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ENTRAPMENT OF PROTEINS IN GLYCOGEN-CAPPED AND HYDRAZIDE-ACTIVATED SUPPORTS

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Abstract

A method is described for the entrapment of proteins in hydrazide-activated supports using oxidized glycogen as a capping agent. This approach is demonstrated using human serum albumin (HSA) as a model binding agent. After optimization of this method, a protein content of 43 (± 1) mg HSA/g support was obtained for porous silica. The entrapped HSA supports could retain a low mass drug (*S*-warfarin) and had activities and equilibrium constants comparable to those for soluble HSA. It was also found that this approach could be used with other proteins and binding agents that had masses between 5.8 and 150 kDa.

Keywords

Immobilization methods; Entrapment; High-performance affinity chromatography; Drug-protein binding; Frontal analysis; Human serum albumin; Glycogen

Affinity chromatography uses a biologically-related binding agent as a stationary phase to undergo specific interactions with a target analyte (1–3). The selectivity of this approach is dependent on the way in which the binding agent is attached to the support (4). It is common for a binding agent such as a protein to be covalently immobilized through amine, sulfhydryl, carboxyl or carbonyl groups (4–6). However, covalent immobilization can produce multisite attachment or improper orientation of the binding agent, which may cause an apparent change in this agent's activity. These issues can be minimized in some cases by coupling the binding agent to the support through functional groups that occur in only a few locations in its structure, such as the use of the carbohydrate chains in antibodies or α_1-acid glycoprotein (AGP) for their site-selective attachment to hydrazide-activated supports (4,6).

Another approach to avoid these immobilization effects is to use the physical entrapment (or encapsulation) of the binding agent within a support. This approach has been of interest for many years in work with materials such as sol gels and has generally been based on the physical containment of binding agents in a highly cross-linked polymer network (7–9). However, past approaches have used materials with pressure or flow rate restrictions that are not easily amenable for use with HPLC. Also, a general entrapment approach is still needed that can be used with common HPLC supports such as porous silica.

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This report used the entrapment method shown in Figure 1(a), as based on the use of a hydrazide-activated support and oxidized glycogen (~2.5×10^5 kDa) as a capping agent. The use of this method with HPLC-grade porous silica and nonporous glass beads was examined using human serum albumin (HSA) as a model protein and binding agent (see Supplementary Material for suppliers of reagents and equipment). This approach was performed by first activating a porous or nonporous support with hydrazide groups according to a previous method (10). Typically, a 0.03 g portion of this support was incubated in a pH 5.0, 0.10 M phosphate buffer containing the protein of interest (50 mg/mL HSA) and 100 μL of a 4.2 mg/mL mildly oxidized form of glycogen (note: this approach can be easily scaled to larger amounts of support by using proportionally larger amounts of added reagents). The oxidation of glycogen in the final method involved the reaction of 10 mL of a 5 mg/mL glycogen solution with 20 mg/ml of periodate for 12 h at room temperature in pH 7.0, 20 mM sodium acetate containing 15 mM sodium chloride. After oxidization, the glycogen was purified using an Econo-Pac 10DG column and pH 5.0, 0.10 M potassium phosphate buffer as the mobile phase, using an approach similar to that described for oxidized antibodies in Ref. (11). The extent of oxidation for the glycogen was monitored using Lucifer yellow CH as a labeling agent, also as described for the labeling of oxidized antibodies (11).

The mixture of support, protein and oxidized glycogen was shaken at 4°C for 24 h. During this incubation step, aldehyde groups on the oxidized glycogen formed a stable covalent bond with the hydrazide groups on the support, resulting in entrapment of the protein. In the final hour of incubation, 200 μL of a 2 mg/mL oxalic dihydrazide solution in pH 5.0, 0.10 M phosphate buffer was added to cap any remaining aldehyde groups on the glycogen. After immobilization, the final support was washed with 0.10 M, pH 7.0 potassium phosphate buffer and water.

Several parameters were varied to achieve maximum protein content in this method, as measured using a bicinchoninic acid assay (4). The first study examined the relationship between the amount of entrapped protein and support porosity, using both nonporous silica and silica with pore sizes that ranged from 50 Å to 1000 Å. It was found that the amount of entrapped protein was similar for each porous support (see Supplementary Material), with an average final content of 27 (± 3) mg HSA per gram silica. It was also noted that HSA could be entrapped on nonporous glass beads, giving a protein content of 30 (± 1) mg HSA per gram support. These results suggested that the mechanism for protein entrapment under the given preparation conditions involved HSA being held by the glycogen within shallow pores or pockets near the support's outer surface. This mechanism was supported by the fact that the total protein content measured in this report for HPLC grade silica was comparable to results obtained with covalent immobilization techniques for HSA (1,5).

The amount of HSA that could be entrapped was next examined as a function of the amount of oxidized glycogen used during the entrapment process. This was studied by incubating fixed amounts of 300 Å silica and HSA with various amounts of 4.2 mg/mL oxidized glycogen. As the amount of oxidized glycogen added to the reaction mixture was increased from 0.8 mg to 17 mg per gram of silica, the amount of immobilized HSA increased from 1.2 to approximately 44 mg/g silica. Increasing the amount of oxidized glycogen up to 35 mg/g silica did not yield any additional increase in the entrapped HSA. Thus, 15 mg oxidized glycogen per gram silica was added in all further experiments.

The relationship between the amount of added protein and the amount of entrapped protein was examined by using fixed portions of 300 Å pore size hydrazide-activated silica and oxidized glycogen reacted with a solution containing various concentrations of HSA. Increasing the amount of added HSA from 20 to approximately 100 mg HSA per gram silica resulted in an increase in the amount of entrapped HSA. However, as larger amounts of HSA were added (i.e., up to 250 mg added HSA/g silica), the amount of entrapped protein reached

a plateau at approximately 43 (± 1) mg HSA per gram silica (n = 21). From this result, 100 mg HSA per gram silica was used in all later experiments.

This method was next used to entrap HSA in Nucleosil Si-300 porous silica, which was then packed into a 1.0 cm × 2.1 mm I.D. column. Two control columns of the same size were also prepared. The first of these control columns was made in the same manner as the entrapped HSA support but with no oxidized glycogen being added. The second control column was made using oxidized glycogen but with no HSA being added. Zonal elution injections of *S*-warfarin (i.e., a drug with strong binding to HSA) were then made on each column at room temperature in the presence of pH 7.4, 0.067 M potassium phosphate buffer (Figure 1b) (12). The peak for *S*-warfarin that eluted from the entrapped HSA column had a retention time of 118.4 s at 0.5 mL/min and a retention factor of 11.8 (± 0.8). However, the peak observed for *S*-warfarin on both control columns was statistically identical to the void time, indicating that no appreciable interactions were occurring between *S*-warfarin and either the hydrazide-activated support or the glycogen (note: less than 5% of the total binding for *S*-warfarin on the entrapped HSA column was due to interactions with these components, as determined by frontal analysis). These results confirmed that HSA had been entrapped by the glycogen when both of these agents were combined with the hydrazide-activated silica and that the HSA was accessible for binding by a small solute such as *S*-warfarin. Similar behavior has also been observed for *R*-warfarin and D/L-tryptophan in their binding to entrapped HSA.

Frontal analysis (12,13) was next used to measure the association equilibrium constants and the total moles of binding sites of the entrapped HSA for *S*-warfarin (see Supplementary Material for typical results). All tested columns were stable over at least one week during these studies. Table 1 lists the equilibrium constants found for S-warfarin on each tested column, which ranged from 1.4–2.0 × 10^5 M^{-1} at pH 7.4 at 37°C. These results were in good agreement with values of 1.9–2.4 × 10^5 M^{-1} that have been reported for the same analyte (13). The average specific activity for the 100 Å to 1000Å pore size supports was 0.99 (± 0.25), a value statistically equivalent to that for soluble HSA (note: the lower activity of the 50 Å pore size support may be a reflection of some steric effects or restricted diffusion but including this value still gave an overall average specific activity of 0.92 (± 0.29) for all supports). Similar work with sulfhydryl-reactive immobilization methods for HSA have given specific activities of 0.81–0.81 (5), and the Schiff base method (i.e., an-amine based coupling method) has given typical values of 0.55–0.57 (13). These results indicated the entrapment method allowed for greater retention of HSA activity for binding with *S*-warfarin. Similar binding studies with other analytes (e.g., D/L-tryptophan and *R*-warfarin) are now in progress.

The use of this method of entrapment was considered for other proteins and biomacromolecules. The other agents examined and the amounts that were entrapped (in nmol/g silica) were as follows: insulin (5.8 kDa), 16,000 (± 300); myoglobin (16.7 kDa), 1,200 (± 200); streptavidin (52.8 kDa), 238 (± 1); avidin (69.0 kDa), 155 (± 1); glycated HSA (71.4 kDa), 209 (± 19); IgG-class antibodies (150 kDa), 105 (± 1); and ferritin (450 kDa), 20 (± 1). These results indicated that binding agents up to at least 150 kDa (as represented by IgG) could be successfully entrapped by this method. Ongoing experiments are examining the activities for many of these entrapped agents.

This method of entrapment was directly usable with HPLC-grade silica or nonporous supports. Other attractive features of this method were the high activity of the entrapped binding agent, its accessibility to small analytes, the good agreement in the binding affinity of the entrapped agent with its soluble form (i.e., as demonstrated for HSA), and the ability to use this method for agents with a variety of sizes. These features should make this approach valuable for the entrapment of other proteins and biomacromolecules for HPLC and affinity separation methods. The results obtained with antibodies, streptavidin/avidin and non-porous glass beads

suggest this method should also be useful in biosensors and other techniques that use immobilized proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AGP	α_1-acid glycoprotein
HPAC	high-performance affinity chromatography
HSA	human serum albumin
IgG	immunoglobulin G

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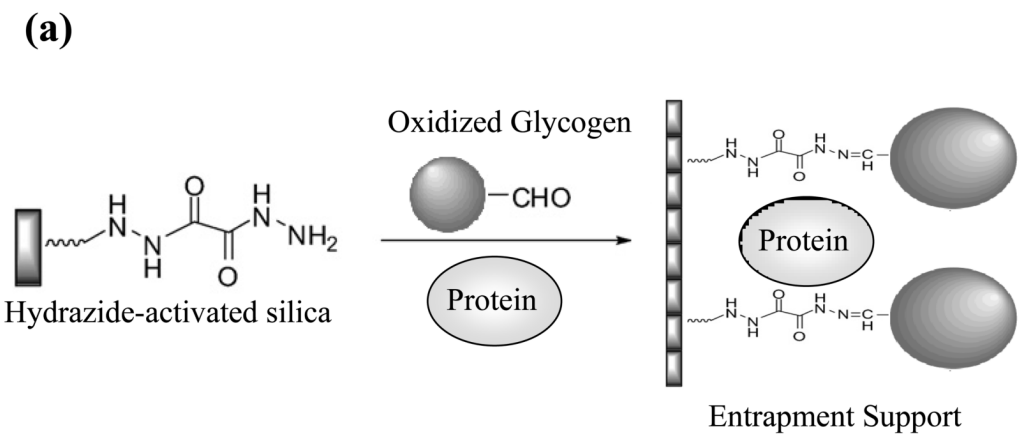
Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version.



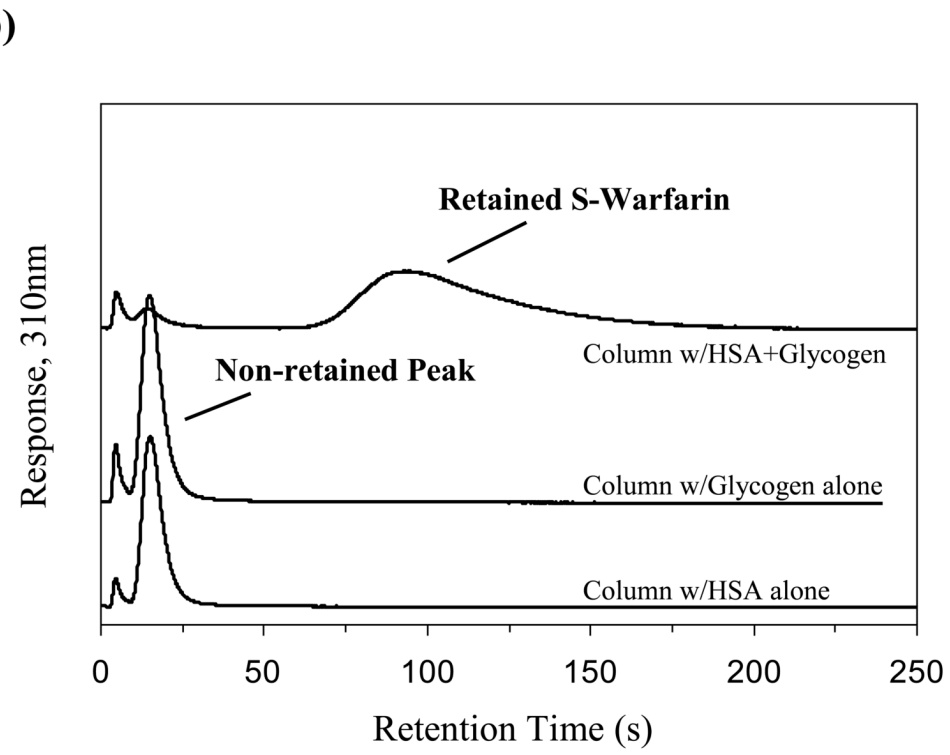


Figure 1.

(a) General scheme for entrapment of a biomolecule such as a protein using a glycogen-capped and hydrazide-activated support, and (b) chromatograms obtained for the injection of *S*-warfarin on an HSA column prepared by entrapment or control columns prepared with the addition of only oxidized glycogen to the support or the addition of only HSA. These chromatograms were obtained by injecting a 25 μL sample of 25 μM *S*-warfarin, which was found to represent linear elution conditions for the HSA column. The broad peak shape noted for *S*-warfarin is typical of what is seen for this analyte on other HSA columns and is a direct result of the strong binding and slow dissociation kinetics for this system [1,5,12].

Table 1

Binding parameters measured for *S*-warfarin with various silica supports containing entrapped HSA[a]

Nominal pore size (Å)	Binding capacity(× 10^{-8} mol)	Specific activity(mol/mol HSA)	Association equilibrium constant (× 10^5 M^{-1})
1000	1.3 (± 0.2)	1.1 (± 0.2)	2.0 (± 0.3)
500	1.8 (± 0.2)	1.29 (± 0.17)	1.4 (± 0.2)
300	1.6 (± 0.2)	0.73 (± 0.11)	1.5 (± 0.2)
100	1.8 (± 0.2)	0.85 (± 0.11)	1.9 (± 0.2)
50	2.1 (± 0.2)	0.56 (± 0.11)	1.3 (± 0.1)

[a]The values in parentheses represent a range of ±1 S.D.