

## Production of glycolipid affinity matrices by use of heterobifunctional crosslinking agents

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**Summary** Photoactivatable heterobifunctional crosslinking agents have been used to prepare glycolipid affinity matrices by nitrene insertion into adsorbed ligand. High degrees of covalent coupling have been obtained and the method has been used to purify antisulfoglycolipid antibodies from immune serum.—**Lingwood, C. A.** Production of glycolipid affinity matrices by use of heterobifunctional crosslinking agents. *J. Lipid Res.* 1984. **25**: 1010–1012.

**Supplementary key words** galactosyl ceramide • sulfatoxygalactosylalkylglycerol • galactosylalkylglycerol

Photoactivatable heterobifunctional crosslinking agents have been widely used in the study of biological recognition at the molecular level (1). However, their use in the preparation of immobilized ligands has not been fully exploited. Guire (2) first described the use of heterobifunctional crosslinking agents for the immobilization of enzymes under mild conditions so as to retain activity. The method was used to crosslink enzymes to cell surfaces (3). No further adaptations of this procedure have been reported, although this is potentially a universal method for the covalent attachment of ligand to a support matrix.

Sulfatoxygalactosylalkylglycerol (SGG) is the major glycolipid of mammalian male germ cells (4, 5). Its synthesis is an early marker of differentiation, and it is maintained in the germ cell plasma membrane throughout the remainder of spermatogenesis (6, 7). Unlike glycosphingolipids which can be coupled after oxidation of the olefinic bond (8) or, in some cases, cleavage of the amide linkage (9), glycolipids cannot be chemically derivatized so as to retain the carbohydrate moiety intact, for covalent attachment to a support matrix. Several non-covalent methods for the production of affinity matrices have been developed (10, 11), but these are not ideal due to either high background absorption or 'bleeding' of ligand from the column. We have used a technique involving coupling with photoactivatable heterobifunctional crosslinking agents for the

covalent immobilization of glycolipids on agarose or glass beads. An SGG affinity matrix thus produced has been successfully used to purify anti-SGG antibodies from crude anti-SGG serum.

## MATERIALS AND METHODS

SGG was isolated from bovine testes as previously described (12). Galactosylalkylglycerol (GG) was prepared from SGG by anchimeric-assisted desulfation in acetone (13). Galactosyl ceramide (GC) was purchased from Supelco, Burkhardt, IN and radiolabeled by reductive tritiation (14). Methyl-4-azidobenzidinate (MABI), hydroxysuccinimidylazidobenzoate (HSAB), aminopropyl and aminoaryl controlled pore glass were purchased from Pierce, Rockford, IL. Aminoethylagarose was purchased from P.L. Biochemicals.

### Derivatization of amino matrix

Amino beads (100 mg) were shaken in the dark for 1 hr at room temperature in methanol–water 1:1 containing an appropriate amount of HSAB (diluted from 100 mM stock in dimethylsulfoxide) or in water containing MABI (added in aliquots at 10, 20, and 40 min). The beads were centrifuged and washed once with water. A solution of the ligand to be coupled was then added as follows. The glycolipids were dissolved in ethanol (1 mg/ml) and an equal volume of water was added. This solution was added to the beads and the beads were then flash-evaporated in the dark. Photosensitive beads with adsorbed ligand were then irradiated 1 cm from a Mineralight UVS II lamp at 260 nm for 2 min with stirring. The beads were then washed extensively as follows. Glycolipids immobilized on glass beads were washed with 30 ml of ethanol (~60 column volumes), 30 ml of chloroform–methanol 1:1, and finally with 30 ml of chloroform–methanol 2:1 (v/v). In the case of agarose supports, the chloroform–methanol 2:1 wash was omitted. The wash fractions were pooled and evaporated and the residue was redissolved in the original ligand volume. In the case of radiolabeled ligands, binding was quantitated by counting an aliquot of the wash and comparing with the original material. An aliquot of the matrix was also counted, but because of the difficulty of gel volume measurement, this value was not used quantitatively. In the case of unlabeled glycolipids, coupling was measured by galactose dehydrogenase assay after acid hydrolysis (15). For glass beads, a minimum value for coupled glycolipid was obtained by measurement of galactose released after acid hydrolysis of an aliquot of the matrix. Control experiments were also performed in which the crosslinking agent was omitted and no coupling of ligand was observed. More-

Abbreviations: SGG, sulfatoxygalactosylalkylglycerol; GG, galactosylalkylglycerol; GC, galactosyl ceramide; MABI, methyl-4-azidobenzidinate; HSAB, hydroxysuccinimidylazidobenzoate.

over, no binding was observed if such a matrix were treated with 10% benzaldehyde, to provide a reasonable control for hydrophobic interaction. Unreacted amino groups were blocked by washing the matrices with 1% formaldehyde (five column volumes).

#### Purification of anti-SGG antibodies from immune serum

SGG (0.4 mg) was covalently linked to 200 mg of aminopropyl glass pretreated with 2 mM HSAB as described above. The 1-ml column (7 mm i.d.) was pretreated with BSA (10 ml, 1 mg/ml) and extensively washed with 1 M KI followed by 1 M KSCN, and finally, phosphate-buffered saline (PBS). Two ml of crude immune serum (12) was applied at 4°C and the column was washed with PBS. The column was batch-eluted in sequence with 20 ml of 1 M KI and 20 ml of 1 M KSCN. The eluted fractions were transferred to dialysis bags and concentrated against dry sucrose crystals and then extensively dialyzed against PBS. Complement fixation versus SGG was performed as previously described (12).

## RESULTS AND DISCUSSION

SGG was successfully covalently bound to a variety of amino supports by the use of photoactivatable heterobifunctional crosslinking agents (Table 1). Coupling was dependent on a crosslinker since no binding of SGG to amino glass alone or to benzaldehyde-treated aminophenyl glass was observed, eliminating the possibility of hydrophobic binding. Some deleterious effect was noted for the agarose supports treated with strong organic solvents, and prolonged washing in ethanol might prove a procedure more amenable to the subsequent use of the affinity column.

Fig. 1 shows the elution profile of a 2-ml aliquot of crude anti-SGG serum (12) applied to an SGG-glass

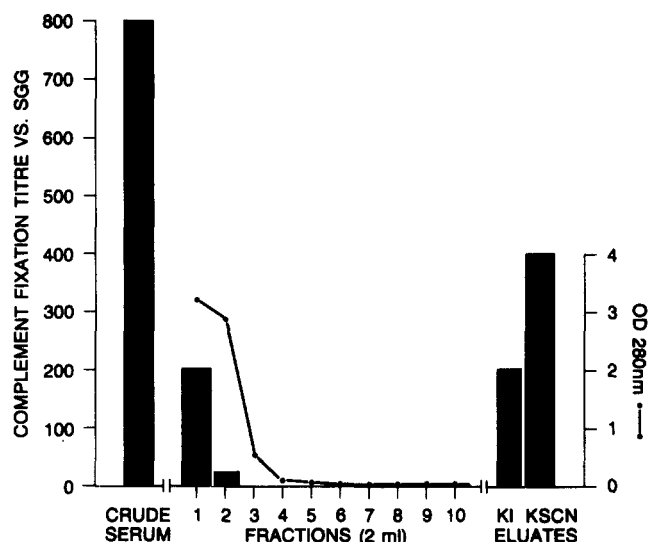


Fig. 1. Purification of anti-SGG antibodies from whole immune serum on SGG-glass.

affinity column prepared as described in Methods. The anti-SGG complement fixation activity in the flow-through fraction was greatly reduced (the column may have been overloaded). The column was washed with PBS and the majority of the anti-SGG activity was later recovered in the high salt eluates. The ligand did not 'bleed' from the matrix during antibody elution and the column could be reused after washing with buffer.

SGG-glass has also been successfully used as immunogen in the *in vitro* stimulation method (16) for the production of monoclonal hybridoma antibodies (E. M. Eddy, C. H. Muller, and C. A. Lingwood, unpublished results) for which particulate antigens are particularly suited.

Table 2 lists the immobilized glycolipids so far prepared by this procedure. In the case of GC, extent of coupling was also monitored by use of <sup>3</sup>H-labeled GC prepared by catalytic hydrogenation (14). Binding was determined by counting an aliquot of the matrix after coupling. These ligands are suitable for affinity chromatography of antibodies or enzymes. Preliminary experiments have shown (K. Oda and C. A. Lingwood, unpublished results) that a testicular galactolipid sulfo-transferase will selectively bind to, and can be released from, GG-glass.

The use of heterobifunctional crosslinking agents is a quick, simple procedure for the immobilization of probably any hydrocarbon ligand. This report demonstrates that immobilized SGG is an effective affinity matrix for purification of anti-SGG antibodies. Potential for other uses has been indicated. The use of nitrene insertion as a method of coupling will result in a wide range of undefined linkage positions. Although the

TABLE 1. Covalent linkage of SGG to a support matrix by use of photoactivated crosslinking agents

	% Coupled
Aminopropyl-glass (100 mg) 2 mM HSAB	
0.1 mg SGG	17.8
0.2 mg SGG	71.0
0.4 mg SGG	40.0
Aminophenyl-glass (100 mg) 0.4 mg SGG	
1 mM HSAB	20
2 mM HSAB	35
4 mM HSAB	39
Aminohexyl-agarose (0.5 ml) 0.4 mg SGG	
1 mg/ml MABI	53
2 mg/ml MABI	42
3 mg/ml MABI	60

TABLE 2. Affinity columns prepared by photoactivated crosslinking

Ligand	Crosslinking Agent	Support	% Covalent Coupling	Immobilized Ligand Density <i>μmol/ml</i>
SGG (0.2 mg)	2 mM HSAB	NH <sub>2</sub> propyl-glass	71	0.34
SGG (0.4 mg)	2 mM HSAB	NH <sub>2</sub> phenyl-glass	35	0.34
SGG (0.4 mg)	1 mg/ml MABI	NH <sub>2</sub> propyl-glass	39	0.3
SGG (3.5 mg)	1 mg/ml MABI	NH <sub>2</sub> -agarose	46	1.23
GG (2.0 mg)	2 mM HSAB	NH <sub>2</sub> -agarose	70	0.87
GG (3.3 mg)	2 mM HSAB	NH <sub>2</sub> propyl-glass	91	0.40
GC (0.4 mg)	2 mM HSAB	NH <sub>2</sub> phenyl-glass	73	0.73

spectrum of linkages may be reduced, for example by electrostatic or hydrophobic interactions, this property is of advantage when immobilizing ligands in which the domain of biological relevance is unknown. From mere probability considerations, some of the immobilized ligand molecules will have the required site free.

The variety of photoactivatable heterobifunctional crosslinking agents commercially available increases the adaptability of the method. Thiol, rather than amino-reactive crosslinkers could be used. Cleavable crosslinking agents containing disulfide (17) or azo (18, 19) groups might be used, when the bound ligand could be released under mild reducing conditions.

This work was supported by a Canadian Medical Research Council Scholarship and by MRC grant no. MA 7712. The technical assistance of summer student Dr. M. Shirley is gratefully acknowledged.

Manuscript received 5 March 1984.

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