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Improved Manufacture and Application of an Agarose Magnetizable Solid-Phase Support

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ABSTRACT

A simple, semiautomated, nonhazardous procedure for the production of a magnetizable solid-phase support (MSPS) has been developed based on the extrusion of molten agarose–iron oxide mixtures, which enables manufacture of a range of differently sized spherical agarose–iron oxide beads. This system has enabled scale-up of an original manufacture procedure and reproducible preparation of kg quantities of MSPS suitable for biomolecular purifications. An improved protocol for the isolation of plasmid DNA directly from cell lysates using this MSPS, derivatized with diethylaminoethyl (DEAE) groups, is reported. This involves a modified alkaline lysis, followed by adsorption to and elution from the support, yielding plasmid DNA of a purity comparable with, or better than, other methods of plasmid isolation. Using the same procedure, plasmid DNA can be isolated from bacterial cell culture volumes of 1.5 mL and 100 mL with equal efficiency and purity.

Index Entries: Magnetizable support; purification; biological molecules; plasmid DNA.

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INTRODUCTION

Purification of macromolecules using column-based affinity or ion-exchange chromatography is now a well-established biochemical technique *(1)*. Using this technique, a solid phase, (e.g. agarose, polyacrylamide, or controlled-pore glass) is derivatized with ligands possessing affinity for biomolecules. The derivatized solid phase is then packed into columns through which the ligate-containing solution is passed to allow specific adsorption and elution of molecules of interest.

In magnetic-affinity chromatography, the solid-phase supports are identical in nature to those employed in column-affinity chromatography, but, additionally, they incorporate a paramagnetic component to allow the material to respond to a magnetic field *(2–5)*. The solid phase does not require packing into a column, or centrifugation, to accomplish its separation from an unwanted liquid phase. As a result, the magnetizable solid-phase support (MSPS) can be employed in many applications addressed by classical column chromatography, with the added advantage that fouling and clogging often encountered during column chromatography of marginally soluble or viscous solutions, such as cell lysates, does not occur, or occurs to a lesser extent.

In particular, magnetic separation technology has proved to be advantageous in many biotechnological applications, including cell sorting and separation, enzyme immobilization and purification, and immunoassay *(6–9)*. The majority of applications have been performed on a small bench-top scale, but larger-scale protein purification using such supports in magnetically stabilized fluidized beds has been reported *(10)*.

In molecular biology, the isolation, purification, and/or separation of nucleic acids using magnetic separation techniques is becoming increasingly common. Most procedures involve the specific isolation of a particular nucleic acid species, including, for example, mRNA, biotinylated DNA fragments from PCR amplification (using streptavidin-coated magnetic beads), or plasmid DNA by triple helix formation using an MSPS-bound capture sequence *(11–13)*. These methods rely on specific affinity interactions or prior modification of the nucleic acid species to allow its isolation. Few methods are currently available for the separation and purification of a general class of nucleic acids using a single magnetizable support *(14)*.

We report here a novel method for the nonhazardous preparation of a magnetizable solid-phase support based on agarose, using a semiautomated system capable of reproducible small- and large-scale manufacture of an agarose-MSPS; we also report use of a derivative of this agarose-MSPS in an improved method for the purification of plasmid DNA directly from cell lysates following a modification of an alkaline lysis procedure. This method can be performed on small (mini-prep) or large scale (maxi-prep) without the need for complex or expensive ancillary equipment.

MATERIALS AND METHODS

Reagents and Equipment

All reagents and nucleic acids were obtained from Sigma (Poole, Dorset, UK) or Aldrich (Gillingham, Dorset, UK). Restriction endonucleases were obtained from Boehringer Mannheim (Lewes, East Sussex, UK). Agarose for MSPS manufacture was type XII, low viscosity for beading, from Sigma. Sunflower oil was obtained from local supermarkets. The BCA protein assay kit was obtained from Pierce and Warriner (Chester, Cheshire, UK). MSPS could be magnetically immobilized in Eppendorf tubes using magnetic particle concentrators, supplied by Dynal (Bromborough, Wirral, UK). For large-scale applications, MSPS was immobilized using sintered ferrite slab magnets, grade FER 3w from Magnetic Development (Swindon, Wiltshire, UK). Ultraviolet spectroscopy was performed using a Perkin-Elmer λ3 spectrophotometer (Perkin-Elmer, Warrington, Cheshire). CHN combustion analyses were performed by Butterworths Laboratories, (Teddington, Middlesex, UK). Triplicate samples for CHN analysis were dried to constant weight in a vacuum oven.

Bacterial Strains

Escherichia coli strain JM109 containing plasmid pUC18 was a gift (G. Bignell, this laboratory) and was routinely cultured in LB broth containing 100 μg/mL ampicillin.

MSPS Preparation

Preparation of molten agarose–iron oxide mixtures for extrusion, and MSPS washing, sizing, and storage, was as previously described *(15,16)*. The semiautomated system for MSPS production consisted of a single-pressure vessel in which the molten agarose–iron oxide mixture was simultaneously heated and circulated, and from which it was extruded. The molten agarose–iron oxide mixture was poured into a cylindrical aluminium chamber that was fitted with a sealing lid attached by wing nuts. The chamber was 75 mm in internal diameter and 140 mm in depth, giving a total approx vol of 600 mL. The molten agarose–iron oxide suspension was mixed within the chamber by a 25-mm diameter propeller driven by a 6-mm diameter shaft passing through a high-pressure seal in the wall near the base. A 6 W motor with dual speed control switch turned the propeller at speeds up to about 200 rpm. The molten agarose–iron oxide suspension was maintained at a temperature of 46°C. The chamber, extrusion nozzle, and its contents were maintained at the required temperature by application of electricity to resistive heating tapes that were wrapped around the chamber and nozzle. The temperature was thermostatically monitored and controlled by a CAL

3200 miniature temperature controller (RS Components, Corby, Northants, UK). The combined total power capability of the heating tapes was 250 W.

The extrusion nozzle (Schlick smooth-jet nozzle, 0.5 or 0.8 mm bore size; Cadar, Market Harborough, Leicestershire, UK) was screwed into the lower face of the chamber, and was removable for cleaning and/or fitting of alternative sizes.

When extrusion was required, compressed air of up to 4 bar was admitted into the chamber, which forced the molten mixture through the nozzle into the stirred, immiscible sunflower oil phase situated in a MSE Atomix homogenizer (Leatherhead, Surrey, UK) placed below the chamber. The distance between the nozzle and the surface of the oil was 150 mm; the oil was maintained at a temperature of 20°C, and was stirred at a rate of 2000 rpm.

An electric solenoid air valve, which was operated by a push-button switch, was used to pressurize the chamber, and the air circuit was fitted with a pressure gage and over-pressure safety release valve.

Preparation and Scanning Electron Microscopy (SEM) of Iron Oxide

Paramagnetic, precipitated iron oxide was prepared by oxidative hydrolysis of iron (II) sulfate at alkaline pH *(17)*. After preparation, the microcrystalline iron oxide suspension was washed several times with deionized water by decantation, then dialyzed against 2 L of deionized water (several changes), until the dialysis solution was measured to be pH 7.0. Aliquots of suspension were removed and dried to constant weight to calculate the amount of iron oxide present. The iron oxide was not isolated as a dried bulk solid because this caused aggregation; instead it was dispensed from suspension as required to prepare molten agarose–iron oxide mixtures.

Samples for SEM were prepared by washing an aliquot of iron oxide suspension with absolute ethanol, and depositing a drop on a glass microscope slide and allowing it to air-dry. SEM was then carried out using a Stereoscan 90B scanning electron microscope (Cambridge Instruments, Cambridge, UK).

Crosslinking of MSPS and Derivatization with *N,N'*-(diethylamino)ethyl (DEAE) Groups

These were carried out as described previously *(15,16)*.

Size-Exclusion Chromatography (SEC)

One mL (settled volume; 20–150 µm diameter) of MSPS was packed into a 1 mL plastic sterile syringe, 6 × 0.5 cm (Plastikpak ®, Becton Dickinson,

Ireland), and equilibrated with 0.8 *M* NaCl, 0.05% Tween-20, using a peristaltic pump P-1 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Four dextrans with average mol wt (M_r) of 580,000 (I), 71,000 (II), 37,700 (III), and 11,300 (IV) were used to characterize the size-exclusion characteristics of the column. Blue dextran (M_r 2,000,000) was used as the void volume marker, and 100% acetone was used as the total volume marker. Proteins were also used and these were horse radish peroxidase/rabbit antigoat IgG conjugate (HRP-IgG, M_r 270,000), IgG (M_r 150,000), bovine serum albumin (BSA, M_r 65,000) and horseradish peroxidase (HRP, M_r 40,000) (all M_r are approximate). Samples (100 µL, 2 mg/ml) were loaded onto the bed surface and eluted with 0.8 *M* NaCl, 0.05% Tween-20 at a flow rate of 45 µL/min, and 45 µL fractions were collected using a Jaytee 5512 fraction collector (Whitstable, Kent, UK).

Eluted fractions containing dextrans were assayed using anthrone by the following method. Samples were diluted to 0.3 mL using sterile distilled water in a 20 mL test tube and concentrated hydrochloric acid (0.3 mL) and 90% formic acid (30 µL) were added, followed by freshly prepared anthrone reagent (2.4 mL), slowly, to prevent excessive frothing. The reagent was prepared by dissolving anthrone (20 mg) in 80% sulfuric acid (100 mL). After stirring on a vortex mixer, the tubes were placed, together with appropriate blanks, in a test-tube heating block at 90°C for 5 min and immediately placed in a cold water bath. Optical density (OD) was read at 630 nm, after stirring on a vortex mixer and standing for 5 min to disperse bubbles.

The OD of blue dextran and acetone was read at 260 nm and 272 nm, respectively, after diluting fractions with 500 µL of deionized water. Eluted fractions containing proteins were assayed using the BCA method.

Estimate of Final Agarose Content of Nonmagnetizable Beads

The method used was essentially identical to that of Ennis and Wisdom *(18)*. Triplicate samples of crosslinked, nonmagnetizable agarose beads (1 mL settled bed volume; prepared using the same methods as described above, with the omission of iron oxide) were washed sequentially with 30, 50, and 70% v/v aqueous ethanol, and 100% anhydrous ethanol, then dried to constant weight at 65°C. The fraction of settled volume occupied by the agarose beads was calculated by the subtraction of the void volume, as determined by size-exclusion chromatography (SEC), from the total settled bed volume. Using this method, for 1 mL (settled volume) of nonmagnetizable agarose beads, the volume actually occupied by the agarose was calculated to be 0.69 mL; this figure was then used to calculate the final agarose content (w/v) of the solid phase support.

DEAE–MSPS Isolation of Plasmid DNA from Cell Lysate

From 1.5 mL of Cell Culture (Mini-prep)

One and one half mL of fresh, late log phase bacterial cell culture (approx 10^8–10^9 cells/mL) of *E. coli* strain JM109 containing plasmid pUC 18 in an Eppendorf tube was pelleted by centrifugation at 12,000*g* in a microcentrifuge for 20 s. The supernatant was removed and the cell pellet resuspended in 100 µL of 50 m*M* Tris-HCl/10 m*M* EDTA, pH 8.0, containing RNase A (400 µg/mL) (soln I). 200 µL of 0.2*M* NaOH/1% sodium dodecyl sulfate solution (soln II) was added, and the tube was inverted 4–5 times and placed on ice for 5 min. To the resulting viscous solution was added 150 µL of ice-cold 3 *M* potassium acetate, pH 5.5 (KOAc soln III). The solution was gently mixed by inversion and placed on ice for a further 5 min. The white flocculent precipitate was pelleted by centrifugation at 12,000*g* for 5 min at 4°C. The cleared lysate solution was carefully removed and added to 250 µL of DEAE–MSPS suspension (100 mg/mL) prewashed in 0.05 m phosphate buffer, pH 7.0, in an Eppendorf tube. The suspension was gently shaken for 5 min at 23°C, the MSPS magnetically immobilized and the supernatant removed. The DEAE–MSPS was washed with 400 µL of 0.2 *M* NaCl/10 m*M* Tris-HCl/1 m*M* EDTA, pH 8.0 (soln IV), then magnetically immobilized and the supernatant removed. 200 µL of 1.0 *M* NaCl/10 m*M* Tris-HCl/1 m*M* EDTA, pH 8.0 (soln V) was added to the MSPS and the suspension shaken gently for 5 min at 23°C to elute plasmid DNA. The MSPS was immobilized and the supernatant removed and transferred to a fresh tube. At this stage, the eluted DNA solution could be directly analyzed by agarose gel electrophoresis, or precipitated by addition of 500 µL of absolute ethanol and 20 µL of 7.5 *M* ammonium acetate, chilling at –20°C for 10 min, then centrifugation at 12,000*g* for 5 min to pellet the precipitated DNA. After air drying, the DNA was resuspended in 50 µL of 10 m*M* Tris-HCl/1 m*M* EDTA, pH 8.0 (TE) buffer.

From 100 mL of Cell Culture (Maxi-prep)

100 mL of fresh bacterial cell culture was centrifuged at 5000*g* for 15 min and 4°C. The supernatant was removed and the cell pellet resuspended in 14 mL of soln I. Eight mL of soln II was added, and the mixture treated as previously described. Four mL of ice-cold soln III was added to the resulting viscous solution and the resultant cleared-lysate solution was carefully removed by aspiration, using a sterile pipet attached to the end of a water-pump vacuum line, and added to 10 mL of prewashed DEAE–MSPS suspension (100mg/mL) in a polypropylene beaker. The suspension was dealt with as described in the previous section, except that the DEAE–MSPS was washed with 28 mL of soln IV, magnetically immobilized, and the supernatant removed by aspiration. 10 mL of soln V was subse-

quently added and the suspension shaken gently for 5 min at 23°C to elute plasmid DNA. Recovery of the DNA was identical to that described in the previous section, except that 7 mL of isopropanol was added to precipitate the plasmid DNA, instead of ethanol and ammonium acetate. The precipitated DNA was then recovered and resuspended as previously described.

Gel Electrophoresis

Plasmid DNA samples were analyzed by electrophoresis in a 0.8% agarose gel at 7 V/cm in TBE buffer. Mol wt markers were λ-phage DNA digested with *Hind*III.

RESULTS

Preparations of MSPS

In keeping with other studies *(19–22)*, our previous work had shown that several factors were important in determining the size and quantity of MSPS produced. MSPS of different diameters had been previously obtained when immiscible phase and stir rate, nozzle bore diameter, and extrusion temperature and pressure were varied. A study was conducted, using the previously optimized parameters of immiscible phase, stir rate and extrusion temperature *(15,16)*, of the effects of extrusion pressure and nozzle bore diameter on the diameters and quantities of the MSPS produced. Initially, MSPS fractions of 20–150 μm diameter, and >150 μm diameter were collected after sieving.

Extrusion pressures of 2, 3, and 4 bar were selected, and it was observed that the amounts of the 20–150 μm MSPS fraction recovered appeared to be independent of pressure. This trend was the same for both nozzles, 0.5 and 0.8 mm diameter (data not shown), because a similar amount of MSPS was obtained in all experiments. Extrusion at pressures of 2, 3, and 4 bar, using the 0.5 mm nozzle, corresponds to flow rates of approx 170, 240, and 300 mL/min; extrusion at 2 bar, using the 0.8 mm nozzle, corresponds to a flow rate of approx 400 mL/min.

It was decided to fractionate the 20–150 μm-diameter MSPS recovered from each of the above experiments into 20–32 μm, 32–50 μm, and 50–150 μm fractions by sieving to see if the size distribution of MSPS within the range 20–150 μm diameter had been altered by either nozzle diameter or extrusion pressure. The results are given in Figure 1A; the general trend was identical for both 0.5 and 0.8 mm nozzles.

It can be seen that as the extrusion pressure was decreased, so the amount of the larger sizes of MSPS (50–150 μm and >150 μm) recovered also decreased. The reverse is true for the recovery of MSPS within the range of 32–50 μm diameter, when the trend appeared to be increasing

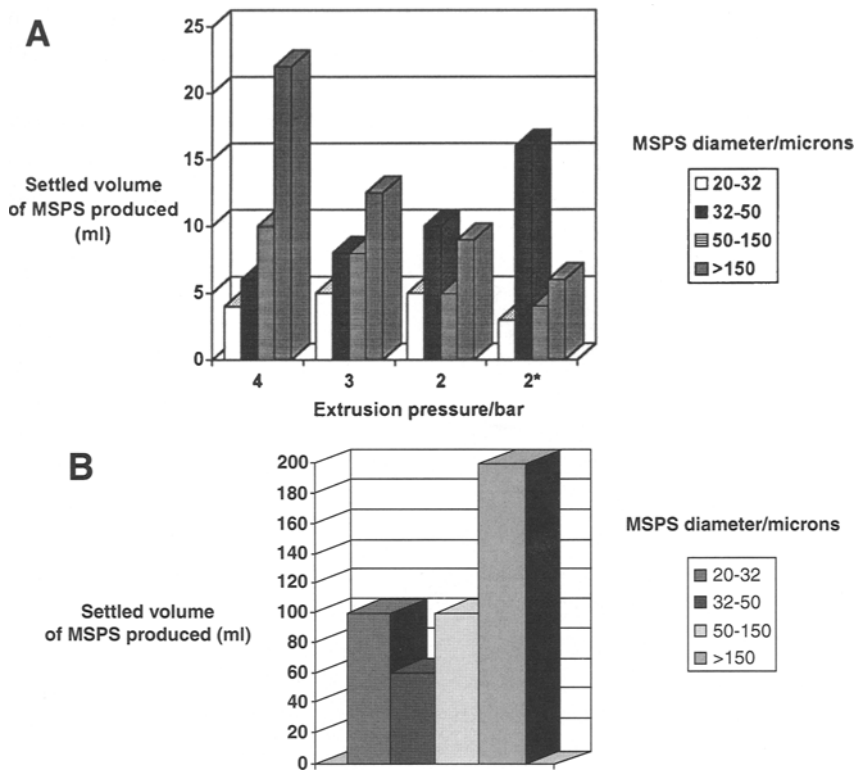

Fig. 1. Effect of extrusion pressure on MSPS size distribution. **(A)** 50 mL of suspension extruded into 500 mL of stirred oil; nozzle bore size for all extrusion was 0.5 mm, except *, in which the nozzle bore size was 0.8 mm. Suspension temperature was 46°C. **(B)** 500 mL of suspension extruded using a 0.8-mm bore diameter nozzle, an extrusion pressure of 2 bar, and a suspension temperature 46°C.

recovery with decreasing pressure. The amount of the smallest fraction (20–32 μm diameter) remains relatively constant over the whole pressure range. It is therefore clear that, although the total amount of 20–150 μm diameter MSPS produced at the various pressures remained relatively constant, the size distribution of particles within this range had changed. The greatest quantity of the 32–50 μm diameter MSPS could be recovered at the lowest extrusion pressure, 2 bar, using either nozzle.

In a comparison between extrusion at 2 bar using both nozzles, it was possible to recover approx one-half again as much 32–50-μm diameter MSPS using the 0.8 μm diameter nozzle, compared to that recovered using the 0.5-μm diameter nozzle.

When MSPS manufacture using these conditions was scaled up, so that 500 mL of mixture was extruded, a high proportion of 20–150 μm diameter bead sizes was again produced, giving the size distribution shown in

Fig. 1B. Given the overall preparation times involved, it is estimated that, under these conditions, the system is capable of producing approx 0.75 L (settled volume) of beaded agarose of 20–150 μm diameter per hour.

Initially, commercially available iron oxide was used as the paramagnetic component, though examination of this material by scanning electron microscopy (SEM) showed that its size range distribution was wide, and that there were significant amounts of very large (up to 10 μm) iron oxide particles present. This led to settling of the iron oxide from the agarose–iron oxide suspension, despite continuous stirring of the mixture by the propellor mounted in the chamber. It also affected the distribution of the iron oxide within the agarose–MSPS matrix after beading. A more homogenous distribution of iron oxide within the MSPS was achieved by using precipitated paramagnetic iron oxide, prepared by oxidative hydrolysis of Fe_2SO_4 in alkaline conditions *(17)*. Examination of this synthetic iron oxide by SEM showed it to have a much narrower size-range distribution, and a mean particle size of 0.2 μm. Furthermore, this smaller-sized iron oxide did not settle at all during the time the molten agarose–iron oxide suspension was held in the chamber, as described in these experiments: Stirring the mixture using the propeller was sufficient to maintain a homogenous agarose–iron oxide suspension.

The pore size distribution of the MSPS was studied by SEC. Techniques such as SEM, adsorption or condensation of nitrogen gas, or mercury porosimetry were not used, because they involve desolvation, evacuation, or high pressures, all of which would have significantly damaged the spherical agarose bead structure, which is maintained by hydrogen bonding between agarose and water molecules within the matrix. Such methods are more suited to the analysis of rigid, dry-matrix supports *(23)*.

The elution profiles of a series of differently sized dextrans and proteins from columns of MSPS were plotted, and the distribution co-efficient, K_D, for each molecule was calculated using the formula

$$K_D = V_e - V_0 / V_t - V_0$$

where V_e = elution volume, V_0 = void volume, and V_t = total column volume.

Crosslinked and noncrosslinked MSPS were tested, as was nonmagnetic beaded agarose prepared using the semiautomated system. These served as controls and comparisons. A 2000 kDa blue dextran was used as the void volume marker, because it eluted considerably earlier than smaller-sized dextrans and proteins.

Results indicated that the inclusion of the iron oxide component within the matrix had little effect on pore size of the MSPS, because the K_D values calculated for proteins or dextrans did not differ significantly between columns prepared from either MSPS or nonmagnetic beaded

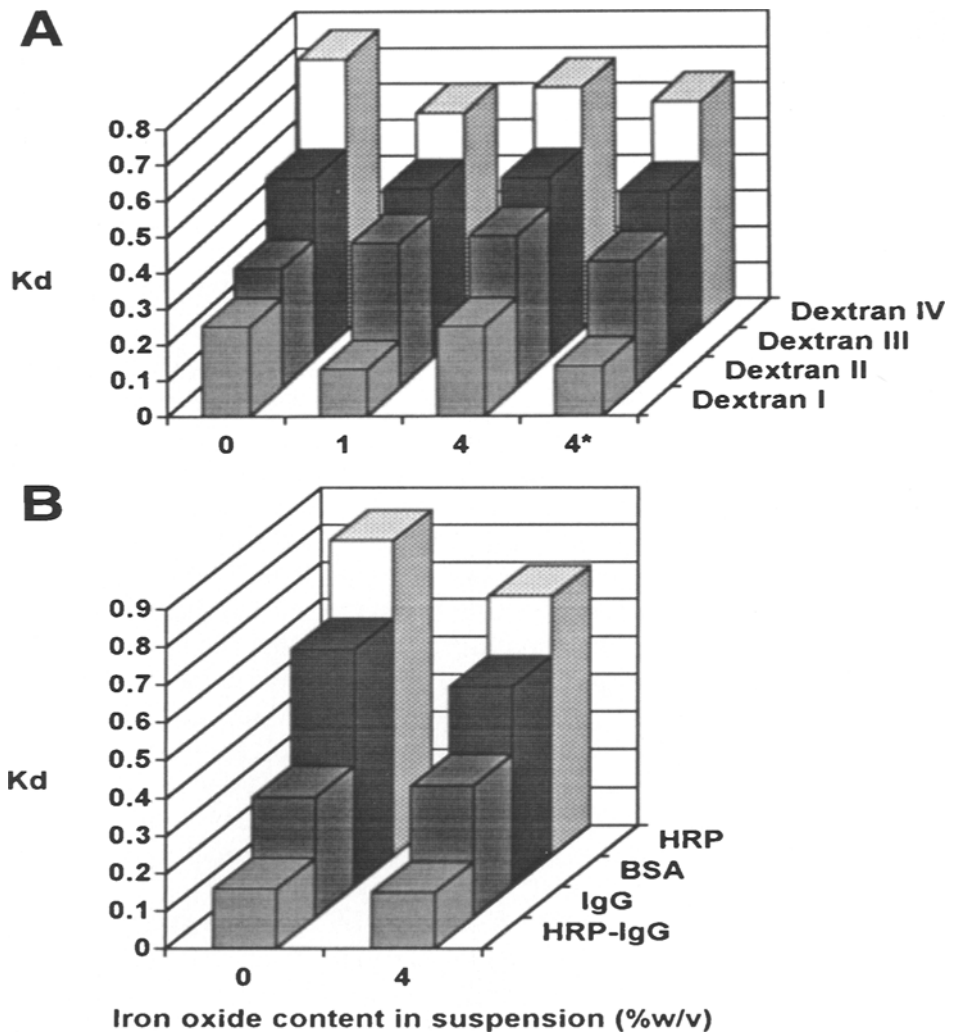

Fig. 2. Distribution coefficients (K_D) for differently sized dextrans **(A)** and proteins **(B)** eluted from columns of beaded agarose and agarose-MSPS. All MSPS samples were prepared using 2% w/v molten agarose, and were crosslinked with epichlorohydrin, except 4*, which was noncrosslinked MSPS.

agarose (Fig. 2A and B). Similar findings were reported by Ennis *(18)* for agarose–iron oxide beads prepared by stirring of molten agarose–iron oxide mixtures in an immiscible phase. It has been suggested that the iron oxide may be situated between the agarose fibers that make up the porous matrix network, but the exact interaction between the two components is not known. Furthermore, crosslinking the matrix also appears to have no significant effect on pore size distribution (Fig. 2A); crosslinking is required to engender thermal, physical, and chemical stability within the matrix.

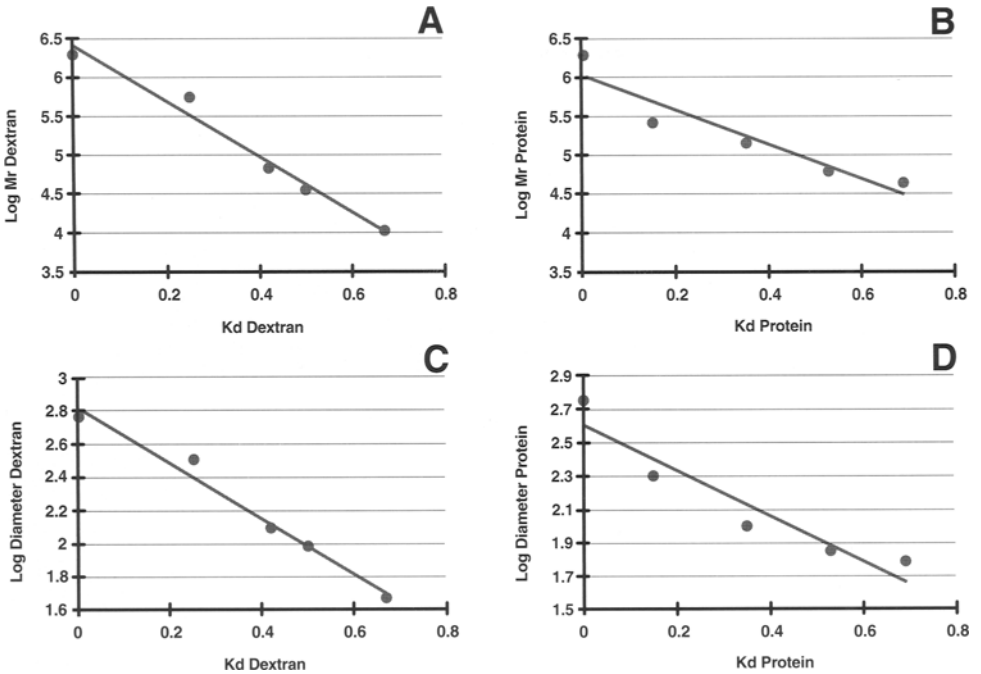

Fig. 3. Calibration curves for MSPS. Plots of K_D(dextrans) vs **(A)** log (relative molecular mass) and **(C)** log (hydrodynamic molecular diameter), and K_D(proteins) vs **(B)** log (relative molecular mass) and **(D)** log (hydrodynamic molecular diameter).

From the above data, calibration curves for the agarose-MSPS were plotted, in terms of both mol wt and estimated hydrodynamic molecular diameter of the molecules concerned (Fig. 3). From these, the size-exclusion properties of the MSPS could be judged. There was an apparent difference observed for results involving experiments in which dextrans and proteins were employed. This was ascribed to differences in the behavior of linear, flexible dextrans and globular protein molecules within the gel matrix. The mol wt exclusion limit was estimated at 2000 kDa from the dextran calibration curve (Fig. 3A), which was also the mol wt of the blue dextran void volume marker used, and 1000 kDa (Fig. 3B) from the protein calibration curve. Similarly, estimates of exclusion limit in terms of molecular diameter were approx 600 Å from the dextran calibration curve (Fig. 3C), and 400 Å from the protein calibration curve (Fig. 3D).

The MSPS size ranges of 20–50 µm, 32–50 µm, and 50–150 µm, collected after sieving, were each crosslinked and converted to the DEAE derivative. CHN combustion analysis of DEAE–MSPS initially did not yield reproducible results for degree of nitrogen (N) content of the MSPS. This was probably because of incomplete combustion of the polysaccharide–iron oxide matrix, making measurement of the relatively small degree

Table 1
Comparison of Yield and Purity of pUC 18 DNA Isolated from 1.5 mL of Bacterial Culture by Alkaline Lysis/MSPS Using Different Concentrations of Potassium Acetate

KOAc/M	Yield of plasmid DNA/μg	A_{260}:A_{280}
4	5.0	2.33
3	7.0	2.08
2	4.9	1.81
1	4.0	1.41

Values are the average of at least three isolations.

of N content difficult. When full combustion was achieved, using elevated temperatures for extended periods, percentage N content values of 0.78% (20–50 μm), 0.61% (32–50 μm), and 0.36% (50–150 μm) per g (dry wt) were obtained, which corresponds to values of 564, 436, and 247 μmol of DEAE ligands per g (dry wt) of MSPS, respectively. The specific surface area of the supports, and hence the ligand loading in terms of μmol m^{-2}, was not calculated, because of the destructive effect that the techniques involved would have on the agarose matrix, as discussed earlier. Combustion analysis was useful as an indication of the outcome of the DEAE derivatization, and served as a quality control process in selecting DEAE-derivatized supports for use in further applications. Consequently, the 20–50 μm and 32–50 μm-sized DEAE–MSPS were combined and used in all subsequent experiments, because they had the highest degree of derivatization.

Using the DEAE–MSPS, a procedure for plasmid DNA isolation has been developed, based on the alkaline lysis method *(24)*: plasmid pUC18, prepared by a modification of the alkaline lysis procedure incorporating an RNase treatment, has been purified directly from the lysis solution by adsorbtion to and elution from DEAE–MSPS. Denatured proteins and chromosomal DNA were precipitated using 3 *M* potassium acetate (KOAc), pH 5.5, rather than 5 *M* KOAc, pH 5.5, used in the classical method. A low-salt (0.2 *M* NaCl) wash step was incorporated into the protocol to wash any RNA fragments, protein, or other cellular contaminants from the matrix prior to elution of plasmid DNA, which is obtained as a mixture of supercoiled and relaxed forms, with a purity equal to that afforded by other methods for plasmid preparation. Table 1 indicates the comparative yield of plasmid DNA from 1.5 mL of *E. coli* pUC18 culture using different KOAc concentrations.

Samples of plasmid pUC 18, prepared using a standard alkaline lysis method and DEAE–MSPS method, from identical culture samples, were analyzed by agarose gel electrophoresis (Fig. 4), which showed that although both methods yielded similar total mounts of DNA, the plasmid DNA solution obtained from classical alkaline lysis was contaminated with

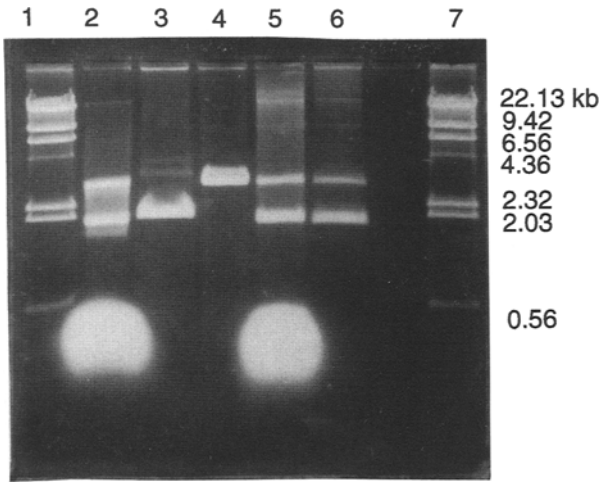

Fig. 4. Agarose gel electrophoresis of plasmid pUC18 obtained from cell lysates. Lanes 1 and 7: *Hind*III digest of λ-phage DNA, mol. wt marker ladder; lane 2: pUC18 obtained from 1.5 mL of bacterial cell culture using classical alkaline lysis; lane 3: pUC18 obtained from 1.5 mL of cell culture using the MSPS method; lane 4: MSPS-purified pUC 18 digested with *Eco*R1 restriction endonuclease; lane 5: pUC18 obtained from 100 mL of cell culture by classical alkaline lysis; lane 6: pUC 18 obtained from 100 mL of cell culture by the MSPS method.

significant amounts of RNA, and that other (nicked and linear) forms of the plasmid and high mol wt chromosomal DNA were present (lane 2). Plasmid DNA prepared using the DEAE–MSPS method was of a higher quality, being isolated largely as the supercoiled form, with only small amounts of other relaxed or linearized forms present (lane 3). These forms of the plasmid could be resolved to a single form upon digestion with *Eco*R1 restriction endonuclease (lane 4). The DEAE–MSPS method provided plasmid DNA that was free of significant protein contamination. The average A_{260}/A_{280} ratio for solutions of the pUC 18 obtained from four independent DEAE–MSPS isolations was 2.08:1; that for pUC 18 obtained from four classical alkaline lyses of the same bacterial culture was 1.33:1. BCA protein assay of the DNA solutions gave values of 0.039 mg/mL of protein for plasmid solutions isolated using the DEAE–MSPS method, compared with 0.15 mg/mL for plasmid isolated using the alkaline lysis. Sequence data obtained using the DNA purified using the DEAE–MSPS method indicated that no damage or alteration of the DNA structure had occurred, and the total length of sequenceable plasmid DNA was similar to that obtained when using high-purity plasmid DNA isolated from other sources. The plasmid DNA has also been digested efficiently with a range of other restriction endonucleases, giving identical results to those provided by pUC18 purified by cesium chloride gradient ultracentrifugation *(25)*.

Precipitation of denatured protein and chromosomal DNA using 3 *M* KOAc was necessary to ensure proper binding of the plasmid DNA via the negatively charged phosphate backbone to the DEAE ligands, which will be positively charged at the pH of the lysate. Less plasmid DNA was adsorbed from a lysate prepared using 4 *M* KOAc, and negligible amounts of DNA were adsorbed from a lysate prepared using 5 *M* KOAc, probably because the ionic concentration was too high to allow binding of DNA to the DEAE ligand. KOAc concentrations of 1 *M* or 2 *M* allowed DNA adsorbtion, but protein precipitation was less efficient, and, subsequently, more protein contamination was found in the eluted solution of DNA, as shown by lower A_{260}/A_{280} ratios (Table 1). Plasmid DNA yields in these latter cases were also lower, probably because of competition for DEAE-binding sites between plasmid DNA and excess unprecipitated protein and chromosomal DNA still present in the lysate. Using 3 *M* KOAc as precipitant offered the best compromise of yield and purity.

Yields of plasmid DNA using the DEAE–MSPS procedure were typical of those expected for a high-copy-number plasmid, such as pUC18 propagated in an *E. coli* host strain grown in LB broth (expected yield 3–5 μg/ml bacterial culture *(24)*). The average yield of pUC 18 obtained from four 1.5 mL samples of cell culture was 7 μg per preparation.

Plasmid DNA was also isolated from large culture volumes, i.e., 100 mL of *E. coli* pUC18 culture, as previously described. Average yield for three separate isolations was 435 μg of plasmid DNA, as measured by UV spectrophotometry. The procedure required only marginally more time than the miniprep.

Agarose gel electrophoresis of the plasmid DNA, isolated using the large-scale DEAE–MSPS method, showed again that the plasmid DNA was free of RNA contamination. A small amount of high mol wt DNA was observed to be present, and other forms of the plasmid were present in greater proportion than before, when performing isolations on the miniprep scale (Fig. 4, lanes 5 and 6). This may be expected, since the shearing forces acting upon the cells and DNA during the isolation procedure are likely to be greater when using larger volumes of solutions. However, the level of protein contamination was still low; the A_{260}/A_{280} ratios of a plasmid pUC18 solution isolated from two 100-mL batches of bacterial cell culture using the MSPS method were 1.92:1 and 1.87:1. The protein content measured using the BCA protein assay was 0.06–0.07 mg/mL of purified DNA solution.

DISCUSSION

The semiautomated system described here allows the large-scale preparation of agarose-MSPS with standardized characteristics and batch-to-batch reproducibility. A set of empirically determined conditions has

been identified that produces large quantities of 20–150-μm diameter MSPS suitable for use in further applications.

The use of dextrans and proteins in SEC studies of the MSPS makes assumptions about the exclusion of flexible, linear molecules or spheres from a three-dimensional array of rigid fibers with pores shaped like open-ended cylinders *(26)*, and serves as a useful first-order approximation. The fact that data differs concerning pore-size distribution for the MSPS from experiments using dextrans and proteins is not unsurprising when considering the flexibility and shape of such molecules, and the comparability of exclusion characteristics of different types of macromolecules has been questioned by other workers *(27)*. Notwithstanding, SEC of swollen gel matrices like agarose gives an indication of apparent, rather than true, chromatographic pore size, and can provide meaningful data about how macromolecules behave within the matrix pore structure and interact with the support. The maximum hydrodynamic molecular diameter of macromolecules able to enter the MSPS matrix, and, by implication, the maximum pore size within the matrix available to these molecules, can be estimated to be within the range of the limits obtained from the calibration curves, i.e., 400–600 Å.

Evidence to support this value range comes from other studies in our laboratory on the use of MSPS for immunoaffinity purification and immunoassay, in which the amount of a rabbit antigoat immunoglobulin (rb antigoat IgG) immobilized to agarose-MSPS has been measured by three different methods: on-bead BCA protein assay, alkaline hydrolysis of all protein from the MSPS, and ELISA. The first two methods gave results that were in good correspondence with one another, but the ELISA indicated that as much as 90% less of the rb antigoat IgG was actually present on the support than in the other two methods. In this assay, the MSPS-bound rb antigoat IgG was reacted with goat IgG, which in turn was complexed with horseradish peroxidase-labeled antibody (HRP-IgG).

The hydrodynamic molecular diameter of one IgG molecule is estimated to be 100 Å *(29)* and that of the HRP-IgG conjugate, in which 3 HRP molecules are bound to each immunoglobulin molecule, is estimated at 280 Å. Therefore, the sum of the (rb antigoat IgG/goat IgG/HRP-IgG ELISA) complex is approx 480 Å. The low levels of rb antigoat IgG detected by ELISA could imply that this complex is not formed easily because of steric constraints on its assembly within the pores of the MSPS matrix, and that the maximum available pore size within the MSPS matrix is similar to the estimated diameter of the ELISA complex, namely, approx 480 Å. This would be in good accordance with our other data.

This result suggests that the final agarose concentration of the MSPS is not the same as that of the starting mixture prior to beading, which is

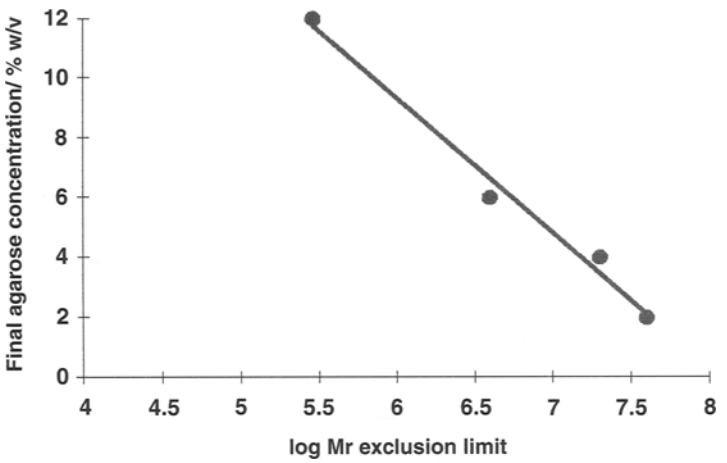

Fig. 5. Graph of log M_r (exclusion limit) vs agarose concentration of commercially available Sepharose and Superose.

2% w/v agarose. Evidence to support this comes from a comparative study of the size-exclusion limits against reported agarose concentrations of commercially available beaded agarose supports (Sepharose and Superose; Pharmacia, Uppsala, Sweden). Crosslinked Sepharose is available as agarose beads of 2% (CL-2B), 4% (CL-4B), and 6% (CL-6B) final agarose concentration, the mol wt (M_r) exclusion limits of which, for globular proteins, are given as 40,000 kDa, 20,000 kDa, and 4000 kDa, respectively; Superose 12 (12% agarose) is a highly crosslinked agarose matrix, with an exclusion limit of 300 kDa. An initial estimate of final agarose concentration of our beaded MSPS was obtained from a plot of log (M_r exclusion limit) vs reported agarose concentration using the published values for the above supports. A linear regression trend line was constructed from these data points, from which the final agarose concentration of our supports, which have an exclusion limit of 1,000 kDa, as judged by SEC *(vide infra)*, was estimated at approx 9% w/v (Fig. 5). This assumes that the degree of matrix crosslinking does not alter pore size *(29)*.

This estimated value was supported, using the method of Ennis *(18)*, to calculate the agarose content of nonmagnetizable agarose beads, prepared in identical fashion to the MSPS, with the omission of iron oxide. The fraction of a known packed volume because of beads could be calculated by subtracting the void volume, V_0, as measured by SEC, from the total packed volume. Using the value calculated for dry wt of agarose present in 1 mL packed volume of beaded agarose supports, the final agarose concentration was calcuated at 9% w/v. This corresponds closely with the figure estimated by comparison of exclusion limits of beaded agarose matrices.

Studies by Hjerten (*29*) have shown that the only factor to significantly affect beaded agarose pore sizes was an increase in the agarose content of the matrices. By using higher concentrations of molten agarose for beading, we have confirmed this by SEC of nonmagnetic agarose beads prepared using 6 and 10% molten agarose mixtures (results not shown).

The large-scale application of DEAE–MSPS will be of particular use for the isolation of very low copy-number plasmids, and for cosmid DNA; the large culture volumes required to isolate useful amounts of these types of DNA are entirely compatible with the modified alkaline lysis/MSPS method described here. The ease of handling of MSPS because of their response to a magnetic field means that large-scale separations of biological molecules are highly economical in terms of time, reagents, equipment, and solutions used, when compared to classical chromatography techniques.

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