome these problems, strategies involving solid phase supported reducing agents were developed.[10–15] Nevertheless, these strategies suffer also from diverse drawbacks consisting either in the poor reducing capacities of the polymer used as the solid support, and/or into its poor swelling abilities regarding different solvents as well as into the non-recyclable supported reducing agents.[10–15] Indeed, the supported reducing agents rely on dihydrolipoyl,[10] phosphine,[13] or on (boro)hydride residues.[14] The phosphine-based resin is the sole one, which is commercially available. However, the fact that the final oxidized supported-species cannot be regenerated easily is a serious drawback of this resin.[13] The poly(4-vinylpyridine) resin supporting zirconium borohydride can only be used with hydride-compatible solvents (THF, ether).[14] Moreover, zirconium tetrahydroborate, which is used for the preparation of the active resin, is a highly volatile solid that decomposes at room temperature. Furthermore, it is inflammable in air and hydrolyzes explosively. Advantage is nevertheless the possibility of recycling the supported reagent. The sole solid phase reducing system being highly and easily recyclable was constructed with the dihydrolipoyl moiety, for example, on the dihydrolipoyl(DHLA)-lipoyl (LA) redox system.[10,12] Next, to the best of our knowledge, the published DHLA-supported solid-phase systems rely

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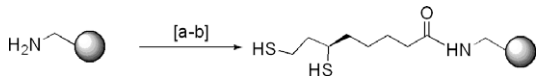


Scheme 1. (a) Coupling step: lipoic acid, DIC/HOBt, CH_2Cl_2 (2×), filtration, washing CH_2Cl_2 (5×). (b) Reduction step: $NaBH_4$ (2×), washing with water (2×), 0.1 M acetic acid (2×), EtOH (2×) then drying.

either on Sephadex,[10] Sepharose,[10,15] polyacrylamide,[10] cellulose resins[10] (for reactions in aqueous media) or on a poly(4-vinylpyridine)[14] resin (for reactions in organic solvents). However, none of these systems can be used in both hydrophilic and apolar solvents.

We describe herein a very simple one-pot, two-step synthesis of a dihydrolipoyl-supported polymer starting from the commercially available Aminomethyl-ChemMatrix® (Scheme 1). This new 'di-thiol' material fulfil the required specifications, that is, (i) high reducing abilities owing to the formation of a stable dithiolane moiety upon oxidation, (ii) good swelling properties in a wide range of solvent composition (aqueous or organic) for a high loading capacity (e.g., up to 1 mmol/g such as conventional PS-based resins;[16] for comparison, Sephadex, Sepharose or cellulose resins once chemically modified to exhibit aminoethyl residues, possess lower capacities, which are in the 0.36–0.29 mequiv/g range), (iii) easy work-up that allows complete recovery of highly valuable thiols from the reduction of the corresponding disulfide substrates without any additional purification steps and (iv) possibility to regenerate quantitatively the di-thiol reducing polymer. The ease of implementation of this procedure as well as its versatility should render this strategy routinely applicable for most biochemists and biologists.

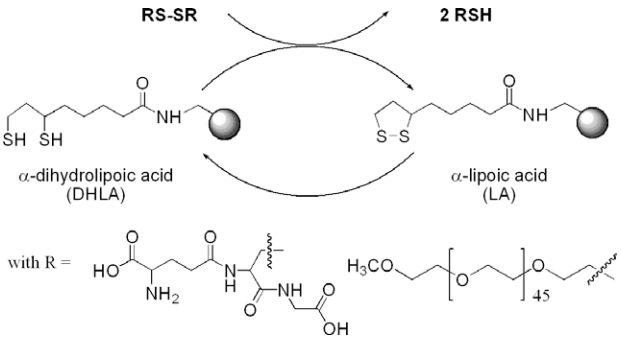
2. Preparation of the resin-supported lipoyl redox

The lipoyl polymer supported conjugate was prepared in a one-pot, two-step procedure (Scheme 1; coupling and reduction step, see Ref. 17). The coupling step was performed by reacting lipoic acid with the PEG Aminomethyl-ChemMatrix® resin using diisopropyl-carbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) as the coupling agent.

This coupling step was repeated twice and completion of the reaction was monitored with Kaiser's test (negative).[18] At this stage, the lipoyl-grafted resin can be conserved indefinitely if kept dry. Reduction of the 1,2-dithiolane ring to generate the potent dihydrolipoyl reductor was accomplished by running two subsequent reduction steps with $NaBH_4$. To prevent from re-oxidation, the resin was acidified with 0.1 M acetic acid. The thiol content determined by Ellman's test was shown to be quantitative (UV titration at 410 nm, data not shown).[19] The reduced di-thiol form of the resin can be conserved for months after EtOH washing and if kept dried in an appropriate dioxygen-free atmosphere. However, reduction should be preferably performed just prior use for better results.

3. Reduction potential of the supported dihydrolipoyl reducing agent

To set up a protocol, as simple as possible, the possibility of using highly volatile buffers was investigated in order to perform the total disulfide reduction that could result into a simple filtration-evaporation step procedure. We thus established two procedures, one for a 100% aqueous environment with ammonium formate buffer and the other running in a 100% organic solvent supplemented with diethylamine. Disulfides to reduce were chosen regarding practical interest for our group but also and more generally for chemists and biochemists. Thus as examples, a small peptide (oxidized glutathione) and methoxypolyethylene glycol



Scheme 2. (a) DHLA-polymer supported reducing agent (15 equiv), formate buffer pH 7.5; (b) filtration, washing and aporation.

disulfide ($[MeOPEG\text{-}S]_2$; $n = 45$) were chosen (Scheme 2). The whole process is described in Refs. 20 and 21.

The optimal conditions were established for the reduction of oxidized glutathione. Freshly regenerated DHLA-polymer supported reducing agent was used in all cases. Completion of the reaction was performed using Ellman's tests by monitoring the UV absorbance at 410 nm. Results showed that for 15 equiv of DHLA-polymer supported reducing agent the reaction was completed in 50 min. To check for an eventual re-oxidation, the thiol content of the sulfide compound obtained was measured again after its recovering. In all cases tested, the thiol content was higher than 98%. Recycled resin obtained after subsequent $NaBH_4$ reduction procedure as mentioned in Scheme 1 was tested and re-used in ten consecutive runs without showing any obvious loss of activity.

Concerning the reduction of $[MeOPEG\text{-}S]_2$ into MeOPEG-SH, it could be done in DMF as well as in acetonitrile. In the latter case, for completion, the DHLA matrix had to be used in larger excesses (30 equiv instead of 15 in DMF).

In conclusion, an efficient, stable and 100% recyclable PEG matrix supporting the dihydrolipoyl residue as the reducing agent was synthesized in a one-pot, two-step procedure. Reduction of disulfide substrates proceeds quantitatively affording the targeted thiols without any purification step. This reduction can be performed either in 100% aqueous volatile buffers or in 100% organic solvents. This very simple protocol can be utilized by researchers that routinely work with cystine containing proteins or organic disulfide and seek for the total recovery of the targeted reduced thiols without setting up heavy strategies.

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20. *Reduction of γ-L-glutamyl-L-cysteinylglycine (aqueous protocol)*: oxidized glutathione (10 mg, 0.016 mmol) was solubilized in 0.1 M ammonium formate solution (pH 7.5). Then excess (15 equiv, 331 mg, 0.245 mmol) of freshly regenerated DHLA-resin previously swollen into buffer was added and the mixture was stirred for 50 min at room temperature. Resulting reduced thiol was isolated upon a simple filtration of resin and it was washed with ammonium formate followed by evaporation. 99.9% of reduction content was established by UV quantification at 410 nm (Ellman's test).[19]
21. *Reduction of methoxypolyethylene glycol disulfide [MeOPEG-S]$_2$ (organic solvent protocol)*: freshly prepared DHLA-resin (20 equiv, 67 mg, 0.050 mmol) was added to [MeOPEG-S]$_2$ (10 mg, 0.0025 mmol) and diethylamine (3 μL, 0.0065 mM) in DMF (5 mL), and the mixture was stirred for 1 h at room temperature. The resin was filtered off and washed with DMF (2 × 5 mL). The MeOPEG-SH was recovered by evaporation under reduced pressure (98.7% of sulfhydryl content).