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Diazotizable arylamine cellulose papers for the coupling and hybridization of nucleic acids

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ABSTRACT

We describe the synthesis of a family of arylamine-substituted papers which can be diazotized and coupled with nucleic acids. The synthesis is simple and uses readily obtainable starting materials. A partial characterization of the nucleic acid binding activity is reported, as well as a demonstration of the utility of the activated paper for the detection of electrophoretically separated RNA by blot transfer and hybridization.

INTRODUCTION

The reaction of DNA and RNA with m-diazobenzyloxymethyl cellulose (DBM cellulose) has been systematically developed by Stark and colleagues into a general technology for the immobilization and detection of fractionated RNAs and DNAs (1-5). One extension of that technology has allowed the rapid immunological detection of proteins fractionated in acrylamide or agarose-acrylamide composite gels (6). In this report we describe alternate methods for the synthesis of arylamine-substituted cellulose papers suitable for diazotization and coupling with nucleic acids and proteins.

Aromatic amine groups have been introduced onto cellulose for the purpose of coupling with proteins and other molecules of biological interest by a wide variety of approaches (for an introduction see Weliky and Weetall (7)). Because unsubstituted cellulose is a weak nucleophile, either drastic reaction conditions or strong electrophiles have been required to derivatize free cellulose hydroxyl groups. These conditions preclude the use of unprotected aromatic primary amine reagents because the amine moiety is generally sufficiently nucleophilic to react with whatever electrophile is to couple with the

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cellulose. The strategy discussed below relies on the prior activation of cellulose paper with oxirane groups. The oxiranes then selectively and rapidly couple with the strongly nucleophilic thiophenoxide anion of a primary amine thiophenol. With this approach mild reaction conditions and easily obtainable reagents can be used throughout. In addition, a large number of different substituents can be introduced onto the cellulose, and their suitability for the experiments of interest explored.

MATERIALS AND METHODS

A. Reagents. All organic chemicals were obtained from Aldrich with the exception of p-aminophenol (technical grade), which was from MCB. Filter papers were obtained from either Schleicher and Schuell or from Whatman, as noted in the text. All solvents were reagent grade or higher.

B. Preparation of Substituted Papers. The following steps were carried out in a well-ventilated fume hood. Precut sheets of filter paper weighing about 20g were placed in a heat-sealable polyester bag (Sears Seal-N-Save) and 70ml of 0.5 M NaOH solution added, followed by 30ml butanediol diglycidyl ether (bisoxirane, (8)). Reactions with epibromohydrin were carried out with 92ml NaOH solution and 8 ml epibromohydrin(9). The bags were sealed,leaving an air bubble of 100 to 200 ml, and attached securely to a homemade rotator having a fixed speed of 30 rpm. The bags were tumbled end over end for either 8 to 16 hours (bisoxirane synthesis) or 4 hours (epibromohydrin synthesis). Horizontal agitation was not sufficient to allow good uniform coupling. After activation, the fluid contents of the bag were drained into a waste container and allowed to inactivate for at least two days before disposal. The activated papers were then reacted with aminophenols or thiophenols in either of the two following ways: (i) the aromatic amine of interest was dissolved or suspended at 10g per 40ml of solvent (ethanol or acetone) and added directly to the bag, which was then resealed and rotated an additional 24 hrs, or (ii), the activated papers were washed for a few minutes to remove the excess bisoxirane in 500ml of a solution composed of equal volumes of ethanol and 0.5M NaOH. The papers were then added individually to a 1% solution of the amine

reagent made by dissolving the amine in ethanol to 2%, and diluting with an equal volume of 0.5M NaOH. Coupling was allowed to procede for 4 hrs. Papers prepared by either of the procedures above were washed by sequential immersion in ethanol and 0.1M HCl, typically for 15 minutes each, with two repetitions of the cycle. The papers were given a final rinse in ethanol, and dried in the dark. p-aminophenylthioether substituents were prepared from p-aminophenyldisulfide by procedure (i) above, except that 1g of sodium borohydride was added to the bag at coupling to convert the disulfide to a thiophenol. The m-aminophenylthioether was prepared from m-nitrophenyl disulfide, using borohydride reduction with coupling as above and by reducing the resulting m-nitrophenylthioether paper with dithionite as described elsewhere (2). Excess thiol reagent resulting from either procedure (i) or (ii) above was oxidized with household bleach before disposal.

C. Diazotization and Coupling with Napthol. Aminophenylether or thioether papers were diazotized by immersion in 1.2M HCl containing 250 micrograms per ml of $NaNO_2$ (2) at 0° for one hour. For routine use the aminophenylthioether substituents were diazotized in the same mixture for 15 minutes. The extent of diazotization was estimated by reaction of the papers with saturated 2-napthol in a 1% aqueous solution of sodium tetraborate. The napthol was dissolved in dimethylsulfoxide and diluted into the borate solution. Thioether papers gave bright red to orange-red derivatives, the ethers pink to salmon. The adduct of p-aminophenol prepared by procedure (i) gave a labile paper which yielded a green color with napthol. When the paper was prepared by procedure (ii), a pink color was observed. This behavior was not investigated further.

D. Conversion to Fluoborate or Hexafluorophosphate Salt. Diazophenylthioether papers prepared as in C. above were converted from the chloride to the fluoborate or hexafluorophosphate form by agitation in a 1% solution of $NaBF_4$ or $NaPF_6$ (10). The paper was either used immediately, or washed with ethanol followed by acetone, dried, and stored at -20°.

E. Transfer of DNA to Activated Diazo Papers. Nick-translated pBR322 DNA was digested with Alu I endonuclease and fractionated

in a 2.5% native agarose gel. DNA fragments were denatured in situ with NaOH, and the gel or gel slices equilibrated with 0.1M acetate buffer pH 4. Transfer to diazotized paper strips prepared as in C. or D. above was allowed to procede for 24 hrs at 4°, after which no counts were found remaining in the gel. Filter strips were dried, autoradiographed, and counted in a scintillation counter. pH dependence experiments were carried out by equilibrating gel slices in citrate-phosphate buffers of constant ionic strength (I=0.25) and varying pH (11). Transfer was for 24 hrs at 4° as above. Acetate or citrate-phosphate buffers were equally effective in allowing transfer to o-diazophenylthioether paper at pH 4, and no difference was observed between the fraction of counts transferred at 4° and at 22°. Radioactivity bound to diazotized papers was not eluted by incubation in prehybridization buffer (see below), followed by exposure to solutions of high ionic strength (I = 2) at 55° for several hours. Counts bound could not be removed by incubation with the diazonium coupling agents azide, iodide, or glycine, nor by immersion in sodium dithionite solution at elevated temperature.

F. Transfer of RNA, Prehybridization and Hybridization. Rabbit reticulocyte RNA of known β-globin mRNA concentration (determined by gel densitometry) was the generous gift of E. Lacy and R. Hardison. Total Sea Urchin RNA was donated by W. Klein. Denaturation of RNA was carried out in 2.2 M formaldehyde in 50% formamide as described by Rave et al. (12). Electrophoresis was carried out in a 1% agarose gel containing 2.2 M formaldehyde, 20mM Na MOPS (3-(N-morpholino)propanesulfonic acid) pH 7.0, 5mM Na acetate, and 1mM EDTA (Seed and Goldberg, unpublished). The electrode buffer lacked formaldehyde. Slices of gel were rinsed several times in 0.1 M Na acetate buffer, pH 4, generally for 20 minutes per rinse. Two rinses at 65° followed. The gel was blotted overnight at 4°, and diazo groups remaining on the paper inactivated by incubating the paper in a prehybridization mix containing 1% glycine (2) at 37° for 18 to 24 hours. The prehybridization mix contained: 10X Denhardt's solution (0.2% Bovine Serum Albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidine), 50% formamide, 50mM Tris-HCl pH7.5, 1M NaCl, 0.1% Na

pyrophosphate, 10% Dextran Sulfate (3), 1% glycine, and 100 μg/ml denatured salmon sperm DNA. Denatured nick-translated (13) pØG-1 DNA (14) was added to the prehybridization mix, and allowed to hybridize for 12 hours at 37°. The hybridized paper was washed at 65° for several hours in 2X SSC, followed by a longer wash in 0.1X SSC.

RESULTS

Figure 1 outlines the overall synthesis, which procedes in two steps. In the first step an epoxide-substituted matrix is created by reacting cellulose filters with either a symmetrical bisoxirane, such as 1,4 butanediol diglycidyl ether, or an epihalohydrin, such as epibromohydrin, under alkaline conditions. The reactions closely follow conditions described for the introduction of oxirane groups onto agarose by Sundberg and Porath (8) and Nishikawa and Bailon (9). However, because cellulose is more resistant to alkaline oxidation than agarose, it is not necessary to include the sodium borohydride used by these workers. In the second step, the oxirane-cellulose is reacted with primary amine phenols or thiophenols in alkaline solution to give primary amine ethers or thioethers. The coupling with aminophenols is considerably less efficient than with aminothiophenols, as reflected in a weaker reaction of the

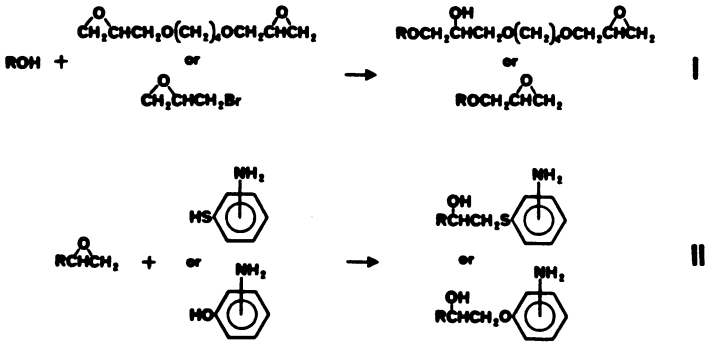

Figure 1. Two-step synthesis of arylamine cellulose. In reaction I ROH represents a cellulose hydroxyl group, while in reaction II R represents the remainder of the oxirane ligand attached in reaction I.

diazotized products with 2-napthol and with single stranded DNA in gel transfer experiments (Table 1). An exception was the the p-aminophenol adduct, which exhibited qualitatively very different behavior from all other ether ligands. However this behavior could not be reproduced in paper prepared under more controlled conditions of synthesis (Materials and Methods).

Although ortho, meta, and para-aminothiophenols all reacted rapidly and strongly with oxirane-cellulose, the stability and reactivity of the adducts differed significantly after diazotization. The rate of spontaneous loss of reactivity with napthol proceded in the order meta > ortho > para. The bisoxirane linked p-diazophenylthioether ligand lost napthol reactivity faster than the epibromohydrin-linked form, and with an attendant color development suggestive of a self-coupling reaction. Self-coupling may account for the decreased DNA binding of the bisoxirane linked form. Of the different thioether ligand papers tested, the ortho isomer had superior DNA binding capacity (Table

Table 1. Blot transfer of pBR322 Alu fragments to diazo papers.

amine ligand	oxirane ligand	BF_4 treatment	% counts bound
o-APT	bisoxirane	+	58
"	"	-	57
"	epibromohydrin	+	54
"	"	-	51
m-APT	bisoxirane	+	5.3
"	"	-	4.5
p-APT	bisoxirane	+	3.0
"	"	-	4.8
"	epibromohydrin	+	32
"	"	-	27
o-APE	bisoxirane	-	1.7
m-APE	"	-	0.6
p-APE*	"	-	49
p-Cl,o-APE	"	-	1.5
p-ASE	"	-	1.1

Electrophoresis and blotting were carried out as described in the Materials and Methods. o-APT is the thioether of 2-aminothiophenol, o-APE the ether of 2-aminophenol, etc. p-Cl,o-APE is the ether of 5-chloro-2-hydroxyaniline, p-ASE is the ether of 4-aminosalicylic acid. *APE paper prepared by procedure (i) of the Materials & Methods. APE paper prepared by procedure (ii) lacked sufficient napthol reactivity to merit testing by DNA transfer.

1). Significant stabilization to spontaneous hydrolysis was obtained by converting the diazochlorides to diazofluoborates or hexafluorophosphates in acid solution (10) (Materials and Methods); the stabilized ortho papers could be kept for a few days at room temperature without significant erosion of either napthol coupling or DNA binding activity, and the epibromohydrin-linked para derivative gave strong napthol reactions for months after diazotization (unpublished results). However stabilization generally had little effect on the percentage of DNA transferred to freshly diazotized paper (Table 1).

Counts bound were resistant to elution under conditions likely to be encountered in molecular hybridization experiments (Materials and Methods). The fraction bound in RNA or genomic DNA transfer experiments may be higher than reported here, since the data in Table 1 were obtained with nick-translated DNA of relatively short single-strand length.

The optimal pH for transfer of labelled DNA fragments was examined after equilibration of gel slices in citrate-phosphate buffers of differing pH and constant ionic strength (Materials and Methods). Figure 2 demonstrates that there is little dependence of transfer on pH over the range pH 3 to pH 7. The

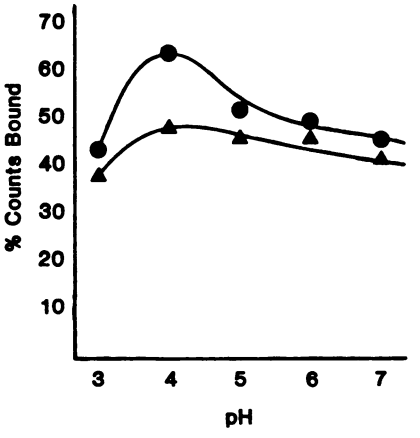

Figure 2. Fraction of DNA applied to gel which was recovered bound to bisoxirane-linked (circles) or epibromohydrin-linked (triangles) diazotized o-APT paper as a function of pH.

greatest transfer takes place at around pH 4 for the ortho-aminophenylthioether of either bisoxirane or epibromohydrin activated paper. Similar experiments assessing the influence of ionic strength on transfer (range, I=.02 to 1.0) showed little effect (data not shown). However when squares of diazotized bisoxirane-linked o-APT paper were exposed to denatured duplex or single stranded (M13) DNA in solution, high ionic strength appeared to have an adverse, albeit somewhat variable effect on the fraction of DNA bound. In 0.1M acetate pH 4, between 10 and 40 μg of DNA were bound per cm^2, depending on the type and concentration of DNA used. Similar values have been reported for DBM paper (4), while values as high as 80 $\mu g/cm^2$ have been reported for nitrocellulose (15).

Scanning densitometry of the autoradiograms of the transferred pBR 322 Alu fragments showed that the fraction bound was essentially independent of length for fragments greater than about 100 basepairs long (Table 2). Some portion of this length independence may be due to the short strand length of the denatured nick-translated DNA.

An investigation of the factors influencing nonspecific binding of labelled DNA to substituted papers led to the observation that highly substituted paper had lower background in mock hybridization experiments, and that the type of paper used played a significant role in the level of background observed

Table 2

Fraction Bound	Fragment Size
0.82	910
0.93	659, 655
0.84	520
0.94	403
1.00	281, 257, 226
0.65	136, 100

Fraction bound is normalized to the highest fraction observed, for the cluster about 257 bp. Net transfer was 57% (Table 1).

(Table 3 and unpublished results). To identify a paper having low inherent DNA binding capacity, a number of underivatized filter sheets were treated with nitrous acid, exposed to acetate buffer at pH 4, incubated with glycine, prehybridized, and mock-hybridized with nick-translated pBR322 DNA. After washing for several hours at 65° in 0.5X SSC, the papers were autoradiographed. Densitometry of the exposed films gave the results shown in Table 3. A great deal of variation was found between the papers tested, but in general the background was uniform over each sheet (data not shown). Of the papers exhibiting low spontaneous binding of DNA, Whatman 50 was chosen for subsequent experiments because it is a thin, high density paper with a small pore size and a high wet strength.

A commercially available form of o-APT paper is prepared by Schleicher & Schuell from 589WH paper, which is not the same as 589W; the commercial paper has an intrinsic background comparable to substituted Whaman 50 paper. We suspect that noncellulose components, such as pulp-derived lignins or agents added as binders in the paper manufacturing process may contribute to nonspecific DNA adsorption. When ordinary letter papers of different rag content were tested, the nonspecific binding decreased with increasing rag content, and 100% rag papers gave

Table 3

Filter Paper	Relative Optical Density
S&S 577	1.0
S&S 589R	.92
S&S 507	.84
S&S 576	.76
W 40	.49
S&S 589W	.33
W 540	.31
W 50	.12
o-APT/W 50	.02-.06

Densities have been normalized between the unexposed film (0) and the darkest sample (1.0) S&S, Schleicher & Schuell; W, Whatman; o-APT/W 50, bisoxirane ligand o-APT Whatman 50 paper

binding as low as unsubstituted Whatman 50 paper. Because increasing substitution with oxirane (Table 3 and unpublished results) yields lower nonspecific binding, extending the oxirane coupling step may reduce background in some cases.

Different blocking agents were also tested for ability to reduce nonspecific binding due to incompletely inactivated diazo groups. Squares of paper were diazotized, washed in 0.1M acetate pH 4, and treated with different blocking reagents for 2 hours at 37°. Subsequent mock prehybridization and hybridization, followed by washing at 70° gave the following relative radioactivity bound for the different reagents: 1% (w/v) Na Borate, $1.13\pm.07$; 0.1M glycine, $1.00\pm.03$; 0.1M NaI, $1.07\pm.02$; 0.1M 2-mercaptoethanol, $0.93\pm.15$. With the exception of the borate (which acts by raising the pH to allow hydrolysis of the diazo groups), all blocking compounds were dissolved in 0.1M potassium phosphate, pH 7.

An example of the use of o-diazophenylthioether paper for transfer and hybridization detection of unlabelled RNA is shown in Figure 3. A sample of rabbit reticulocyte poly(A)-containing RNA estimated to contain 50 pg of β-globin message was mixed with 10 μg of total Sea Urchin RNA to mimic the RNA composition of cells bearing small amounts of an RNA of interest. After denaturation in formamide/formaldehyde at 55°, the RNA was electrophoresed through a 1% formaldehyde agarose gel (12, Seed and Goldberg, unpublished results). The bulk of the formaldehyde was removed with several washes in 0.1M acetate buffer at room temperature followed by two washes at 65°. The RNA was then transferred to diazotized paper, the paper prehybridized and hybridized with nick-translated (13) pβG-1 (14) plasmid DNA, washed at 65° in 0.1X SSC, and autoradiographed. Figure 3 shows the image obtained after two days exposure at -70°. The probe specific was ca. 5×10^7 cpm/μg. Parallel transfer of denatured RNA to nitrocellulose using a formaldehyde/agarose gel system (Materials and Methods; Seed and Goldberg, unpublished results) gave similar intensity of signal when nonstringent washing conditions were used. However the best signal to noise ratio (shown above) was obtained under condition which reduced the nitrocellulose signal relative to the DPT signal.

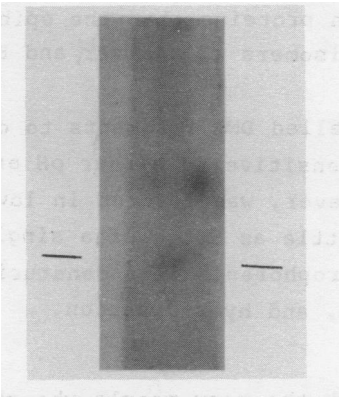

Figure 3. Detection of 50 pg β-globin mRNA by hybridization following transfer to diazotized o-aminophenylthioether paper

DISCUSSION

In the results section of this paper we have described the synthesis and characterization of several arylamine cellulose papers. In the first step of the synthesis an oxirane-substituted cellulose is created by reacting filter paper with a symmetrical bisoxirane or epibromohydrin. In the second step, an unprotected aromatic primary amine is attached to the substrate, taking advantage of the highly nucleophilic character of the thiophenoxide anion. The kinetically favored product is an aromatic amine thioether. The strategy does not extend well to the homologous aminophenols, which are considerably less reactive than the aminothiophenols. Ortho, meta, and para isomers of the aminophenylthioether-coupled cellulose papers were diazotized and reacted with nucleic acids eluted from agarose gels by blotting. The ortho isomer had the highest capacity for DNA in these experiments. The highly labile meta isomer may have been insufficiently stable for good transfer under the conditions employed, while the para form may have been insufficiently reactive. The observed differences in lability and reactivity are not unexpected since in general, meta-substituted arenediazonium salts are less stable, and para-substituted salts more stable than their cognate unsubstituted salts (10). The stability conferred is largely independent of the electron donating or withdrawing character of the substituent (10). Epibromohydrin-linked p-diazophenylthioether paper also reacted

less efficiently with proteins than the epibromohydrin- or bisoxirane-linked o-isomers (D. Balzer and B. Seed, unpublished observations).

Transfer of labelled DNA fragments to o-DPT paper was found to be relatively insensitive to either pH or ionic strength. The transfer of RNA, however, was favored in low salt (unpublished observations). As little as 50 pg of a single specie of RNA was detected after electrophoresis in a denaturing agarose gel, transfer by blotting, and hybridization.

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REFERENCES

1. Noyes, B. E., and Stark, G. R. (1975) Cell 5, 301-310
2. Alwine, J. C., Kemp, D. J., and Stark, G. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5350-5354
3. Wahl, G. M., Stern, M. and Stark, G. R. (1979) Proc. Natl. Acad. Sci. USA 76, 3683-3687
4. Goldberg, M. L., Lifton, R. P., Stark, G. R. and Williams, J. G. (1979) Methods in Enzymology 68, 206-220
5. Alwine, J. C., Kemp, D. J., Parker, B. A., Reiser, J., Renart, J., Stark, G. R., and Wahl, G. M. (1979) Methods in Enzymology 68, 220-242
6. Renart, J., Reiser, J. and Stark, G. R. (1979) Proc. Natl. Acad. Sci. USA 76, 3116-3120
7. Weliky, N., and Weetall, H. H. (1965) Immunochemistry 2, 293-322
8. Sundberg, L. and Porath, J. (1974) J. Chromatog. 90, 82-98
9. Nishikawa, and Bailon (1976) J. Solid Phase Biochem. 1, 33-49
10. Wulfman, D. S. (1978) in The Chemistry of Diazonium and Diazo Groups (Patai, S., ed.) pp. 247-339, J. Wiley, New York, NY
11. Elving, P. J., Markowitz, J. M., and Rosenthal, I. (1956) Analyt. Chem. 28, 1179-1180.
12. Rave, N., Crkvenjakov, R., and Boedtker, H. (1979) Nucl. Acids Res. 6, 3559-3567
13. Maniatis, T., Jeffrey, A., and Kleid, D. G. (1975) Proc. Natl. Acad. Sci. USA 72, 1184-1188
14. Maniatis, T., Sim, G.K., Efstratiadis, A., and Kafatos, F. C. (1976) Cell 8, 163-182
15. Thomas, P. (1980) Proc. Natl. Acad. Sci. USA 77, 5201-5206