J. Pharm. Pharmacol. 1996, 48:147–149
Received April 21, 1995
Accepted April 28, 1995

Magnetizable Solid-phase Supports for Purification of Nucleic Acids

I. J. BRUCE, M. J. DAVIES, K. HOWARD, D. E. SMETHURST AND M. TODD

School of Biological and Chemical Sciences, University of Greenwich, Wellington Street, London SE18 6PF, UK

Abstract

A simple, small scale non-hazardous procedure for the production of magnetizable solid-phase support (MSPS) beads has been developed based on the extrusion of agarose/iron oxide mixtures.

The MSPS beads were derivatized using various amine ligands. Derivatized MSPS beads were used to adsorb nucleic acids from aqueous solution and to separate RNA/DNA mixtures.

Techniques for purification of biological macromolecules using column based ion exchange and affinity chromatography (Dean et al 1990) are well established. However, in recent years the emergence of magnetizable solid-phase supports (MSPS) for the purification of biological molecules has led to the development of a new generation of materials for biomolecular separations. MSPS, when derivatized with suitable ligands, can be used for the routine separation and purification of biomolecules with the aid of only a simple magnet (Fig. 1). Magnetic separation technologies have proved to be advantageous for many biotechnological problems, including cell-sorting, enzyme immobilization and the purification of antibodies. The isolation of nucleic acids using magnetic separation is also becoming increasingly common. Procedures have been developed for specific isolation of nucleic acid species such as messenger RNA, biotinylated DNA fragments from PCR amplifications and plasmid DNA by specific triple helix affinity capture (Jakobsen et al 1990; Hawkins 1992; Wahlberg et al 1992; Ji & Smith 1993). However, few methods are currently available for the general separation and purification of nucleic acids using a single support.

We have developed a simple method for the manufacture of beaded MSPS based on agarose/iron oxide mixtures (Ennis & Wisdom 1991). Agarose was chosen as the support material because of its porosity and biological inertness. It also has an abundance of hydroxyl groups which can be linked to a wide range of ligands. We have succeeded in producing highly uniform spherical particles of a size suitable for biomolecular purifications. In particular we have addressed the general problem of isolation and purification of nucleic acids using MSPS beads derivatized with a range of ion-exchange ligands which possess affinity for nucleic acids.

Materials and Methods

MSPS preparation

A solution of molten agarose (10 mL; 2% w/v; type XII, low viscosity for beading, Sigma) containing paramagnetic iron oxide, Fe_3O_4 (4% w/v; Aldrich), and sodium azide (0·02% w/v) was extruded from a hole drilled at the end of a sealed 10-mL syringe into vegetable oil (100 mL), rapidly stirred by an overhead paddle stirrer. Stirring was continued for 1 min after extrusion was completed, then deionized water (100 mL) was added and the two-phase mixture left to stand on a slab magnet for 16 h. The majority of the cleared oil phase was poured off and the aqueous phase containing the beaded MSPS re-washed with water. The resultant suspension of MSPS was initially sized by sieving through a series of Endecott sieves (mesh sizes 500, 250, 200, 180 and 150 μm) using a Fritsch sieve shaker. All fractions collected were then washed successively with 30:70 v/v acetone:-water, 70:30 v/v acetone:water anhydrous acetone. The amount of each sized fraction produced was measured in terms of its moist weight, i.e. MSPS was filtered under water pump suction on a sintered-glass funnel until the surface of the MSPS began to crack and no more filtrate was collected. MSPS beads were stored in 20% aqueous methanol to act as an anti-bacterial agent.

Cross-linking and derivatisation of MSPS

Cross-linking was carried out using epichlorohydrin and the affinity ligands covalently linked to the MSPS by standard procedures (Dean et al 1990). The cross-linked MSPS was finally washed successively with deionized water, 1 M sodium chloride, deionized water, then suspended in 20% aqueous methanol and stored at room temperature until needed. Derivatized MSPS beads were prepared using standard procedures (Dean et al 1990)

General procedure for adsorption and elution of nucleic acids using MSPS

Twenty-five milligrams (250 μL of 100 mg mL^{-1} suspension) of the agarose-MSPS derivative was placed in an Eppendorf tube. The MSPS was magnetically immobilized using a magnetic stand/concentrator and the supernatant removed. Sterile distilled water (1 mL) was added, the suspension shaken, then the water removed. The nucleic acid solution (1 mL) was added, and the suspension shaken gently at 23°C

Correspondence: M. Todd, School of Biological and Chemical Sciences, University of Greenwich, Wellington Street, London SE18 6PF, UK.

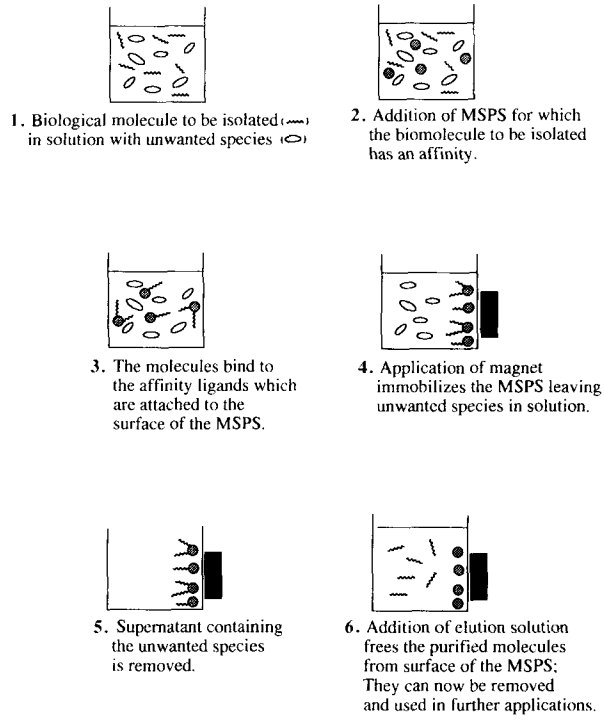

FIG. 1. Separation and purification of biomolecules with the aid of a simple magnet.

for 30 min. The MSPS was magnetically immobilized, the supernatant removed and its A260 and A280 values recorded. These were compared with those of the stock solution of nucleic acid, and the amount of nucleic acid adsorbed by the MSPS calculated and expressed as a percentage of the theoretical maximum. To elute adsorbed nucleic acids, the relevant elution solution (1 mL) was added to the MSPS and the suspension incubated at 65°C for 30 min with occasional shaking. The MSPS was immobilized, the supernatant removed and its A260 and A280 values recorded to calculate the amount of adsorbed nucleic acid eluted (expressed as percentage of the theoretical maximum).

Separation of RNA and DNA using DEAE-MSPS

A sample of DEAE-MSPS (2–3 mg) was immobilized in the well of a microtitre plate using a BeadPrep magnetic microtitre plate concentrator/shaker (Techne (Cambridge) Ltd). The MSPS was washed with sterile distilled water, then incubated with a mixture of λ phage DNA/Hind III digest (0·1 μg μL^{-1}; 10 mL) and total RNA (type IV from Torula yeast; 5 μg μL^{-1}; 3 mL) for 30 min at 23°C with shaking. The MSPS was then immobilized and the supernatant removed. The MSPS was washed with sterile distilled water. The RNA was eluted by adding 50 μL of 0·3 M NaCl/50 mM arginine (free base) and shaking for 30 min at 23°C. The DNA was then eluted by adding 50 μL 1.0 M NaCl/50 mM arginine (free base) and shaking in an oven at 65°C for 30 min. The supernatants from each step were analysed directly by agarose gel electrophoresis.

Results and Discussion

The MSPS beads were examined using a light microscope set up for Köhler illumination (Fig. 2) and were found to be highly spherical with the iron oxide incorporated regularly throughout the bead matrix. A range of bead sizes (>500–<150 μm) was produced (Table 1). MSPS beads of a diameter less than 150 μm were selected for affinity purification applications as their greater surface area/volume ratio enhanced their ligand loading capacity and subsequent ability to take up and release ligates during the purification process. Thermal and chemical stability of the matrix was improved by cross-linking (Dean et al 1990). Once cross-linked, the beads could be autoclaved (121°C, 15 pounds in^{-2}, 15 min) without damage or alteration to their structure. This chemical stability allowed the derivatization of agarose-MSPS under a range of conditions, and the thermal stability provided by cross-linking allowed purification applications to be performed at temperatures above that at which agarose would normally melt.

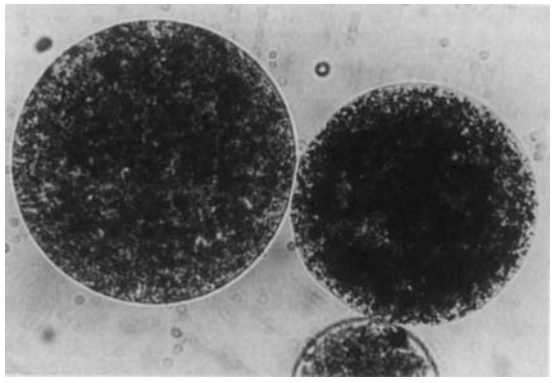

FIG. 2. MSPS beads examined under a light microscope set up for Köhler illumination.

The MSPS beads were functionalized with a range of nucleic acid binding ligands. Diethylaminoethyl- (DEAE), epichlorohydrin/triethanolamine- (ECTEOLA) and spermine-derivatized beads were readily formed. Other ligands based on hexane-1,6-diamine (HDA), tris(aminoethyl)amine (TAEA) and epichlorohydrin/2-hydroxyethylpiperazine (EHEP) were also linked to the beads (Fig. 3).

CHN combustion analysis of derivatized MSPS did not yield reproducible results for indicating the degree of MSPS surface ligand coverage. This may have been due to the relatively high molecular weight of the iron oxide significantly increasing the overall weights of samples for analysis, thereby diminishing (as a percentage) the proportion of nitrogen to limits below that of the baseline for detection.

Table 1. Range of MSPS beads formed by extrusion of 10 mL molten agarose/iron oxide mixture into oil.

Diameter of MSPS (μm)	Moist weight (g)
>500	2·78 ± 0·30
250 500	1·87 ± 0·20
180 250	1·01 ± 0·21
150 180	0·75 ± 0·10
<150	1·99 ± 0·38

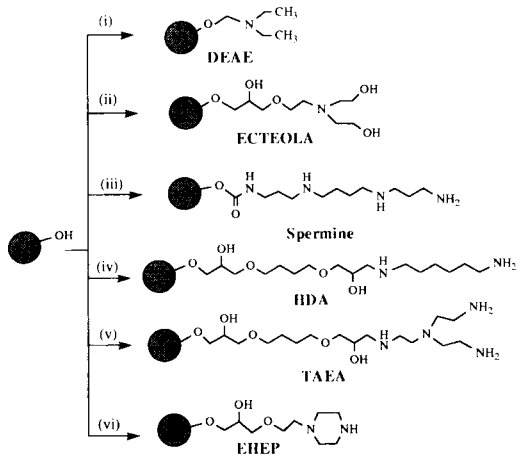

FIG. 3. The functionalization of MSPS beads with a range of nucleic acid binding ligands. Diethylaminoethyl- (DEAE), epichlorohydrin/triethanolamine- (ECTEOLA) and spermine-derivatized beads were readily formed. Other ligands based on hexane-1,6-diamine (HDA), tris(amino-ethyl)amine (TAEA) and epichlorohydrin/2-hydroxyethylpiperazine (EHEP) were also linked to the beads.

Titration of the MSPS against 0·1 M hydrochloric acid gave inconsistent results which appeared to be due to the presence of basic iron oxide. Surface analysis techniques such as secondary ion mass spectrometry (SIMS) and X-ray photo-electron spectroscopy (XPES) were also employed to attempt to measure the level of surface derivatization, but these too proved inconclusive due to their very high sensitivity to other surface contaminants. Instead, the degree of ligand loading was expressed as the ability of a known weight of MSPS to adsorb a known amount of DNA, as measured by ultraviolet spectroscopy. This also served as a method of quality control for the MSPS to check that surface derivatization had proceeded satisfactorily. Typically, for DEAE-MSPS a figure of at least 95% uptake of salmon sperm DNA was obtained. Similar results were observed for ECTEOLA-, TAEA-and spermine-MSPS. Use of the 2,4,6-trinitrobenzenesulphonic acid (TNBS) titration method (Dean et al 1990) to determine the concentration of primary amine ligand attached to the MSPS gave the following results for at least three experiments in each case: TAEA 298 ± 28 mmol g^{-1} (dry weight); spermine 382 ± 41 mmol g^{-1}; HDA 291 ± 31 mmol g^{-1} (dry weight). All of the ligands used contain amine groups, which cause the MSPS beads to have a positively charged surface under aqueous conditions due to the protonation of the nitrogen atoms. All will bind to nucleic acids in solution (via the negatively charged nucleic acid phosphate backbone) to varying degrees. The affinity for nucleic acids was highest with tertiary amine-containing ligands (such as DEAE, ECTEOLA and TAEA), and was lower with secondary and primary amine ligands. Thus the DEAE-, ECTEOLA- and TAEA-MSPS beads performed best in tests of uptake of salmon sperm DNA from solution (Table 2). However, the spermine-MSPS beads displayed good affinity for DNA, possibly because the long polyamine chain enables the ligand to bind DNA molecules via groove binding.

Table 2. Efficiency of adsorption of salmon sperm DNA by derivatized MSPS beads.

Ligand	Adsorption (%)
DEAE	95 ± 5
ECTEOLA	89 ± 5
TAEA	95 ± 5
Spermine	86 ± 2
EHEP	67 ± 7
HDA	40 ± 4

Twenty-five milligrams MSPS incubated with 1 mL salmon sperm DNA (50 μg mL^{-1}), 30 min, 23°C. Values calculated from optical density readings at 260 nm. (Average values of at least three experiments.

The MSPS derivatized beads investigated in this study were non-specific in their uptake of nucleic acids, but they could be used specifically for the separation of different forms of nucleic acids. Using DEAE-MSPS, mixtures of DNA and RNA were separated into their component parts upon application of different eluant concentrations.

These procedures for nucleic acid purification can be scaled up or down according to the amount of material to be isolated. Our procedure can be performed in a microtitre plate which reduces greatly the amounts of MSPS, reagents and eluants required. Alternatively, large-scale isolation of nucleic acids could theoretically be carried out by increasing the amount of MSPS used to provide milligram quantities of nucleic acid, which would be of particular use for large scale isolation of plasmid DNA from cell lysates. Therefore, MSPS beads can be used either for analytical or preparative purposes. The versatility of agarose MSPS beads is such that the separation and purification of other important biological molecules may be possible. We are currently investigating the use of our MSPS beads for the purification of messenger RNA, and for the isolation of specific gene sequences by capture using MSPS-bound oligonucleotide probes.

Acknowledgements

SIMS and XPES analyses were performed at the Laboratory of Biophysics and Surface Analysis, Department of Pharmaceutical Science, University of Nottingham, UK.

References

Dean, P. D. G., Johnson, W. S., Middle, F. A. (1990) Affinity Chromatography - a Practical Approach. IRL Press, Oxford

Ennis, M. P., Wisdom G. B. (1991) A magnetizable solid phase for enzyme extraction. Applied Biochem. Biotech. 30: 155–164

Hawkins, T. (1992) M13 single-strand purification using a biotinylated probe and streptavidin coated magnetic beads. DNA Seq. 3: 65–69

Jakobsen, K. S., Breivold E., Hornes, E. (1990) Purification of mRNA directly from crude plant tissues in 15 minutes using magnetic oligo dT microspheres. Nucl. Acids Res. 18: 3669

Ji, H., Smith, L. M. (1993) Rapid purification of double-stranded DNA by triple-helix-mediated affinity capture. Anal. Chem. 65: 1323–1329

Wahlberg, J., Holmberg, A., Bergh, S., Hultman, T., Hulen, M. (1992) Automated magnetic preparation of DNA templates for solid phase sequencing. Electrophoresis 13: 547–551