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Review

Hydrazido-derivatized supports in affinity chromatography

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CONTENTS

1. INTRODUCTION

Biospecific affinity chromatography is now a common technique in all areas of biomedical research. Over the years, much effort has been focused on the development and understanding of affinity chromatographic systems and the practical uses thereof. In particular, the chemistry and "structure" of the support and the chemistry of immobilization of ligands have received considerable attention.

The two major requirements for the immobilization of ligands onto insoluble matrices for subsequent use in affinity chromatography are (a) a stable linkage between the matrix and the ligand and (b) retention of specific binding characteristics of the immobilized ligand. Numerous immobilization chemistries have been developed in an attempt to fulfill these requirements. However, because of the extreme diversity of the ligands being immobilized, not one methodology can be considered as universal. On the other hand, certain generalizations may be made and it is one such generalization that serves as the basis for the methodology described in this article, which concerns the immobilization of glycoconjugates specifically via their glycosylation.

Glycoconjugates are ubiquitous in nature and are involved in many cellular and extracellular events including enzymatic activities, the immune system, cell–cell recognition, hormone–receptor interactions etc. From this point of view, glycoconjugates are finding increasing importance in affinity chromatographic systems. The common feature of all glycoconjugates is the presence of one or more sugar moieties covalently

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linked to a non-sugar moiety. In addition, the oligosaccharide moiety(ies) is, in many instances, not involved in the biological activity one wishes to preserve or investigate in a chromatographic system[1–3]. This non-involvement of the oligosaccharide in ligand binding forms the basis of the methodology described below for the specific and site-directed immobilization of glycoconjugates. Particular reference is given to glycoproteins since the immobilization of proteins in general is more problematical with respect to retention of activity than the immobilization of small ligands.

2. PRINCIPLES OF THE METHOD

Specific and site-directed labelling of the oligosaccharide moieties of glycoproteins with fluorescent dyes, biotin etc. is not new and has been reviewed recently[3,4]. The methodologies employed for the site-directed immobilization of glycoproteins via their glycosylation are of course the same as those used for "tagging" glycoproteins. Surprisingly, however, this approach to the immobilization of glycoproteins, or glycoconjugates in general, has not been widely reported.

The method relies on the oxidation of glycoproteins (or other glycoconjugates), specifically on the oligosaccharide moieties. Basically two procedures are available. Galactose oxidase can be used to form a C_6 aldehyde on terminal galactose or N-acetylgalactosamine residues[5]. Since in many instances the terminal sugar of a glycoprotein is a sialic acid, particularly for glycoproteins of mammlian origin, neuraminidase treatment is required, prior to oxidation with galactose oxidase, to expose the penultimal galactosyl residue. The second procedure used to generate aldehydes on the oligosaccharide moieties is chemical oxidation of vicinal diols using sodium metaperiodate[6]. Under suitably mild conditions, periodate oxidation is reported to be specific for the generation of an exocyclic C_7 aldehyde on sialic acids[7]. The aldehydes thus generated, by either enzymatic or chemical means, may then be condensed with nucleophiles such as primary amines or hydrazine derivatives. The chemistry involved is shown schematically in Fig. 1.

The choice between a primary amino-derivatized support or a hydrazido-derivatized support is again one of specificity. Primary amines exist on lysine residues of the protein moiety of glycoproteins and are available for condensation with the aldehydes generated on the oligosaccharide moieties. This results in a competition between the support and the ligand-associated primary amines for the ligand-associated aldehydes and may cause inter- or intra-molecular cross-linking of the ligand. In addition, the Schiff base that is formed from the condensation of an aldehyde and a primary amine is unstable and needs to be reduced, preferably with sodium cyanoborohydride, to a secondary amine. The use of this reagent in itself may have a deleterious effect on the bioactivity one wishes to preserve[8].

Greater specificity can be achieved using hydrazido-derivatized matrices. This is due to the low pK of a hydrazido function (*ca.* 3, ref. 9) compared to the pK of a primary amine which is *ca.* 9–10. Both primary amines and hydrazines will only condense with aldehydes when in the unprotonated form. Therefore the marked difference in the pK values of these two groups allows one to significantly reduce the formation of Schiff bases between the oligosaccharide moieties and the protein moiety of the ligand by performing the coupling reaction under mildly acidic conditions, *ca.* pH 4.5–5.5, where the ligand-associated primary amines are protonated and unreac-

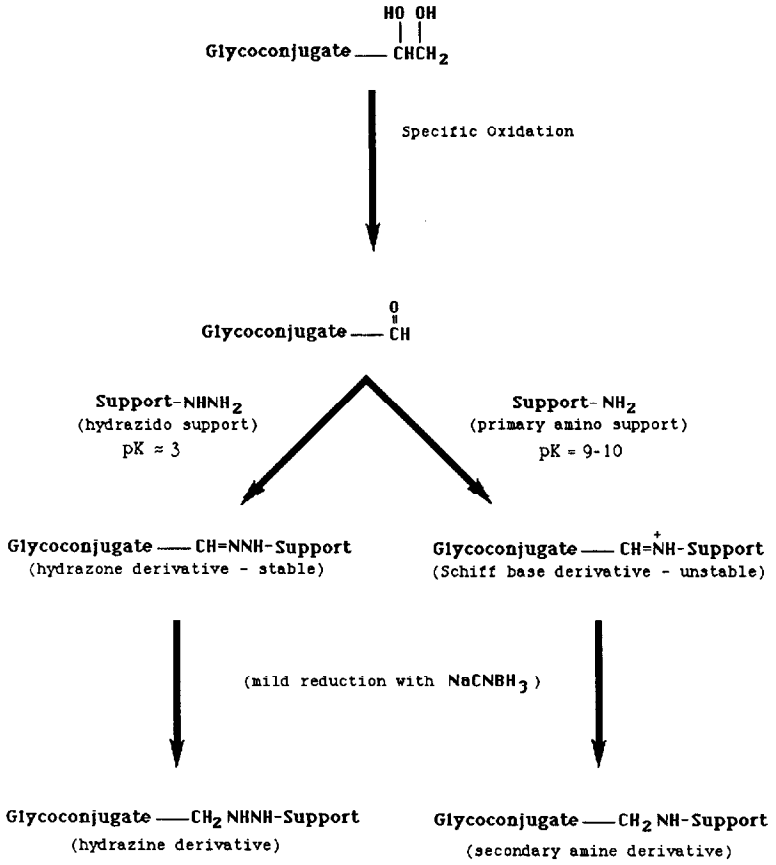

Fig. 1. Flow diagram summarizing the chemistry involved in the site-specific immobilization of glycoconjugates via their glycosylation onto hydrazido-derivatized and amino-derivatized matrices.

tive. In addition, the product of condensation between a hydrazine and an aldehyde is a stable hydrazone, obviating the need for reduction (although this can be performed if desired). The increased nucleophilicity of a hydrazine also results in an increased rate of reaction with an aldehyde, which may be advantageous in some situations. Another important advantage of hydrazido supports is that the linkage to the immobilized ligand, whether in the non-reduced or reduced forms, is non-ionizable and therefore does not add ion-exchange properties to the matrix as happens with the primary amino-derivatized supports[10].

With these points in mind, the use of hydrazido-derivatized supports is recommended over the use of primary amino-derivatized supports. Further discussion will therefore be restricted to hydrazido supports and specific examples from the literature on the uses of these supports for the immobilization of glycoconjugates will be described.

3. IMMOBILIZATION OF GLYCOPROTEINS

The first description of the immobilization of glycoproteins to hydrazido supports appears to be the immobilization of glucoamylase onto carboxymethylcellulose hydrazide[11]. This approach to immobilization was reported to be superior to immobilization via amino acid side-chains with respect to retention of enzymatic activity. However, some loss of activity as well as some precipitation of the oxidized glucoamylase was reported, as had previously been described for horseradish peroxidase[12] and α-amylase[13]. Similarly, some loss in affinity has also been described for avidin immobilized via its oligosaccharide moieties[14]. These results contrast to a report by Zaborsky and Ogletree[2] in which oxidation of glucose oxidase resulted in complete retention of both enzyme protein and enzymatic activity. Junowicz and Charm[15] also reported complete retention of activity of DNAase B after oxidation and coupling to hydrazido supports. Some question therefore remains as to the efficacy of this approach to the immobilization of glycoenzymes. However, no systematic study on the effect of oxidation on the activity of glycoenzymes has yet been presented and it is most likely this reaction which results in a loss of enzymatic activity, rather than the immobilization *per se*. All of the studies presented so far have used chemical oxidation of the oligosaccharides which may result in oxidation of some amino acid residues, thereby leading to a decrease in enzymatic activity. It is conceivable that the more specific enzymatic oxidation of the oligosaccharides would prove effective in studies on glycoenzymes.

4. IMMOBILIZATION OF ANTIBODIES

Unlike the immobilization of glycoenzymes, the results obtained for the immobilization of antibodies clearly demonstrate that site-directed immobilization via the oligosaccharide moieties is superior to amino acid-directed immobilization chemistries. Quash *et al.*[16] were the first to describe the immobilization of polyclonal immunoglobulin G (IgG) via the oligosaccharide moieties. In this report, IgG was immobilized onto hydrazido-derivatized latex particles for use in agglutination experiments. Unfortunately, no quantitation of residual activity or comparison to other methods of immobilization were described.

Recently, however, several authors have presented comparative studies on the immobilization of polyclonal IgGs for use as immunoaffinity matrices. Prisyazhnoy *et al.*[17] reported a 300% increase in activity of rabbit anti-mouse IgG immobilized onto Sepharose-hydrazide when compared to the same antibodies immobilized through SH groups onto maleimide-Sepharose. A similar increase in activity was reported by Hoffman and O'Shannessy[18] for rabbit anti-human IgG immobilized onto the hydrazide derivative of Affi-Gel 10. These authors also showed that the moles antigen bound per mole of immobilized antibody varied depending on the molecular weight of the antigen. Little *et al.*[19] also showed increases in specific antigen-binding activities from 35 to 400%, depending on the antigen/antibody pair, and demonstrated the stability of the immobilized antibodies to various eluting agents such as potassium thiocyanate, urea and high and low pH.

In each of these studies, therefore, a significant increase in antigen binding capacity of the immobilized antibodies was demonstrated for "oriented" *versus*

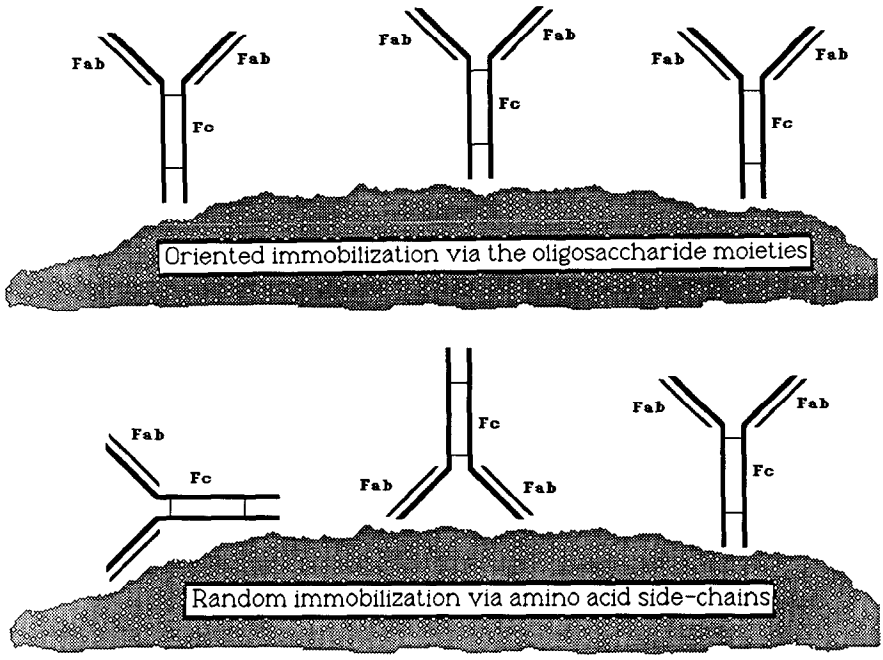

Fig. 2. Schematic depicting the inferred differences in the orientation of antibodies immobilized via "random" amino acid-directed chemistries and via "oriented" oligosaccharide specific chemistries.

"random" coupling procedures (see Fig. 2). The oligosaccharide moieties of polyclonal IgGs are primarily located on the Fc portion of the molecule such that site-directed immobilization via the oligosaccharide moieties should result in the antigen binding Fab regions being oriented away from the matrix, resulting in greater accessibility of antigen. This is similar to the immobilization of IgG onto protein A supports, followed by cross-linking of the IgG to the protein A (ref. 20). Although the oriented *versus* random immobilization model is somewhat oversimplified, the increased retention of antigen binding activity, coupled with the excellent stability of such conjugates, demonstrates the efficacy of this technique for the immobilization of antibodies.

Several other reports on the use of this procedure have been published but no quantitative data presented. Interestingly, two reports on the immobilization of monoclonal antibodies by this procedure did not demonstrate an increase in antigen-binding activity as would be expected from the results reported for the immobilization of polyclonal antibodies, although the activity was comparable to other immobilization techniques[21,22]. The reasons for the lack of increase in activity with monoclonal antibodies are not known. Table 1 lists the known examples from the literature of the site-directed immobilization of glycoproteins via their glycosylation.

5. GENERAL COMMENTS ON THE IMMOBILIZATION OF GLYCOPROTEINS

The immobilization of glycoproteins onto hydrazido supports requires prior oxidation of the oligosaccharide moiety(ies), an indication of the specificity of the

TABLE 1

IMMOBILIZATION OF GLYCOPROTEINS ONTO HYDRAZIDO-DERIVATIZED SOLID SUPPORTS

Glycoprotein	*Support*	*Ref.*
Glucoamylase	Cellulose	11
DNAase B	Cellulose	15
Polyclonal IgG, viral agglutinins	Latex	16
Avidin	Sepharose	14
Glucose oxidase, glucoamylase	Polyethyleneimine	23
Polyclonal IgG	Agarose	24
Polyclonal IgG, avidin, HRP[a]	Agarose	25
Polyclonal IgG	Agarose	18
Invertase	Cellulose	26
Polyclonal IgG	Sepharose, Separon	17
Monoclonal IgG	Matrex-Pel-102	21
Polyclonal IgG	Agarose	19
Polyclonal IgG	Methacrylate	22
Monoclonal IgG_1	Agarose	27
Polyclonal IgG	AvidGel AX	28

[a] HRP = Horse radish peroxidase.

reactions. In addition, a number of authors have shown that the product formed on reaction of an oxidized glycoprotein with a hydrazido support is stable, without reduction, to a number of common eluents such as urea and thiocyanate, as well as to extremes of pH (2–10). The immobilization of glycoenzymes via their glycosylation has also been shown to increase their stability, particularly with respect to temperature.

The reaction of an oxidized glycoprotein with a hydrazido support is a specific acid-catalysed reaction and shows a pH maximum at around 3 (ref. 25). Acetate buffer appears to be the most suitable for the immobilization and buffers containing primary amines, such as Tris, should be avoided. In addition, immobilization is independent of the p*I* of the glycoprotein since fetuin (p*I* = 3.3), human IgG (p*I* = 5.8-7.3) and avidin (p*I* = 10.5) have all been shown to bind to the hydrazide derivatives of Affi-Gels (Bio-Rad)[25]. However, the rate of immobilization of a glycoprotein does appear to be dependent on the degree of glycosylation and possibly the "type" of glycosylation. In this respect, the increased binding of glycoproteins at lower pH values may in part be due to a partial unfolding of the protein, thus exposing the oxidized oligosaccharide and facilitating binding to the hydrazido support.

6. IMMOBILIZATION OF NUCLEOTIDES, NUCLEOSIDES AND RNA

Periodate oxidation of RNA has been shown to be specific for the 3′-terminal *cis*-diol resulting in the formation of a reactive dialdehyde, which may then be condensed with primary amines, hydrazines or other suitable nucleophiles. This technique has long been used for the isolation, purification and analysis of tRNAs[29]. Similarly, RNA species immobilized onto hydrazido supports have been used for the affinity purification of, for example, hybridizable DNA[30], ribosomal proteins[31–34] and the C_5 protein sub-unit of RNAase P[35]. Since only one reactive site (dialdehyde) is

generated per RNA molecule, this technique leads to an extremely site-specific and oriented immobilization of RNA, with the result that the RNA is literally projecting into the liquid phase, anchored only by the 3′-terminal sugar.

Nucleosides, nucleotides and coenzymes possessing vicinal hydroxyls have also been immobilized onto hydrazido supports following oxidation with sodium periodate[36]. Examples of the use of such supports include the purification of glucose-6-phosphate dehydrogenase on agarose-NADP and the adsorption of heavy meromyosin onto agarose-ATP[36]. Pyridoxal 5′-phosphate has also been immobilized onto various hydrazido supports and used for the purification of apo-aspartate aminotransferase[15]. In the case of pyridoxal 5′-phosphate, no oxidation step is required as this compound contains an aldehydic function.

7. MISCELLANEOUS COMPOUNDS IMMOBILIZED ONTO HYDRAZIDO SUPPORTS

The immobilization of sugars onto hydrazido supports for the affinity purification of lectins has been described[37]. In this case, use is made of the linear–cyclic equilibrium of the reducing end sugar, which in the linear form exists as an aldehyde. The aldehyde will condense with the hydrazido support and the hydrazone produced is stabilized by performing the reaction in the presence of sodium cyanoborohydride (see Fig. 1). In a similar manner, heparin has been immobilized onto hydrazido supports and used in studies on heparin-binding proteins[38]. It is worth noting at this stage that multi-site attachment of ligands to the hydrazido support is thought to stabilize the ligand–matrix complex. Single-site attachment of ligands may result in a less stable bond, depending on proximal function groups, and reduction of the hydrazone in such situations is recommended.

Parikh and Cuatrecasas[39] described the preparation and use of ganglioside-agarose derivatives. The gangliosides were oxidized with periodate, allowed to couple to a polyhydrazido-agarose [poly(L-lysyl-DL-alanyl-hydrazido)-agarose] and subsequently reduced with sodium borohydride. Such preparations were useful for the affinity purification of cholera toxin. The use of polyhydrazido-agarose derivatives has been reported to decrease the "leakage" of ligands from such supports. Neoglycoproteins, such as glycosyl albumin, have also been immobilized in a site-specific manner onto hydrazido derivatives of cellulose[40].

Ligands containing functional groups other than aldehydes may also be immobilized onto hydrazido-derivatized supports. Examples of this include the immobilization of the tresyl ester of T-2 fungal toxin for the affinity purification of anti-T-2 antibodies[41] and the immobilization of proteins and other ligands through carboxylic acid functions using carbodiimide activation of the ligand[42,43]. It is worth noting that hydrazido derivatives of a number of support matrices have been used as intermediates in the synthesis of other "activated" supports, such as acyl azides[43].

8. CONCLUDING REMARKS

In the foregoing brief discussion, the methodology for the site-directed immobilization of glycoconjugates onto insoluble matrices, and the uses thereof, were presented. As previously stated, no single immobilization chemistry can be considered as universal. However, it is clear from the literature examples cited that site-directed

immobilization of glycoproteins, particularly antibodies, has inherent advantages not the least of which is retention of biological activity. In the case of nucleosides, nucleotides and RNA, immobilization onto hydrazido-derivatized supports would appear to be the method of choice. Hydrazido-derivatized matrices, along with the associated chemistries, should therefore be added to the repetoir of chemistries available for immobilization of ligands onto insoluble matrices in the preparation of affinity supports.

9. ABSTRACT

Many chemistries have been developed for the immobilization of ligands onto insoluble matrices for subsequent use in affinity systems. One such chemistry which has received little attention involves the use of hydrazido-derivatized solid supports. Hydrazine derivatives are strong nucleophiles which will react with a number of functional groups including aldehydes which may be generated on the oligosaccharide moieties of glycoconjugates by specific oxidation reactions. This paper presents a brief overview of the chemistries involved and the uses of hydrazido-derivatized solid supports for the site-directed immobilization of glycoconjugates. Specific examples from the literature on the uses of affinity matrices prepared by this method are cited.

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