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Immobilization of DNA on Poly(Glycidyl Methacrylate-Co-Ethylene Dimethacrylate), Bead Cellulose and Sepharose

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Summary

The copolymer of glycidylmethacrylate with ethylene-dimethacrylate, its derivatives, bead cellulose and Sepharose were used for immobilization of DNA via new coupling methods: reaction with oxirane, acylhydrazide hydrazide, and diazonium salt. The susceptibility of immobilized DNA for DNAse was tested. The most efficient immobilization was achieved on carriers with acylhydrazide groups. The binding capacity of the carrier and the susceptibility of immobilized DNA for the DNase depends on the length of the spacer. The immobilized DNA is stable in solution for several weeks, and in the lyophilized state for several months.

Introduction

Affinity chromatography on carriers with immobilized DNA is a potent method for separation and purification of DNA binding proteins, nucleic acids, anti-DNA antibodies, etc. (WEISSBACH and POONIAN, 1974; ARNDT--JOVIN et al., 1975). The immobilized DNA for these purposes should be stable in wide range of pH values, should be accessible to combination with other macromolecules, which, on the other hand, should not exhibit nonspecific sorption on the carrier. The amount of immobilized DNA should be reasonably high (0.5 to 5 mg DNA per 1 ml of the gel) (ARNDT-JOVIN et al., 1975).

DNA has been immobilized on a great number of natural and synthetic matrices by many methods. It has been entrapped in polyacrylamide gel (CAVALIERI and CARROL, 1970) or agarose gel (SCHALLER et al., 1972), linked to cellulose after activation of the terminal phosphate group with carbodiimide (GILHAM, 1968), coupled with CNBr-activated Agarose (ARNDT-JOVIN et al.,1975), trichlor-triazine-activated cellulose (BIAGIONI et al., 1978) or attached to porous glass (SKRYOBIN et al., 1976). Some other techniques of DNA immobilization have been reviewed by WEISBACH and POONIAN (1974).

It has been found during the search for DNA sequencing through specific base modification that bases of DNA and RNA could be copulated with diazonium salts prepared from aryl amines (MOUNDRIANAKIS and BEER, 1965).

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This reaction is 60 times faster with guanine than with other bases. It was described by the same group of authors (GAL-OR et al., 1967) that cytidine reacted selectively with acyl hydrazides. These reactions are applied in the present work.

In our previous contribution, oxirane groups containing macroporous copolymer of glycidyl methacrylate with ethylene dimethacrylate (G-gel) were reported as a very versatile carrier for protein immobilization (DROBNÍK et al., 1979). Various derivatives of G-gel as well as bead cellulose (PEŠKA et al., 1976) and Sepharose modified with different spacer chains are used for DNA immobilization via the methods mentioned above.

Materials and Methods

All chemicals were purchased from Fluka unless stated otherwise and were of p.a. quality. N-(N-Glycyl -glycyl)glycine ethyl ester was prepared by esterification of the tripeptide (FISCHER, 1903). Carriers used for immobilization of DNA are schematically presented in Fig.1. Carrier GI was the copolymer (glycidyl methacrylate with ethylene dimethacrylate 70:30) as described previously (DROBNÍK et al., 1979) and also its modifications were accomplished by the technique described in this paper: Carrier GII was prepared by treatment of G-gel with hydrazine hydrate, carriers GIII to GIV were prepared by a reaction of G-gel with the ethylesters of ω-aminoacids or their oligomers with subsequent treatment with hydrazine hydrate. Carriers GVII and GVIII were obtained by reacting 4-amino-N-(2- -aminoethyl)benzamide with G-gel and with G-gel previously treated with ethylester of 6-aminohexanoic acid, respectively.

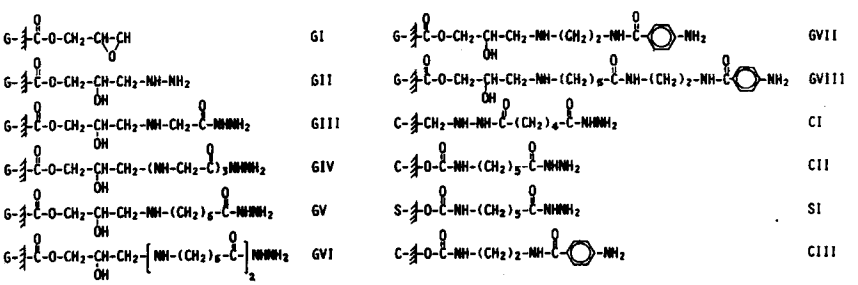
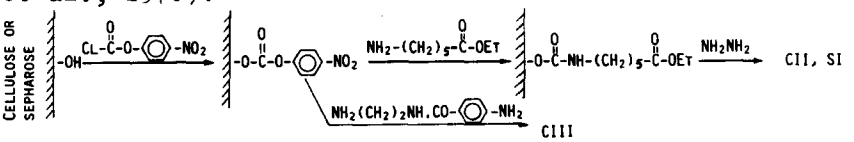


Fig. 1 Carriers used for immobilization of DNA
G - glycidyl methacrylate-co-ethylene dimethacrylate; C - bead cellulose; S - Sepharose 4B Cl

Carriers CI to CIII were based on bead cellulose, the fraction with diameter 0.3 - 0.75 mm (PEŠKA et al., 1976); PEŠKA et al., 1978), carrier SI on Sepharose 4B Cl (Pharmacia). The carrier CI was prepared by the reaction of oxidized cellulose (PARIKH et al., 1974)

with adipolyl dihydrazide (SMITH, 1956) and subsequent reduction with BH_4 (PARIKH et al., 1974). Carriers CII, CIII and SI were prepared by the activation with p-nitrophenyl chloroformiate as described elsewhere (DROBNÍK et al., 1978):



The solution of DNA was prepared by dissolving 175 mg of highly polymerized DNA from calf thymus (Sigma) in 100 ml of 0.15 M NaCl containing 0.01 M borate pH 7.5 and 0.01% sodium azide. The total amount of DNA used for immobilization is given in Table 1.

Denaturation of DNA was performed by heating the solution of DNA for 2 min. in a steam bath, and cooling at 10°C. It was used for immobilization without delay. The hyperchromic effect was 25%.

The Immobilization Procedures

Reaction with acylhydrazides (method A). The solution of DNA was added to a suspension of the carrier (Table 1) in 10 ml of 0.2 M acetate buffer of pH 4.2 which contained 0.15 M NaCl. After gentle shaking for 24 h at constant temperature the supernatant was removed and solution of 25% glucose in 0.1 M acetate pH 5.5 was added. The mixture was shaken for another hour at the same temperature.

Copulation of diazonium salts with DNA (method B). The carrier (Table 1) was suspended in 7.5 ml of 80% acetic acid, cooled in an ice bath, and 0.3 g of $NaNO_2$ were added. The mixture was shaken at 4°C for 15 min. The carrier was then separated, washed with 0.05 M ice cold borate buffer containing 0.15 M NaCl, and a solution of DNA diluted with 7.5 ml of the above buffer was added. The shaking at 4°C continued overnight.

Direct reaction with oxirane groups (method C). 1 g of G-gel (GI) was gently shaken in 0.05 M borate buffer pH 7.5 containing 0.15 M NaCl and 0.01% sodium azide with the solution of DNA for 10 days.

The azide method (D). 1 g of the carrier GIII, GIV, GV, or GVI was suspended in 7.5 ml of 10% HCl, cooled with ice and treated with 0.3 g of $NaNO_2$. After 15 min the carrier was quickly separated, washed with ice cold 0.05 M borate buffer pH 7.5 with 0.15 M NaCl, and transferred into the above buffer with the solution of DNA. The suspension was shaken at 4°C overnight.

All the preparations of immobilized DNA were washed with 2 litres of 1 M NaCl pH 7.5 with 0.01 M borate.

The flow rates of all carriers after modifications and immobilization of DNA remained essentially unchanged.

The yield of the immobilized DNA was estimated by measurement of phosphorus by the modified method of MURPHY and RILEY (1962). This method is able to detect 0.01 μmol of phosphorus in 1 g of carrier. 10 to 100 mg of dry carrier was mineralized in 10 ml volumetric flask by heating with 10 drops of concentrated H_2SO_4 and 20 drops of $HClO_4$ (Merck). The clear colourless solution was diluted with water, neutralized with NaOH (phenolphthalein), heated for 20 min in a steam bath, mixed with freshly prepared reagent, diluted with water exactly up to 10 ml and its optical density was measured at 890 nm. The amount of phosphorus was calculated from calibration curve constructed with KH_2PO_4. The reagent was prepared from 8 ml of 2.5 M H_2SO_4, 4.8 ml of 0.1 M ascorbic acid, 0.8 ml of a 0.28% solution of $K/C_4H_2O_6Sb(OH)_2/.1/2\,H_2O$ and 2.4 ml of 4% solution of ammonium molybdate. It was found that 1 g of G-gel, bead cellulose and Sepharose contained less than 10 n-mols, 33 n-mols and 40 n-mols of phosphorus respectively.

The fraction of immobilized DNA susceptible to pancreatic DNase (EC 3.1.4.5) was determined according to POTTER et al. (1952). The wet carrier was shaken in 4 ml of 0.05 borate buffer pH 7.1 containing 0.1 M $MgSO_4$ and 0.1 mg DNase (Sigma) for 24 h at 37°C. The optical density of the supernatant was measured at 260 nm. The amount of released nucleotides was calculated from the calibration curve constructed with different concentrations of DNA hydrolyzed by DNase. Blank without the enzyme was treated in a similar way. The exact weight of the carrier was determined after the treatment with DNase.

Lyophilisation was performed as follows: 3 ml of wet carrier were washed with water, stirred in 25% sucrose solution, the excess of the liquid was removed and the rest was lyophilized.

Results and Discussion

The results of immobilization experiments are summarized in Table 1. Some general conclusions can be drawn from them.

(1) No nonspecific sorption of DNA was observed with either carrier, as can be seen from methods D and even C. Washing with 1 M NaCl is sufficient for removal of uncoupled DNA. Therefore, all carriers tested can be used in experiments with nucleic acid hybridization. With proteins, the nonspecific sorption may be different from protein to protein. In general, the sorption decreases in the sequence G-gel >> cellulose > Sepharose.

(2) The capacity of the binding reaction with hydrazides (method A) grows with increasing temperature. The denaturation of DNA should be considered besides the increase of the binding reaction efficiency. The DNA is partly denatured in the acid buffer already at

room temperature; a higher temperature extends the strands separation, exposing in this way more bases for the binding reaction. This effect can be clearly seen from the results of the copulation with diazotated carriers (method B). The binding of the denaturated DNA is twice as effective as that of the native one.

(3) Hydrazine groups as in GII are also present in some extent in all carriers based on G-gel and prepared by hydrazine hydrate treatment in the last step of modification (DROBNÍK et al., 1979). Since there is immobilization on GII, we may expect some contribution of hydrazine groups to immobilization on carriers GIII to GVI as well.

(4) The important factor affecting the immobilization yield is the length of the spacer chain between the carrier and the reactive group.

(5) The susceptibility of immobilized DNA to the DNase is also highly influenced by the length of the spacer. The chain composed of 6 atoms in a row is sufficient to provide complete accessibility. However, all preparations derived from G-gel modified with acylhydrazide contain some hydrazine groups which also bind DNA, but being too close to the matrix, do not provide full accessibility. Probably, this is why 100% accessibility cannot be reached even with such a long spacer as in GVIII. These results can be used as model for the interaction of immobilized DNA with medium-size proteins in general, which is critical for many affinity separations.

The stability of the immobilized DNA was tested with the preparations No 15, 26, and 28 listed in Table 1 (carriers GV, CII and SI). The preparations were kept under various conditions and washed afterwards with the same buffer as after the immobilization procedure. The amount of DNA was estimated by the cleavage with DNase. The results are given in Table 2. It can be seen that the immobilized DNA is not detached by exposures to pH 3 and pH 10 for several hours. Its accessibility is only slightly affected by storage in solution and in lyophilized state. Drying almost completely blocks the access to DNA on carriers based on polysaccharides, whereas the G-gel owing to its rigid structure (high degree of crosslinking) preserves quite a high fraction of DNA accessible to the enzyme molecule.

On evaluating all the methods tested, we may conclude that the reaction with acid hydrazides is the most promising binding procedure. The only disadvantage may be seen in partial denaturation and possible depurination of DNA during binding. It can be relaxed by lowering the temperature. An immobilization yield of 50% and 2 mg of DNA per 1 ml of the gel is obtained in this case. When singlestranded DNA is to be immobilized, 90% yields and up to 7 mg of DNA per ml can be reached.

TABLE 1

Results of Immobilization of DNA

Sample No	Carrier	Binding groups[a]	DNA in reaction	Method[c]	Temperature	Immobilized DNA		
		μmol/g	mg[b]		°C		Yield of immob. %	DNAse susceptible %
1	GI	4450	8.75	C	25	0.13	3	
2	GII	1110	8.75	A	25	0.7	18	18
3	GII		8.75	A	60	1.57	39	22
4	GII		52.25	A	60	1.59	7	21
5	GIII	1160	8.75	A	25	0.67	17	55
6	GIII		8.75	A	60	1.26	32	53
7	GIII		52.25	A	60	1.72	7	48
8	GIII		52.25	D	4	0	0	-
9	GIV	540	8.75	A	25	1.91	48	86
10	GIV		8.75	A	60	2.39	60	89
11	GIV		52.25	A	60	2.29	10	88
12	GIV		52.25	D	4	0	0	-
13	GV	880	8.75	A	25	2.03	51	89
14	GV		8.75	A	60	2.54	64	97
15	GV		52.25	A	60	7.22	30	100
16	GV		52.25	D	4	0	0	0
17	GVI	620	8.75	A	60	3.62	91	98
18	GVI		52.25	A	60	6.75	28	97
19	GVI		52.25	D	4	0	0	-
20	GVII	460	8.75	B	4	0.29	7.3	85
21	GVII		8.75	B	25	0.28	7	88
22	GVII		8.75	B[d]	25	0.69	17	94
23	GVIII	790	8.75	B	25	0.35	9	93
24	GVIII		8.75	B[d]	25	0.71	18	89
25	CI	3030	8.75	A	60	0.19	5	100
26	CII	160	8.75	A	60	0.53	15	100
27	CIII	150	8.75	B	4	0.18	5	100
28	SI	180	8.75	A	60	0.33	9	100

[a]Calculated from elemental analysis DROBNÍK et al.,1979. [b]DNA added to 1 g of carrier derived from G-gel or to 2.5 ml of bead cellulose or Sepharose. [c]Methods: A reaction of hydrazine or acylhydrazide, B copulation of diazonium salt, C reaction of oxirane, D azide method. [d]Denaturated DNA was used. [e]Calculated from P-content on the bases of 8% w/w of phosphorus in the DNA. Swollen Sepharose contained 4% of dry gel, cellulose 9%. 1 g of G-gel gave 2.2 ml of swollen gel.

TABLE 2
The Stability of the Immobilized DNA

Sample No[a]	% of DNA ausceptible to DNase after following treatment[b]					
	pH 3[c]	pH 10[d]	Storage in solution	Lyophiliz-ation[f]	Lyophil. and storage[g]	Drying[h]
15	100	100	100	91	90	83
26	100	100	94	93	90	3
28	100	100	90	88	88	1

[a]see Table 1. [b]Determined by hydrolysis with DNase. [c]The gel was washed for 3 h with 0.1 citrate buffer pH 3 with 0.15 M NaCl. [d]0.05 M borax pH 10 was used in the same procedure as ad[c]. [e]The gel was stored for 1 month in 0.15 M NaCl with 0.01 M borate, pH 7.5 and 0.01% sodium azide. [f]The gel was lyophilized with 25% sucrose. [g]The lyophilized gel was stored at room temperature for 6 months. [h]The gel was dried in vacuum over H_2SO_4.

These figures can be compared with the best results published: BIAGINI et al.(1978) referred 20 mg of DNA (yield 90%) per 1 g of cellulose (about 2 mg per 1 ml of swollen carrier) and ARNDT-JOVIN et al.(1975) obtained 8 μmols of phosphorus per 1 ml of Sepharose (about 3 mg of DNA).

It was the G-gel which was examined in detail in our experiments. The other carriers were taken as reference. Therefore, optimal conditions for binding were not looked for. Particularly, the density of binding groups can be tuned up. Their high content (CI) distorts the porosity, whereas low substitution brings low yields (CII).

The reasonable preservation of accessibility on G-gel after drying opens interesting ways to chemical modifications of attached DNA, as G-gel can be used in almost any organic solvent without any substantial change in porosity. In some solvent systems the same is true with bead cellulose (PEŠKA et al., 1976).

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