A novel organic solvent-based coupling method for the preparation of covalently immobilized proteins on gold

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Abstract

A novel organic solvent-based coupling method has been developed for the covalent immobilization of biological material to gold surfaces. The method employs the polar organic solvent anhydrous 2,2,2-trifluoroethanol as the reaction medium and involves dissolution of the protein (catalase) in the solvent allowing protein coupling to proceed under basic conditions in a dry organic environment. The advantage of this method is that protein attachment is favored over hydrolysis of the coupling reagent. We have shown qualitatively and quantitatively that following attachment to the gold surface a significant proportion of the enzyme catalase remains catalytically active (at least 20–31%).

Keywords: catalase; immobilization; protein; organic solvent; self-assembled monolayer

The recent interest in the development and production of advanced biosensors, bioreactors, and chromatographic separation materials has resulted in significant effort being directed towards the investigation of both effective and non-destructive methods for immobilizing biological material at interfaces. These areas of research require the ability to rigidly anchor biomolecules to an inert support under non-denaturing conditions. It is also desirable that homogeneous surface coverage is achieved. Following the work of Nuzzo and Allara (Nuzzo and Allara, 1983) showing that dialkyl-disulphides form oriented monolayers on gold surfaces, the phenomena of molecular self-assembly, in which thiol compounds adsorb spontaneously onto metallic surfaces to form self-assembled monolayers (SAMs), has been an area of intense research for molecular immobilization (Bain et al., 1989a, 1989b; Biebuyck et al., 1994). The SAMs are formed spontaneously by the immersion of a gold surface into a solution of a thiol dissolved in an appropriate solvent. Through variation of the thiol compounds terminal group the surface properties exhibited by the SAM may be modified. For example, functionality may be introduced that allows the SAM to be coupled to a range of biomolecules (Leggett et al., 1993). However, despite the attraction of this approach low surface coverage may result from a combination of factors including the rapid hydrolysis of coupling reagents in aqueous media.

In this study we have developed a SAM biomolecular immobilization strategy to provide a high degree of protein surface coverage. The main advantage of the described method is the use of an organic solvent as the reaction medium; such a dry environment allows synthesis to be favored over hydrolysis of the coupling agent, resulting in a high degree of protein coupling.

Results and discussion

SPDP is a bifunctional cleavable crosslinker (Carlsson et al., 1978) containing a *N*-hydroxysuccinimide ester and a 2-pyridyl disulphide group. As shown in the reaction scheme (Fig. 1), a SAM of SPDP is first formed on the gold surface through the disulphide, forming a strong dative bond with the gold surface (Nuzzo and Allara, 1983; Biebuyck et al., 1994). This results in the creation of a two-dimensional array of the SAM (Bain et al., 1989a, 1989b). Following immobilization, the hydroxysuccinimide ester reacts with primary amino groups on proteins to provide a stable amide bond. While the reaction proceeds under mild conditions in aqueous media, the *N*-hydroxysuccinimide ester was rapidly hydrolyzed; as observed by a spectrophotometric assay, the half-life for the ester (23 °C) in water at pH 7.5 was approximately 12 min, and at pH 9.1, the half-life was 1 min.

The method that we have developed is the first example of a dry organic solvent-based coupling strategy that provides the opportunity to couple proteins to surfaces using SPDP where the effect of competing hydrolysis is obviated. The low level of water in the solvent (measured at <0.15% wt) results in the reaction equilibrium favoring synthesis. The rate of decomposition of the ester in the dry organic solvent was found to be very much lower than in water. For example, SPDP dissolved in anhydrous trifluoroethanol,

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Fig. 1. Schematic diagram of the immobilization method employed on gold. *N*-Succinimidyl-3-(2-pyridyldithio) propionate (SPDP) is first adsorbed to a clean gold surface as a self-assembled monolayer. The protein solution in basic 2,2,2-trifluoroethanol (TFE) is then introduced and coupling allowed to proceed. The protein monolayers were then imaged using scanning tunneling microscopy (STM).

typically retained >80% of its ester content for a period of up to four weeks at room temperature (21 °C). These findings were similar to those previously reported (Carlsson et al., 1978) for SPDP stored in 99.5% ethanol.

The gold substrate with bound SAM was introduced into the vial containing the protein solution and left for a period of up to 24 h allowing the coupling reaction. In our experiments, protein coupling to SPDP in trifluoroethanol was allowed to proceed by the addition of a hindered tertiary nucleophilic base, *N,N*-diisopropylethylamine ($pK_a \sim 11$). The addition of an organic base was necessary in order to deprotonate lysine residues on the surface of catalase, allowing covalent coupling to the SAM of SPDP. This finding correlates well with the literature, where the base has been used in a similar capacity for conventional peptide synthesis (Yuan et al., 1982). No immobilized catalase could be detected if this base was not present in the solution.

Based upon the fact that catalase has an extremely high catalytic efficiency for the decomposition of hydrogen peroxide ($k_1 = 4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$) (Boyer, 1976), a qualitative colorimetric assay was developed that could rapidly determine if the enzyme remained active after coupling to the gold surface. Undecomposed hydrogen peroxide oxidizes sodium iodide crystals to iodine to produce a color change upon addition of starch indicator. In repeated assessments of catalase activity no color change was detected, indicating that all hydrogen peroxide had been decomposed by the active immobilized catalase. Controls containing gold-coated slides with no protein present were simultaneously performed, in all of which on addition of the indicator a change to the solution color to blue/black was detected. Hydrogen peroxide at 0.24 μM was determined to be the minimum quantity necessary for a visible color

change upon addition of starch indicator in the control reaction. To ensure that catalase immobilized to the gold ball was active, an assay was also performed using one of the gold balls prepared with immobilized protein. The gold ball with immobilized protein was immersed in the stirring solution and left for a period of 10 min. A control vial, free of catalase, was treated in exactly the same way. After this time a spatula tip of sodium iodide was added to both vials followed by starch indicator. In the control vial a faint blue color was immediately detected; in the vial containing the gold ball with immobilized catalase no color change was apparent. In addition, a quantitative estimation of catalytic activity of the catalase was determined by measuring the rate of decomposition of hydrogen peroxide with the amount of immobilized protein. Assuming a complete monolayer coverage of catalase forms on the gold surfaces, the number of molecules immobilized to the surface, each occupying 100 nm², was calculated. For the control solution, one molecule of catalase decomposed 3.02 million molecules of hydrogen peroxide per minute. Comparison of the activity of the immobilized catalase with the control demonstrates that between 20 and 31% of the catalase molecules remain active after immobilization. This is a minimum assessment of activity, since we have assumed complete protein coverage of the gold.

After immobilization, the gold samples were routinely assessed by STM (Parker et al., 1995). On the prepared flat gold surface areas protein molecules were easily detected in a high proportion of cases and observed to be of monolayer coverage. Figure 2 displays typical high resolution STM images of the immobilized catalase protein on the gold surface. The images reveal a dense surface coverage of protein, clearly resolving individual protein molecules. The observed diameter of the catalase molecules was 10 nm, which corresponds well with the 10.5 nm value obtained from X-ray crystallography (Murthy et al., 1981). Control samples prepared without the addition of protein or gold substrates alone did not show such surface features.

Organic solvents can dissolve significant amounts of protein (Singer, 1962; Chin et al., 1994), and the retention of enzyme catalytic activity in a number of both polar and apolar solvents have been well documented (Burke et al., 1989; Klibanov, 1989; Halling, 1990; Parker et al., 1994). Indeed, following dissolution, alpha-chymotrypsin (Paradkar and Dordick, 1994) and other proteins (Mozhaev et al., 1991) have been shown to be catalytically active in non-polar organic media, and using UV, CD, and fluorescence measurements were shown to be spectrally identical to that in aqueous media (Paradkar and Dordick, 1994). It should be noted that while Paradkar and Dordick used Aerosol OT to promote protein dissolution, the Aerosol concentration employed was too low to form reverse micelles, and therefore, dissolution of the protein resulted via ion pairing. Mozhaev et al. used only prolonged weak shaking at room temperature and achieved a concentration of 0.1–0.3 μM of chymotrypsin into octane. In addition, solid-state NMR studies (Burke et al., 1989) have shown that the integrity of the active site of a protein (α -lytic protease) was retained in a number of organic solvents. Therefore, using a dry polar protic solvent such as TFE, which can dissolve small amounts of protein while maintaining secondary structure, is a valid approach for preparing immobilized protein.

We have demonstrated that a dry organic solvent such as TFE is a suitable medium in which to carry out protein coupling and protein immobilization reactions. The method described in this paper is a useful alternative for the preparation of protein conjugates and immobilized proteins, which involve the use of hydro-

Fig. 2. High-resolution scanning tunneling microscope (STM) images of immobilized catalase molecules. (a) A 190×190 nm STM image showing globular features that correspond to immobilized catalase protein molecules. (b) A higher resolution image of the lower left-hand portion of (a). The average diameter of the observed catalase molecules is 10 nm.

lytically sensitive coupling reagents. Equally, the method may also be useful for the covalent coupling/immobilization of more hydrophobic molecules that have only limited solubility in aqueous solution.

Materials and methods

Preparation of protein

Catalase (E.C.1.11.1.6) from bovine liver was received as a crystalline powder (Sigma, Poole, Dorset, UK). The protein was dissolved in sodium phosphate buffer (10 mM, pH 7.8) to a concentration of 5 mg/mL. The solution was flow-dialyzed against deionized water at 4 °C and lyophilized. The resulting freeze-dried powder was then stored in a sealed container over freshly activated 3 Å molecular sieves.

Covalent coupling in anhydrous organic solvent

The coupling reagent, *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) (Pierce, Luton, UK) was dissolved in anhydrous 2,2,2-trifluoroethanol (TFE) (99%+, Aldrich, Gillingham, Dorset, UK) to a concentration of 0.95 mM. A gold ball prepared by flame annealing gold wire in a Bunsen burner was rigorously washed in deionized water. The gold ball was then suspended in the SPDP solution using clampable tweezers for a period of up to five hours, removed, and transferred to a solution containing approximately 0.4 µM catalase in TFE. The same volume of *N,N*-diisopropylethylamine (DIPEA, 99.5%, Aldrich, Dorset, UK) was then added. The gold ball was incubated in this solution for approximately 21 h and washed exhaustively in flowing deionized water in order to remove any non-covalently bound protein. Assessment of the degree of surface coverage by scanning tunneling microscopy (STM) imaging of the immobilized protein was carried out within 48 h.

Scanning tunneling microscopy analysis

The samples were scanned in at 59% humidity with mechanically prepared platinum/iridium (80:20) tips using a VG STM 2000 system (VG Microtech, Uckfield, UK). Images were collected in

constant current mode. Typical experimental conditions comprised a tunnelling current between 5 to 20 pA and a tip bias voltage range of +0.5 to +1.0 V. The image presented is a grey scale representation, which shows the lowest points as dark pixels and the highest points as light pixels.

Coupling reagent hydrolysis

Solutions of SPDP (0.01–0.1 mM) in aqueous Tris buffer of different pH values were prepared; these were at pH 7.0, 7.5, and 9.1. SPDP has an absorption maximum at 280 nm and an extinction coefficient of $5 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The degree of hydrolysis was measured by determining the amount of *N*-hydroxysuccinimide released after a measured amount of time, typically every two to three minutes. The amount of *N*-hydroxysuccinimide was measured at 260 nm (extinction coefficient $8.1 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$).

Qualitative assessment of catalytic activity of catalase after immobilization to gold

Glass microscope slides were cut into sizes of approximately 4 cm². The slides were then coated with dimethyl dichlorosilane by immersing them in a stirring 50 mM dichloromethane solution. They were then removed, rinsed exhaustively in flowing distilled water, and allowed to air dry. Sputter coating with gold was achieved in a SC 510 coater (VG Microtech, Uckfield, UK) operated at 0.1 mbar, with a sputtering current of 30 mA and a coating time of four to six minutes. The gold slides were treated with the disulphide coupling reagent and then exposed to the catalase solution in the same manner as described above. Non-covalently bound protein was removed by rinsing exhaustively in deionized water. Gold slides with covalently immobilized protein were immersed in a vial containing a solution of 0.8 mM hydrogen peroxide (Aldrich) and a few crystals of sodium iodide. The vial was shaken gently in order to cover the slides with solution, and after a few minutes a drop of starch indicator solution (1 wt% solution in water, Aldrich) was added. Starch indicator was used to test for the presence of iodine formed by the reaction of undecomposed hydrogen peroxide.

Quantitative assessment of catalytic activity of catalase after immobilization to gold

Catalase was immobilized on gold surfaces, epitaxially grown gold on mica (Morozov et al., 1993), of a range of sizes upto 20 mm². The samples were immersed in a vial containing 30 mM hydrogen peroxide solution. The decomposition of hydrogen peroxide by catalase was determined by measuring the UV absorbance at 240 nm after 10 min. The decomposition of a control solution containing 30 mM hydrogen peroxide and 33 pM catalase was also determined in a similar manner.

Karl-Fischer water titration

The optimized Karl-Fischer water titration method (Laitnen and Harris, 1975) (Metrohm 684) was used to determine the amount of water present in TFE. The average water content of the solvent was measured colorimetrically.

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