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Journal of Chromatography B, 738 (2000) 217–223

JOURNAL OF CHROMATOGRAPHY B

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Immobilization of antibodies onto glass wool

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Received 12 March 1999; received in revised form 28 October 1999; accepted 1 November 1999

Abstract

The immobilization of antibodies onto solid phases in an efficient and activity-retaining form is an important goal for both research and industry. Methods have been developed for the site-directed attachment of antibodies to agarose by oxidation of the carbohydrate moieties in their Fc region. Similar attachment to silianized supports have not been as successful. Here we describe a novel combination protocol for the site-directed attachment of periodate oxidized, goat polyclonal antibodies to glass wool fibers activated with 3-aminopropyltriethoxysilane. The study demonstrates that this procedure results in effective immobilization of polyclonal antibodies that retain their antigen-binding capacity. This protocol should prove useful in the development of more efficient and effective glass-based immunosupports. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Glass wool; Antibodies

1. Introduction

The immobilization of biological proteins such as enzymes and immunoglobulins has now found wide application in both analytical [1,2] and preparative systems [3,4]. Two important goals in developing these applications are: (i) maximum covalent attachment of the protein to the solid phase and (ii) maintenance of protein functionality following immobilization. Despite the availability of different solid surfaces and immobilization chemistries, it is still not a simple matter to achieve these goals. This may not be surprising given the variety of factors that influence immobilization such as buffer composition, protein stability, three-dimensional structure of the protein, the availability of additional functional groups on glycoproteins or lipoproteins and the predicted distance from the site of immobilization to the protein's active site [1]. Indeed, these considerations have severely limited the development of simple and standard procedures for the immobilization of enzymes [5,6].

With regard to antibodies, there may be some applications where ‘‘inefficient’’ immobilization is acceptable. For instance, in solid-phase immunoassays in which the antibody is adsorbed to a plastic microtiter plate, the relatively large surface area to sample volume ratio allows binding of excess antibody which probably compensates for those molecules functionally inactivated by the binding process.

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PII: S0378-4347(99)00514-9

Increasing immobilization efficiency by the control of antibody orientation becomes important as assay sensitivity increases [7,8], where the reagents are not readily available and for cost-effective preparative chromatography and immunobioreactors.

Several chemistries have been used to immobilize antibodies to solid surfaces, usually based on the strategy of activating the solid matrix and then binding pre-treated or native antibody. Popular methods include activation of the solid matrix with cyanogen bromide or *N*-hydroxysuccinimide esters [9–12]. These procedures couple antibodies covalently to the solid phase via the ϵ-amino groups of approximately 70 lysine residues in the molecule, resulting not only in multiple-site attachments but also multiple orientations [13] and a decrease in antigen binding capacity [14].

As a means of overcoming these limitations, methods were developed for the site-directed immobilization of antibodies. One approach is to oxidize the carbohydrate moieties of the antibody with periodate to form aldehyde groups which are then chemically bound to hydrazide-activated solid supports such as agarose [15–18]. As antibodies are only glycosylated at a single site in the CH_2 domain of each heavy chain, this approach leaves the two Fab (antigen binding) regions of the antibody free to interact with the mobile phase. Hydrazide-activated silica-based particles have also been used for site-directed antibody attachment [14]; however only low levels of active, immobilized antibodies were obtained. The authors attributed this result to excessive non-specific interactions between the antibody and support surface, which had to do more with physical adsorption than site-specific attachment. To improve on this method Ruhn et al. [18] attached dihydrazides to oxidized, diol-bonded silica. The resulting activated support was used successfully to immobilize oxidized proteins such as goat and rabbit IgG, horseradish peroxidase and tRNA. An advantage of this approach is that it permits immobilization though site-directed attachment.

The use of silica-based supports and controlled pore glass for immobilization of enzymes, antibodies and other proteins has been known for some time [12,19]. Silanization using 3-aminopropyltriethoxysilane (ATES) for antibody immobilization has also been developed. For instance, ATES was used to pre-treat glass surfaces [20] or quartz crystals [21] and acted as a bridge to which gluteraldehyde was bound, allowing chemical attachment of the terminal aldehyde to amine groups in antibodies. While this method efficiently results in a silica-activation chemistry similar to that developed by Ruhn et al. [18] and might reduce non-specific attachment to the solid phase, there is no site-directed attachment of the antibody.

We have studied the possibility of combining the advantages of these previous methods by designing an antibody immobilization protocol in which periodate-oxidized antibodies are covalently coupled to an ATES-activated silica-based support. As the solid phase we used glass wool, a cheap and widely available material. We show here that this simple procedure provides an economic, site-directed and efficient method for antibody immobilization.

2. Experimental

2.1. Glass wool treatment

Glass wool fibers (Sigma, St. Louis, MO, USA) were soaked in a solution of ATES (5%, w/v) in water and held for 30 min at 37°C. The glass wool was isolated from the solution on a Buchner filter and washed five times with distilled water, five times with acetone, once with 0.1 *M* acetate buffer (pH 5.5) containing 0.15 *M* NaCl (buffer A) and drained [20].

2.2. Oxidation of antibodies

A 10-μl volume of a 0.2 *M* solution of sodium metaperiodate (Sigma) was added to 200 μl of affinity purified goat anti-biotin antibody solution (Sigma) (0.9 mg/ml) in buffer A. The oxidation was carried out for 30 min at room temperature in the dark with gentle mixing. Afterwards the antibody solution was diluted ×10 in buffer A and dialyzed at 4°C overnight against the same buffer [15,17].

2.3. Immobilization of antibodies

The procedure is outlined in Fig. 1. Silianized and drained glass wool was soaked in the solution of

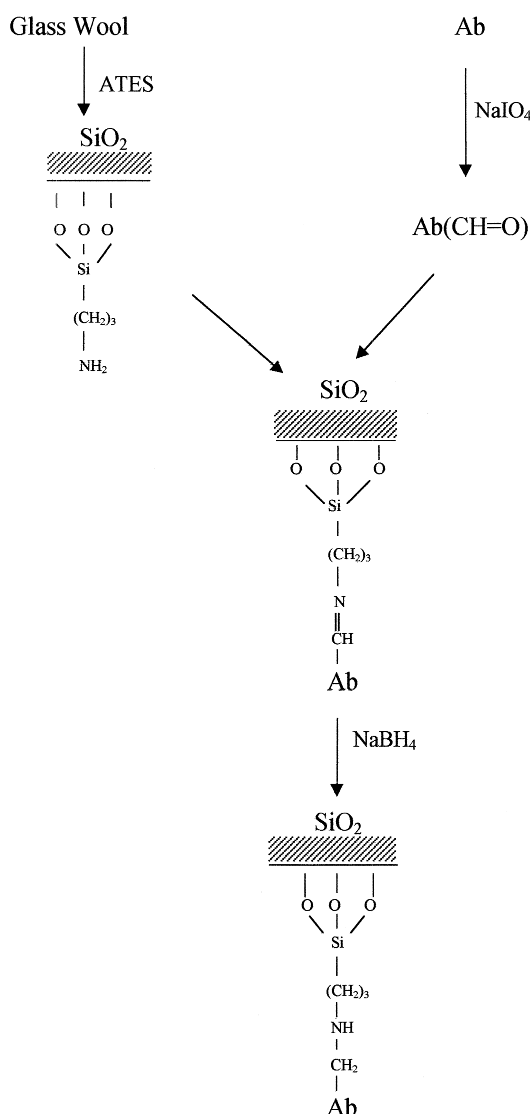

Fig. 1. Schematic protocol for immobilization of oxidized antibody onto ATES-activated glass wool fibers.

oxidized antibodies (0.4–7.6 μg/ml) in buffer A (150 mg of glass wool for 1 ml of antibody solution) and left overnight under rotary mixing at 4°C. Strict precaution was taken to mechanically eliminate air bubbles from the glass wool during the soaking. Afterwards, the glass wool was washed three times with 0.1 *M* phosphate buffer, pH 7.3, containing 1 *M* NaCl, and five times with 0.01 *M* PBS, pH 7.3, and drained. The glass wool was then soaked in 0.01% sodium borohydride in 0.1 *M* borate buffer, pH 7.3 for 1 h at 37°C, and washed with 0.01 *M* PBS, pH 7.3, containing Tween 20 (0.5 mg/ml) [20].

2.4. Enzyme-linked immunosorbent assay (ELISA) for goat anti-biotin antibody activity

Standard ELISA plates (Maxisorb, Nunc, Denmark) were coated with 100 μl of native goat or oxidized anti-biotin antibodies in 0.01 *M* phosphate-buffered saline (PBS), pH 7.3 (buffer B) in concentrations 0.01–1 μg/ml for 16 h at 4°C. After discarding the antibody solution, the plates were washed three times in buffer B and blocked with 150 μl of solution containing 0.2 *M* glycine, 2 *M* NaCl, 40 m*M* $MgCl_2$ and 0.05% Tween 20 in buffer A for 1.5 h at room temperature. After washing the plates with buffer A, 100 μl of biotinylated alkaline phosphatase (Sigma), diluted to 0.635 μl/ml in buffer A, were applied for 1.5 h at room temperature. The plates were washed three times with buffer A and then incubated with 100 μl of substrate pNPP (Sigma) (one tablet for 5 ml of 0.1 *M* glycine buffer, containing 1 m*M* $MgCl_2$, 1 m*M* $ZnCl_2$, pH 10.4) for 1 h at 37°C. The absorbance (A_4) was read at 405 nm with an ELISA reader (Biotech Instruments).

2.5. Evaluation of immobilization efficiency

In order to estimate the amount of antibody immobilized to glass wool, antibodies were immobilized as outlined in Section 2.3 above. A 100-μl volume of pre-immobilization mixture or post-immobilization supernatant was diluted from 1/4–1/100 in buffer A, coated to ELISA microplates and used to determine the level of free anti-biotin as described in Section 2.4. The difference in anti-biotin activity between the pre and post immobilization samples was used as an indirect measure of the level of antibody immobilization to glass wool. Thus, the fraction of antibodies immobilized at each starting concentration was calculated using the formula $P = A_b - A_a / A_b$, where A_b=optical density measured in ELISA before immobilization, A_a=the corresponding sample after immobilization. To ensure that non-specific or weakly adsorbed antibodies would not distort these calculations, the post-immobilization washes of the glass wool (see Section 2.3 above)

were also examined for antibody activity. These washes showed absolutely no anti-biotin activity, indicating that non-immobilized antibodies were not bound to the glass wool. The resulting quantity of antibody immobilized on the glass wool (m) was calculated as, $m = m_o P / m_g$ where m_o = amount of antibodies added to a quantity of glass wool (μg), P = fraction of antibodies bound, m_g = mass of the glass wool (g).

2.6. Stability of immobilized antibodies

The glass wool–antibody preparation was separated from the buffer solution, drained, divided into equal portions of 30 mg each and stored in fresh buffer A for 7 days at 4°C. The amount of active antibody retained on the glass wool was estimated by draining the glass wool, washing three times in buffer A and incubating it with 200 μl of biotinylated alkaline phosphatase conjugate as described above in Section 2.4. After washing with buffer A to remove any non-specifically absorbed conjugate and incubation with pNPP substrate solution, the glass wool was separated and absorbance of the supernatant was read at 405 nm. The absorbance reading of a similarly tested aliquot taken at zero time was used as 100% activity standard of the immobilized antibodies.

3. Results and discussion

The aim of this work was to develop an effective method for the covalent, site-directed attachment of antibodies to glass wool fibers, a cheap, readily available and potentially useful solid support for immunoaffinity chromatography and immuno-bioreactors.

Glass wool fibers have been used successfully for many years as a solid phase for the separation of lymphocytes [22] and neutrophils [23] and more recently for the isolation of viruses [24] and sperm [25,26]. While some results are available for immobilization of antibodies on to glass surfaces [12,14,16,18,20,27], we are not aware of published data concerning attachment of antibodies to glass wool fibers.

Immobilization of goat polyclonal antibodies was achieved by combining two previously described methods for covalent attachment of antibodies to solid phases; one for the silianization of glass surfaces using ATES [21,27] and the second for the site-directed oxidation of antibody carbohydrate moieties [13–15,17,18]. The combination protocol so developed (Fig. 1), allows covalent attachment via carbohydrates present only in the second constant domain (CH_2) of the antibody's Fc region to the glass wool in a site-directed manner. This leaves the antigen-binding Fab arms of the antibody oriented towards the aqueous phase and free to interact with antigen. Previous methods designed to attach antibodies to glass surfaces resulted in low immobilized antibody activity due to the effect of either additional non-specific attachments [14] or random attachment via free amine groups on the protein's surface [20]. Such non-directed orientation of the antibody may potentially cause significant loss in activity though participation of the Fab regions in attachment to the solid phase or at least in their steric hindrance.

We used polyclonal goat anti-biotin antibodies to demonstrate the effectiveness of the coupling procedure. Antibodies were oxidized by sodium periodate, which provides the active aldehyde groups in the Fc region by gentle oxidation of the carbohydrate present there. Incubation of the oxidized antibodies with ATES-activated glass wool and subsequent reduction by sodium borohydride leads to immobilized antibodies that retain their antigen-binding activity.

The conditions used for polyclonal antibody oxidation were based on those described earlier by Hoffman and O'Shannessy [15] and optimized by Wolfe and Hage [17]. The latter study demonstrated that effective oxidation of polyclonal antibodies and preservation of antibody activity is dependent of several variables such as periodate concentration, reaction pH, time and temperature. Their data shows that several combinations of these conditions, including the combination used in this study, achieve good results. Based on this information, Hage et al. [28] recently developed a mathematical model to describe the kinetics of antibody oxidation. We tested the effect of oxidation on the pre-immobilized goat anti-biotin antibodies by ELISA, using treated and untreated antibodies for coating and enzyme-labeled biotin. Fig. 2 demonstrates that oxidation resulted in

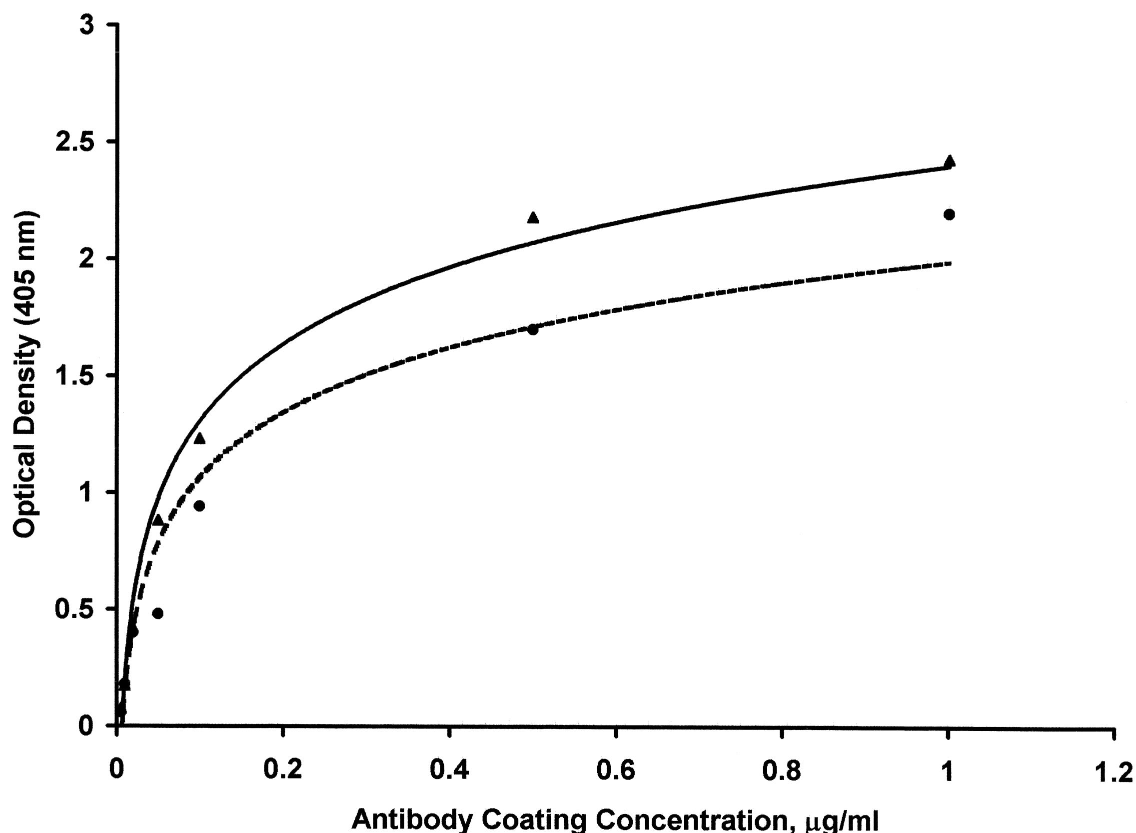

Fig. 2. The influence of periodate oxidation on antibody activity. Goat anti-biotin antibodies were oxidized as described in Experimental and then tested with ELISA by coating microplates with oxidized or native antibody and adding enzyme-conjugated biotin (▲ native anti-biotin; ● oxidized anti-biotin).

an average of 15% decrease in antibody activity. Both native and oxidized antibodies showed maximum activity at similar coating concentrations. These results compare favorably with previous studies on periodate oxidation of antibodies under similar conditions which showed an average effect of 0–22% activity on both polyclonal [29] and monoclonal [30,31] antibodies.

The optimal concentration of oxidized antibody used for coupling to the ATES-activated glass wool was then determined. Various concentrations of antibody were added to a constant amount of glass wool and the amount of bound antibody determined as described in Experimental. Fig. 3 shows that antibody coupling to the glass wool increased linearly with the amount of oxidized antibody added, reaching a plateau at 25 μg oxidized antibodies/g solid phase. This corresponds to a binding efficiency of approximately 70%. It is difficult to directly compare this result to other processes as a previous study on immobilization of monoclonal antibodies to glass test-tube surfaces did not report binding efficiency data [21]. In a different study the binding of oxidized goat antibodies to a hydrazide activated gel was reported to have an efficiency of 85% [13].

Some caution is needed however in the choice of glass wool fibers used for immobilization. Our initial experiments with a low-grade product indicated that this material required washing with sulfuric acid to remove adherent particles prior to ATES activation. This treatment almost completely abolished the antibody-binding capacity of the fiber (data not shown).

Initial studies were carried out to determine the

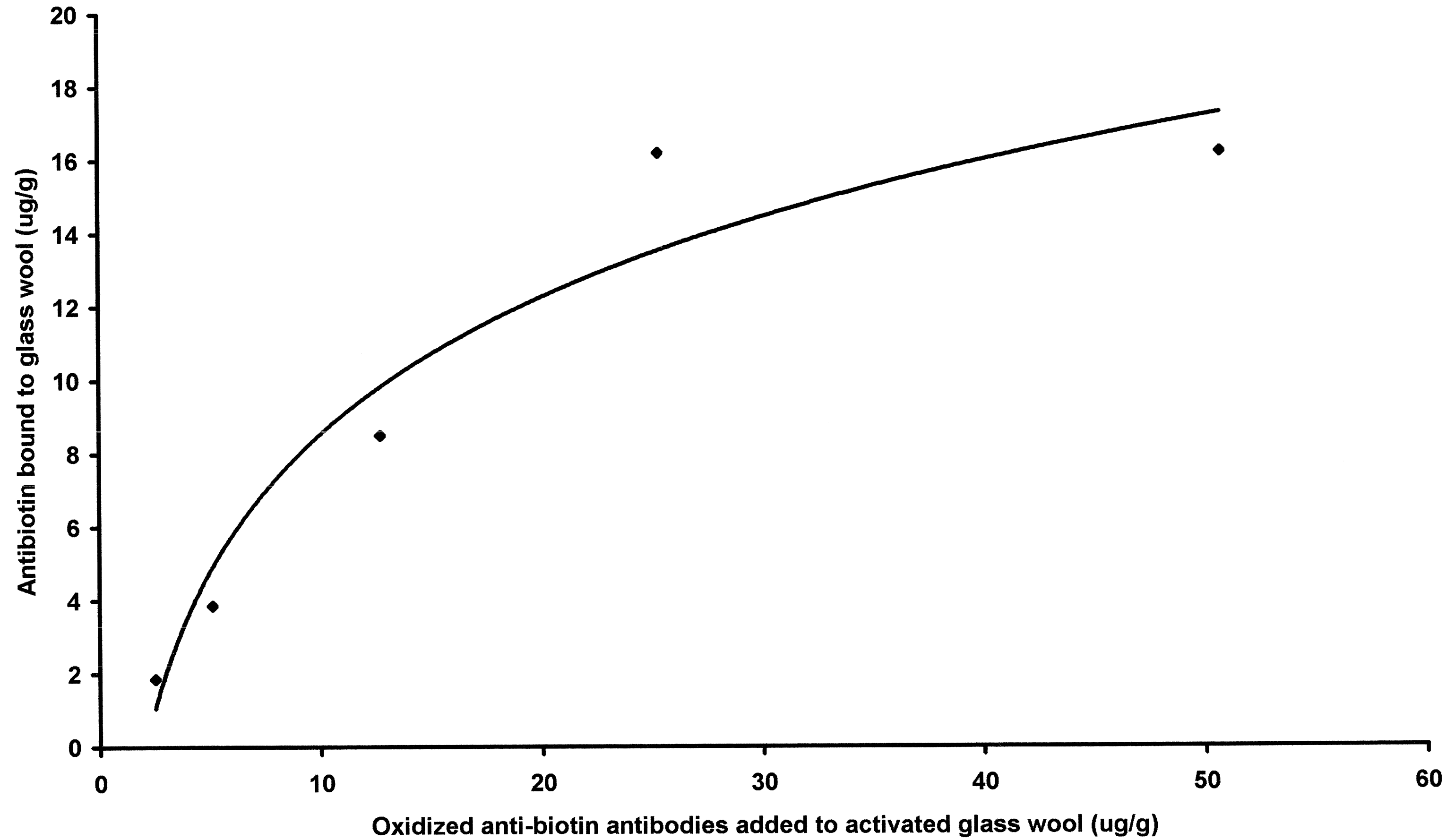

Fig. 3. Immobilization of oxidized goat anti-biotin antibodies to ATES-activated glass wool fibers. Different amounts of oxidized antibodies were added to activated fibers and the amount of bound antibody calculated (see Experimental for calculation details).

stability of the immobilized antibodies. A quantity of antibody coated glass wool was maintained at 4°C and samples removed periodically for activity testing. These tests showed that immobilized antibodies retained up to 90% of there initial activity over a period of at least 7 days. This result compares well with stability data reported previously for antibody immobilized to glass surfaces [14].

This study has demonstrated the effectiveness of a new protocol for the site-directed immobilization of polyclonal antibodies to glass wool polymers. The combination of ATES activation of the solid matrix with gentle periodate oxidation of antibodies combine for the first time the advantages provided by each of these steps alone. The method provides for efficient immobilization of antibodies that retain their antigen-binding capacity. In addition, the demonstration of this protocol with glass wool fibers offers the use of a cheap, effective and readily available solid support, bearing in mind the polymer quality conditions discussed above. The model system described here can now be used to expand the procedure for development of targeted applications. For example, we have used the activated glass wool to prepare chromatography columns and plan to test its use as an affinity adsorbent in bioreactors for sequestration of secreted products during cell growth.

Acknowledgements

This work was supported in part by the Israel Ministry of Trade and Industry.

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