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Surface derivatization with spacer molecules on glutaraldehyde-activated amino-microplates for covalent immobilization of β-glucosidase

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ABSTRACT

Protein molecules immobilized on a hydrophobic polystyrene microplate by passive adsorption lose their activity and suffer considerable denaturation. In this paper, we report a thorough evaluation of a protocol for enzyme immobilization on a microplate with relatively inexpensive reagents, involving glutaraldehyde coupling and spacer molecules, and employing β-glucosidase as a model enzyme. The recommended conditions for the developed method include 2.5% glutaraldehyde to activate the reaction, 1% chitosan in an HAc solution to increase the binding capacity, 2% bovine serum albumin to block non-specific binding sites, and 0.1 M $NaBH_4$ to stabilize Schiff's base intermediates. Using this method, the amount of β-glucosidase immobilized on amino-microplate was 24-fold with chitosan than without spacer molecules. The procedure is efficient and quite simple, and may thus have potential applications in biosensing and bioreactor systems.

1. Introduction

The microtiter plate has become a common tool in analytical research and clinical diagnostic testing laboratories because it is easy to handle and adaptable to automatic microplate readers. A very common use for this plate is in enzyme-linked immunosorbent assays (ELISAs), which are the bases of most modern medical diagnostic testing in humans and animals. Biological macromolecules such as proteins are immobilized on the surface of polystyrene microplates through passive adsorption if the surface has not been otherwise treated. Passive adsorption primarily involves multiple hydrophobic interactions between the solid phase and the biomolecule, which may interfere with the structure of the latter and lead to conformational changes [1] and alterations in their functions [2]. Thus, it is necessary to design a proper surface and rational conjugation for the controlled placement of biomolecules on polystyrene microtiter plates [3].

The surfaces of polystyrene can be modified to introduce specific functions. In general, treatments result in the surface incorporation of hydroxyl, carbonyl, and carboxyl functional groups [4,5]. Since amine groups can be readily used for the covalent linking of bioactive molecules, the introduction of these groups is most often described in the literature [6]. The most commonly used treatments for coating or covalently linking amine groups to polystyrene include polymers, such as phenylalanine–lysine [7], nitration–reduction [8], gamma irradiation [9], plasma treatments (nitrogen or ammonia plasma) [10], and carbodiimides [11,12]. The aminated surface is activated and covalently coupled to the functional groups (primary amines, thiols, and carboxyls) of biomolecules via bifunctional crosslinkers (i.e., glutaraldehyde and carbodiimide). Glutaraldehyde activation of aminated supports is one of the most popular techniques for immobilizing enzymes [13]. The proposed methodology is rather simple and efficient and, in some instances, even allows for the improvement of enzyme stability through multi-point or multi-subunit immobilization [14].

Since a microplate consists of a large number of molecules within a small well, the density of the active surface amino groups is a key factor in increasing their binding ability to surfaces and providing higher signals [15]. To create a three-dimensional structure that generates sufficient spacing on surfaces and avoids lateral steric hindrances between immobilized biomolecules, surface derivation with a spacer molecule has been applied for biomolecular immobilization on the surface of various solid supports [16]. The most commonly used spacer molecule includes dendrimers [15,17], polyethyleneimine [18], poly(ethylene glycol) [19,20], chitosan [21], and poly(carboxybetaine methacrylate) [22], as well as self-assembled monolayers [23,24].

Herein, we demonstrate the suitability of commercially available amine-graft polystyrene microwell plates for enzyme immobilization via a relatively inexpensive glutaraldehyde activation reaction. To improve the enzyme binding efficiency of the amino-microplate, spacer molecules are employed for surface

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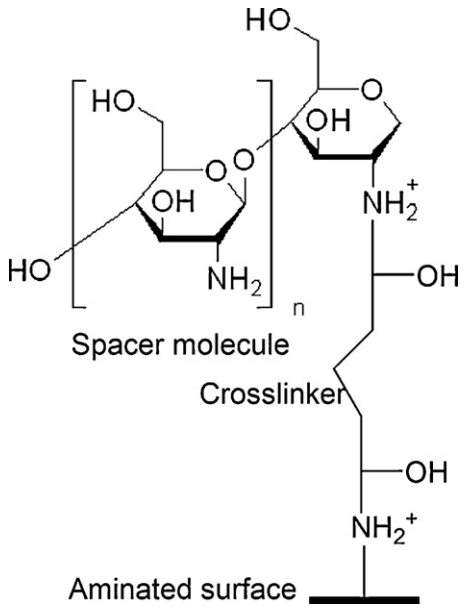

Fig. 1. The scheme of strategy for surface derivatization with spacer molecules on amino-microplate for protein immobilization. Crosslinker: glutaraldehyde, spacer molecule: chitosan.

derivatization (Fig. 1) and some conditions for immobilization, such as enzyme–support contact time and initial enzyme amount in the attachment solution, among others, are optimized. A comparative study between the glutaraldehyde and bis(sulfosuccinimidyl) suberate (BS^3) methods is also undertaken.

2. Experimental

2.1. Materials

Amine-graft polystyrene microtiter plates were purchased from Corning (96-well, Cat. No. 2388). Sodium borohydride and Tween 20 were purchased from Alfa Aesar. β-Glucosidase (E.C.3.2.1.21), 4-nitrophenyl β-D-glucopyranoside (pNPG), acetylcholinesterase (E.C.3.1.1.7), 5,5′-dithiobis-(2-nitrobenzoic, acid), acetylthiocholine iodide, BS^3, chitosan, glutaraldehyde, bovine serum albumin (BSA), and polyethyleneimine were all purchased from Sigma–Aldrich.

2.2. Microplate assay of β-glucosidase activity

Color development in microplate wells was determined using a microplate reader (LMR340M, Labexim). A straight line of the average change absorbance versus time for each detected well was constructed. The change in absorbance per unit time per well (Δabs at 405 nm/min/well), which represents the quantity of the immobilized enzyme, was calculated from the slope of each straight line.

β-Glucosidase activity was assayed in 200 μL of a 0.1 M sodium phosphate buffer containing 5 mM pNPG at pH 5.0. After the solution was incubated for 15 min at room temperature, the absorbance increase was recorded at 405 nm. A blank containing substrates but no immobilizing enzyme was prepared to control for the nonspecific hydrolysis of substrates in the microplate wells.

2.3. Protocol for immobilizing β-glucosidase using glutaraldehyde

The amino-microplate wells were placed in 2.5% glutaraldehyde and 0.1 M Na phosphate buffer (pH 8.0) at room temperature for 15 min, after which they were washed thoroughly in more pH 8.0 phosphate buffer. The plates were incubated for 60 min at 4 °C in 1% chitosan in HAc (1 vol. conc. HAc + 99 vol. H_2O), and then washed thoroughly with H_2O and 0.1 M Na phosphate pH 8.0 buffer. Nonspecific binding sites on the plates were blocked by incubation for 2 h at 4 °C for in a solution of 2% bovine serum albumin in 0.1 M Na phosphate pH 8.0 buffer. Excess blocking solution was washed away with phosphate buffer. After applying the first step of the procedure to the plates once more, they were activated with glutaraldehyde. Afterward, they were incubated in the enzyme solution at 4 °C for a minimum of 2 h, although overnight incubation is preferred. Washing with 0.1 M Na phosphate pH 8.0 buffer followed. The plates were incubated for 60 min at room temperature in 0.1 M $NaBH_4$ in 0.1 M Na phosphate pH 8.0 buffer. Finally, the microplate wells were thoroughly and sequentially washed in H_2O, 0.1 M NaCl in 0.1 M Na phosphate buffer pH 8.0 containing 0.5% (v/v) Tween 20, and 0.1 M Na phosphate pH 8.0 buffer.

2.4. Immobilization of β-glucosidase using BS^3

Immobilization was carried out according to the manufacturer's recommendations. About 200 μL of a 1 mg/mL solution of BS^3 in 20 mM phosphate buffered saline (pH 7.4) solution were added to the detected wells of an amine surface strip plate. After being rinsing thrice with PBS, the solution was incubated in a β-glucosidase solution at 4 °C for a minimum of 2 h, although overnight incubation is preferred. The amount (activity) of β-glucosidase in the 0.1 M Na phosphate pH 5.0 buffer was the same as in the glutaraldehyde activation method described above.

2.5. Storage stability

An experiment was conducted to determine the stabilities of the immobilized β-glucosidase preparations after storage in a phosphate buffer (100 mM, pH 5.0) at 4 °C for a predetermined amount of time. The residual activities were then determined as described above. The activity of each preparation was measured in batch operation mode and expressed as a percentage of the residual activity relative to the initial activity.

3. Results and discussion

The aminated surface of the polystyrene 96-well plates was specifically designed to be used with bifunctional crosslinkers to covalently couple to the functional groups of biomolecules. In this study, β-glucosidase was used as a convenient model enzyme to develop the protocol, as shown in Section 2. Each of the steps in the procedure is examined individually.

3.1. Glutaraldehyde activation

Glutaraldehyde activation of aminated supports is one of the most popular techniques for immobilizing enzymes [13]. A multistep procedure for immobilizing proteins and enzymes on nylon has also been developed [25]. The precise control of the conditions during support activation with glutaraldehyde has enabled the modification of the amino groups of the matrix with one or two glutaraldehyde molecules [14]. In this study, amino-microplates were activated with various concentrations of a glutaraldehyde solution and incubated for 15 min at room temperature in a 0.1 M phosphate buffer, pH 8.0. The glutaraldehyde concentration varied from 0.5 to 10%. As can be seen from Fig. 2, the amount of β-glucosidase bound to the surface in the final stage of the procedure increased with increasing glutaraldehyde concentrations up to 2.5%. At concentrations over 2.5%, however, minimal increases in amount of bound β-glucosidase were observed. Thus, in consideration of toxicity, 2.5% (v/v) was chosen as the optimal amount for maximum enzyme immobilization.