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Tailor-Made Agarose-Based Reactive Beads for Hemoperfusion and Plasma Perfusion

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ABSTRACT

Composite beads of approximately 1mm diameter, made of cross-linked agarose and containing Fuller's Earth or zirconium oxide powders, were prepared and used in extracorporeal systems for blood detoxification. The former was used for the removal of Paraquat, while the latter was used to remove inorganic phosphate from hyperphosphatemic animals with or without acute renal failure. The high surface area of the powder, combined with the low resistance to diffusion in the cross-linked agarose matrix, are highly advantageous. The crosslinking provides high mechanical strength, heat stability, prolonged shelf life, good blood flow characteristics, and prevents the release of fine particles into the blood.

Crosslinked agarose beads of 1 mm diameter, containing chemically-bound heparin were also prepared, and used as a model for direct contact removal of LDL–cholesterol from the blood of familial hypercholesterolemic patients by hemoperfusion. The high capacity of these beads (over 5 mg LDL/mL beads) indicates that this clinical modality can replace the highly expensive plasmapheresis procedure presently used.

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Index Entries: Hemoperfusion, on agarose beads; composite sorbents, in hemoperfusion; encapsulation, and hemoperfusion; agarose beads, in hemo- and plasma perfusion; Paraquat, agarose beads for extracorporeal removal of; phosphate, agarose beads for extracorporeal removal of; LDL-cholesterol, agarose beads for extracorporeal removal of; plasma perfusion, with agarose beads.

INTRODUCTION

Yatzides (*1*) demonstrated that a column of activated charcoal particles can be used to remove uremic metabolites by hemoperfusion (HP). Chang (*2, 3*) was first to propose that microencapsulation and coating sorbent particles with a biocompatible membrane greatly improves blood compatibility and decreases the danger of embolism by fine particles release. Albumin–collodion (*3*), hydrogels (*4*), cellulose acetate (*5*), and albumin (*6*) were among the materials suggested as coatings in hemoperfusion (HP) columns. Uncoated polymeric sorbents, such as non-ionic polystyrene and polymethacrylate resins (XAD4 and XAD7, respectively) and others have been used (*7, 8*) in the removal of lipophilic drugs and metabolites.

Table 1 presents some of the composite sorbents suggested by various authors. These different designs are aimed at a safe contact between blood and sorbent. Thus, for example, membranes consisting of a cuprophan layer, backed by a layer of cellulose impregnated with activated charcoal, were used the for the combined dialytic and adsorptive removal of solutes (*9–11*). A HP system composed of a collodion film in which charcoal or XAD resin particles were embedded, was developed (*12, 13*) to remove protein-bound cholephilic anions. An active, rather than adsorptive, system was proposed by Chang (*14–16*), and involves semipermeable microcapsules containing enzymes and other biologically active materials. In a variation of the latter concept, a multienzyme system and cofactors were bound to soluble macromolecules such as dextran (*17*).

The application of a permeable matrix, which allows the transfer of plasma components but prevents the transfer of cellular blood particles through it, is an attractive and promising mode of operation (*18*). Thus, by utilizing beads of desired size and properties, efficient and safe HPs can be performed. Of the various gel-type matrices considered, such as cellulose, poly(hydroxyethyl methacrylate) and agarose, the latter (a natural polysaccharide) is probably the most versatile. The main constituent of agarose is a chain of D-galactose units joined by β-D(1 → 4) linkages, and 3,6-anhydro-L- galactopyranose units, joined by α-L-(1 → 3) linkages. The molecular structure of agarose is that of a left handed double helix with a threefold screw axis, a pitch of 1.90 nm, and a central cavity containing water molecules (*19*). The gel has solid-like characteristics, such as gelling and melting temperature. A gel with 2% agarose has noticeable

TABLE 1
Composite Systems Used in Blood Detoxifying Units

Material	Toxin eliminated	Tested	Ref.
Collodion film with: Charcoal XAD-2 XAD-4 XAD-7	Bile acids Chenodeoxycholylglycine Cholylglycine	In vitro and in vivo	Tangerman et al. (1980) (*12*) A. van Berlo et al. (1982) (*13*)
Cuprophan and cellulose-encapsulated charcoal	Urea, creatinine, phenobarbital vitamin B_{12} Inulin	In vitro and clinical	Randerson et al. (1982) (*11*)
Trisaccharide antigenic determinant of blood Group B, bound to silica	Anti-B antibodies	In vitro	Chang (1980) (*18*)
Tyrosinase in collodion microcapsules	Tyrosine	In vitro	Shu and Chang (1980) (*16*)
Microcapsules containing malic dehydrogenase, alcohol dehydrogenase, and dextran-bound NAD^+	Ethanol, oxaloacetate	In vitro	Grunwald and Chang (1980) (*17*)

mechanical strength (up to 2000 g/cm^2) and this can be further increased by crosslinking the agarose chains with epichlorohydrin (*20–22*) or dibromopropanol (*23, 24*). The crosslinked gel can then be steam sterilized at 121°C. The effective diffusivity through the agarose matrix is high, and the molecular size exclusion limit reaches 20×10^6 daltons for the 2% gels.

Figure 1 is a schematic representation of the agarose-based beads, in which the polymeric network performs as an encapsulating material for fine powders as well as a matrix for attaching chemically active ligands. Table 2 summarizes most of the reported studies involving either of these two options.

Agarose-based reactive systems were utilized experimentally for quite a while, but their clinical application in a recirculating extracorporeal system was restricted by the high pressure drop and low blood flow rates attainable through the agarose gel bed (*25–27*). These restrictions can now be overcome by following Brunner's (*28, 29*) simple procedure to produce beads in a wide size range. The characteristics of the

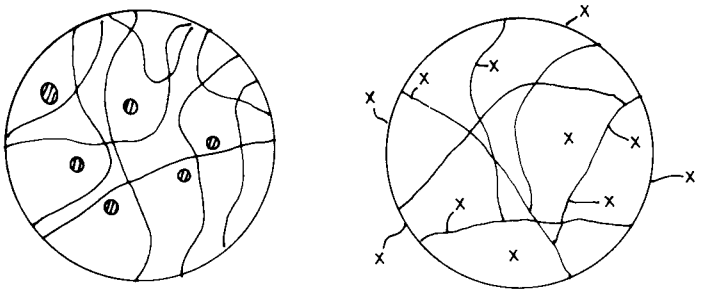

Fig. 1. Schematic representation of agarose-based beaded materials for use in extracorporeal blood detoxification units.

TABLE 2
Tailor-Made Agarose-Based Composite Materials Used in Blood Detoxifying Units

Material used	Toxin eliminated	Tested	Ref.
UDP-Glucuronyl transferase, NADPH-cytochrome-c-reductase, and cytochrome-P_{450} on Sepharose	Phenols Benzphetamine Hexobarbital Hexane Octanoic acid	In vitro and in vivo	Brunner and Loesgen (1977) (*28*)
Albumin on agarose	Chenodexycholic acid Unconjugated bilirubin	In vitro	Hughes et al. (1977) (*25*)
Protein A on agarose	IgG	In vivo and clinical	Jonsson and Hakansson (1981) (*31*) Jonsson et al. (1981) (*32*)
Heparin on agarose	LDL-cholesterol	Clinical	Lupien (1976) (*26*)
	LDL-cholesterol	In vivo	Moorjani (1977) (*27*)
	LDL-cholesterol	In vivo	Burgstaler (1980) (*33*)
	LDL-cholesterol	In vivo	Schmer et al. (1981) (*34*)
	LDL-cholesterol	In vitro	Sideman et al. (1983) (*35*)
LDL Antibody on Sepharose	LDL-cholesterol	In vitro and in vivo	Stoffel and Demant (1981) (*36*)
		Clinical	Stoffel et al. (1981) (*37*)

(continued)

TABLE 2 (*continued*)

Material used	Toxin eliminated	Tested	Ref.
Amberlite XAD-2 in agarose Dowex 1 × 4 in agarose Charcoal in agarose	Phenobarbital Methaqualone Glutethimide Phenol Bile Acid Thyroxine	In vitro	Brunner et al. (1980) (*29*)
Charcoal in crosslinked agarose	Orange II Vitamin B_{12} Inulin	In vitro	Xu et al. (1981) (*30*)
Amberlite XAD-4 in crosslinked agarose Charcoal in agarose	Phenol Creatinine Methyl orange Vitamin B_{12} Inulin	In vitro	de Koning et al. (1982) (*24*)
Fuller's Earth in crosslinked agarose	Paraquat	In vitro	Sideman et al. (1983) (*21*)
		In vivo	Sideman et al. (1983) (*22*)
Zirconium oxide in agarose	Phosphate	In vitro	Sideman et al. (1980) (*38*)
Zirconium oxide in crosslinked agarose	Phosphate	In vivo	Sideman et al. (1983) (*39*)

ensuing, mechanically weak beads were subsequently improved by crosslinking (*21, 22, 24, 30*), thus allowing for the production of mechanically stable, steam-sterilizable beads of desired size.

As also seen in Table 2, recent studies (*24, 29, 30*) report the encapsulation of finely divided activated charcoal particles. These composite materials exhibit the advantage of the high surface area of the powdered sorbent. Yet, their application is limited by the lack of specificity of the charcoal. Here, we briefly report our studies with agarose-based composite beads that are designed for specific tasks and serve as a practical model for tailor-made active sorbents.

MATERIALS AND METHODS

Agarose type II (Sigma Chemical Co., USA) was predominantly used here. Fuller's Earth (Surrey Finest Powder) was obtained from ICI (UK). Hydrated zirconium oxide (chloride form) was obtained from Organon Technika (USA).

Composite beads containing sorbents were prepared by melting a 4% agarose gel at 90°C, homogenizing it with 17 wt% sorbent powder (5–50 μm particles), and ejecting the mixture (through a dispensing/

vibrating mechanism) into a cold organic solvent [toluene:chloroform:hexane, 5:2:1 (v/v/v)]. Noncomposite, 2% agarose beads were prepared by ejecting the molten gel with compressed N_2 through a spray drying-type nozzle. These techniques yield beads from 0.25 to 2.5 mm diameter. The beads were crosslinked with epichlorohydrin in NaOH media, boiled in ethanol, and then steam sterilized (*21*).

Coupling of heparin to crosslinked agarose beads (*35*) was performed in an acetone medium, according to Nilsson (*40*).

RESULTS AND DISCUSSION

Removal of Paraquat

Fuller's Earth (FE), a calcium montmorillonite clay, has very high capacity for specific cations. FE adsorbs Paraquat more selectively than activated charcoal (*41*), and is used as an oral antidote in Paraquat intoxications (*42*). The performance of FE-containing composite agarose beads is shown in Fig. 2. It can be seen that the FE in the agarose gel retains its high capacity for Paraquat. As seen in Fig. 3, the rate of Paraquat removal increases with the crosslinking, probably because of a favorable modification of the porosity of the agarose network. Table 3 summarizes the changes of electrolytes concentration in plasma after contact with the composite beads for 3 h. It is evident that, except for Ca, no significant changes in plasma components take place. Consequently, the composite beads were calcium depleted by prewashing them with *3M* NaCl (*21*). Plasma Ca was unchanged after this treatment.

In vivo experiments conducted on rats showed very good Paraquat clearance, particularly with the smaller-sized beads. The data are presented in Fig. 4 as the percentage of inlet Paraquat removed by the HP

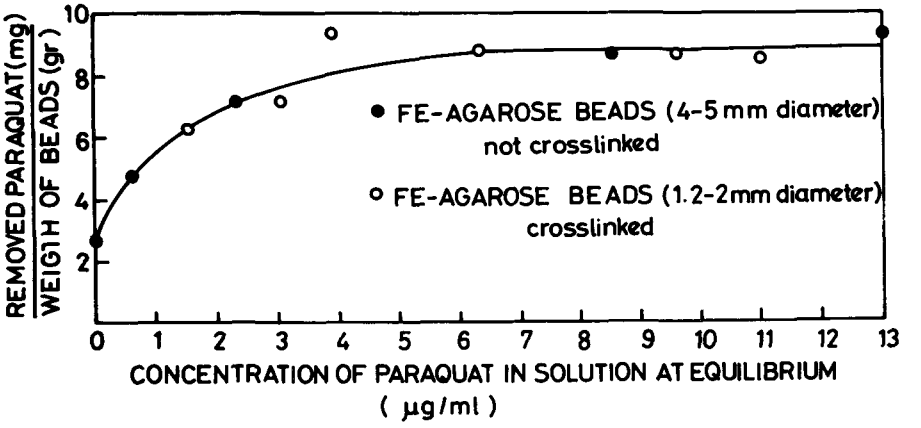

Fig. 2. Equilibrium isotherm for Paraquat adsorption by Fuller's Earth–agarose composite sorbent beads.

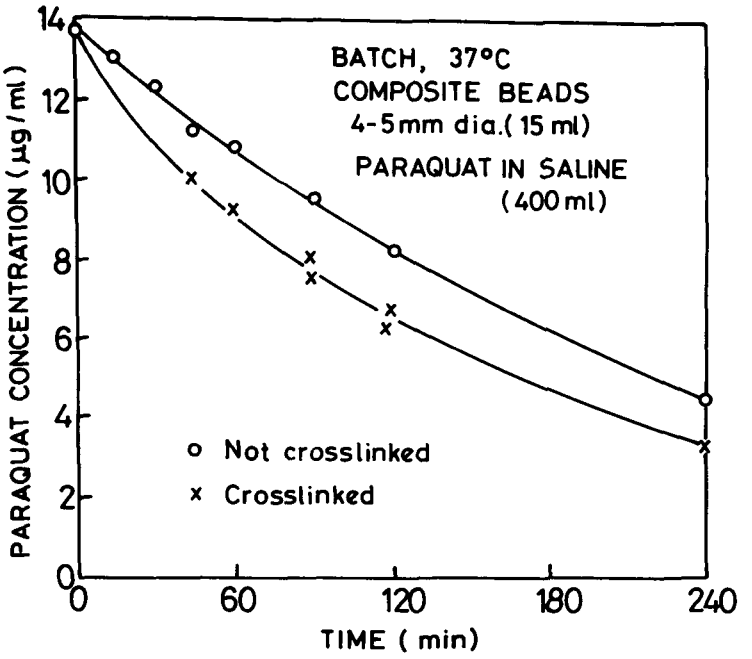

Fig. 3. Paraquat removal by Fuller's Earth–agarose composite beads. Effect of agarose crosslinking.

column. As seen, the FE composite beads perform better than the activated charcoal particles (from Adsorba^{R}-300 column) of similar size.

Additional in vivo studies on dogs (non-intoxicated) and one monkey (baboon) reveal that HP with FE composite beads can be performed essentially free of side effects. Some of the results obtained in these experiments are presented in Fig. 5. As can be seen, the depletion of blood platelets can be almost completely prevented when citrate (0.02 mL ACD/min/mL perfused blood) is infused at the column inlet.

Removal of Phosphate

Aluminum oxide, either in granular form (*43*) or as a powder packed into hollow fibers (*44,45*), was suggested earlier as a HP sorbent for phosphate, but was subsequently discarded when the dialysis dementia syndrome was related to increased concentrations of aluminum in plasma and in brain tissue (*46, 47*). Hydrous zirconium oxide (HZO) is presently used in dialysate recovery systems for the removal of phosphate ions from the dialysate fluids (*48*). The nontoxicity of zirconia compounds encouraged us in their use for blood treatment (*49, 50*).

The zirconia–agarose composite beads were prepared with NaOH-preconditioned HZO (*39*). Their good performance is demonstrated in Fig. 6 which shows phosphate removal by HP from a dog which is continuously infused with a phosphate solution (5 g P/mL solution infused at 0.07 mL/min/kg bw). Hyperphosphatemia was also induced by an in-

TABLE 3
Major Blood Components in Plasma Before and After 3-h Contact with Fuller's Earth–Agarose Composite Beads[a]

	Plasma #1			Plasma #2		
Component[b]	Before	After	% Δ	Before	After	% Δ
Na, meq/L	141	140	−1	154	143	−7
K, meq/L	23.8	22.2	−7	18.9	15	−20
Cl, meq/L	63	64	+2	74	64	−13
Glucose, meq/L	135	117	−13	194	180	−7
BUN, mg%	12	9	−25	9	8	−11
Ca (total), mg%	8.5	12.5	+51	8	12.5	+56
Phospate, mg%	19	19	0	11.6	9.8	−15
Cholesterol, mg%	195	176	−10	140	140	0
TG, mg%	95	85	−10	62	72	+16
Uric acid, mg%	4.5	4.5	0	4.1	3.8	−7
Creatinine, mg%	1.3	1.2	−8	1.3	1.2	−8
Total protein, g%	5.8	5.5	−5	6.8	6.5	−4
Albumin, g%	3.7	3.6	−3	4.6	4.5	−2
CPK, Units/mL	28	32	+14	20	26	+30
LDH, Units/mL	67.5	61.8	−8	259	272	+5
Alk. phos., Units/mL	96	113	+18	105	104	−1
SGOT, Units/mL	14	17	+21	9	11	+22

[a]The results were obtained with a batch of beads from which Ca^{+} was not removed prior to contacting the plasma.

[b]BUN, blood urea nitrogen; TG, triglycerides; CPK, creatinine phosphokinase; LDH, lactic dehydrogenase; Alk. phos., alkaline phosphatase; SGOT, serum glutamic oxaloacetic transaminase.

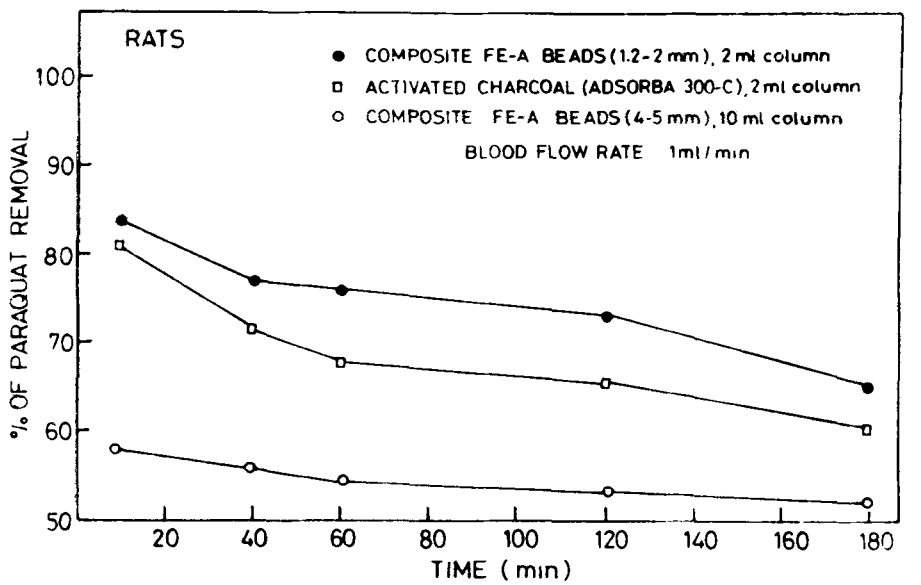

Fig. 4. Efficiency of various hemoperfusion columns for Paraquat removal from rats.

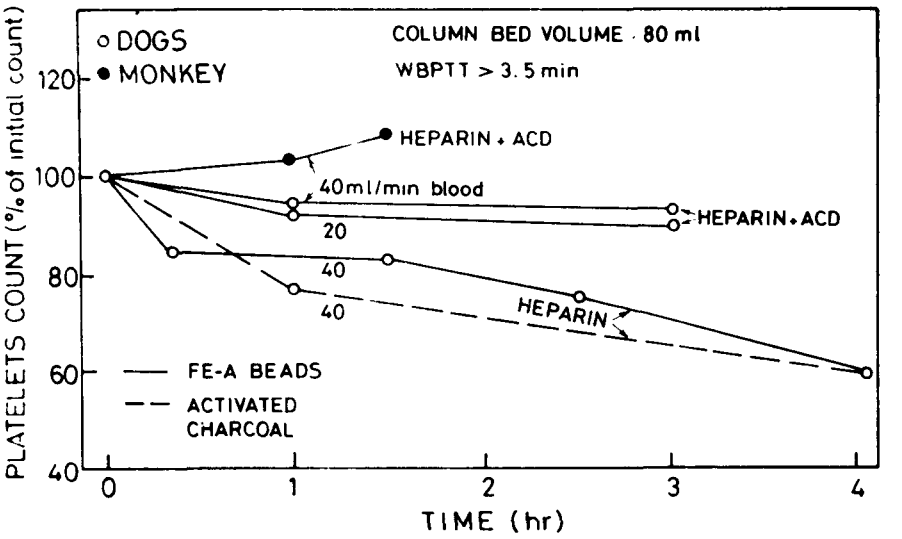

Fig. 5. Change of platelets count, during hemoperfusion of non-intoxicated animals under various conditions. Note the beneficial effect of ACD.

tramuscular injection of glycerol. Figure 7 shows the results of one of these experiments. As can be seen, phosphate removal is very efficient, and biocompatibility is acceptable. However, calcium ions were also removed by the HZO-containing beads (Table 4). This phenomenon is also demonstrated by our in vitro studies summarized in Table 5. This undesired side effect seems, however, to be transient, confined to the

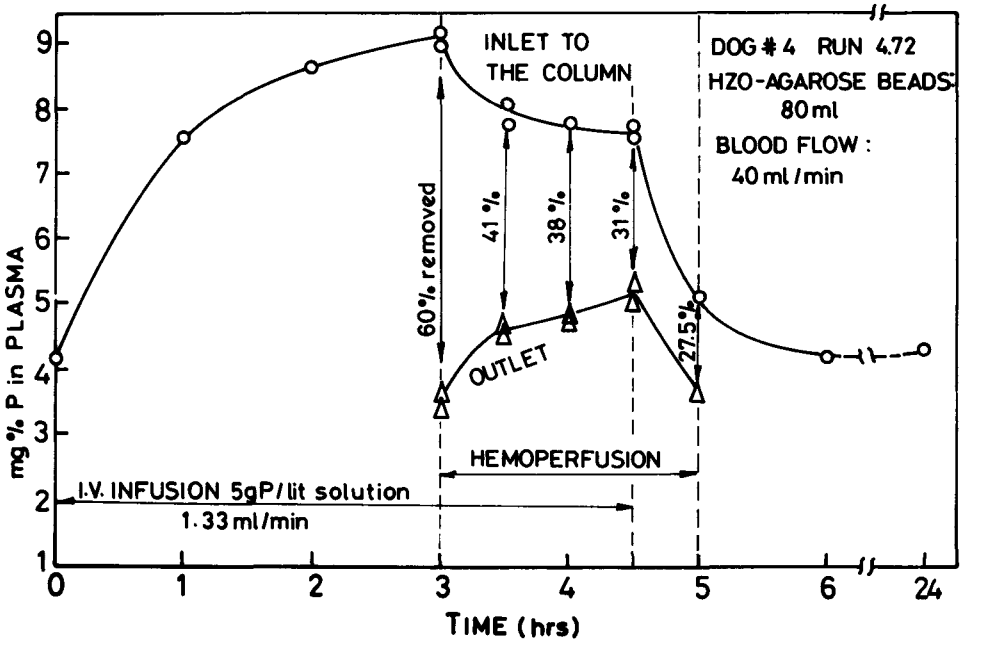

Fig. 6. Phosphate removal by hemoperfusion, from a phosphate-treated dog.

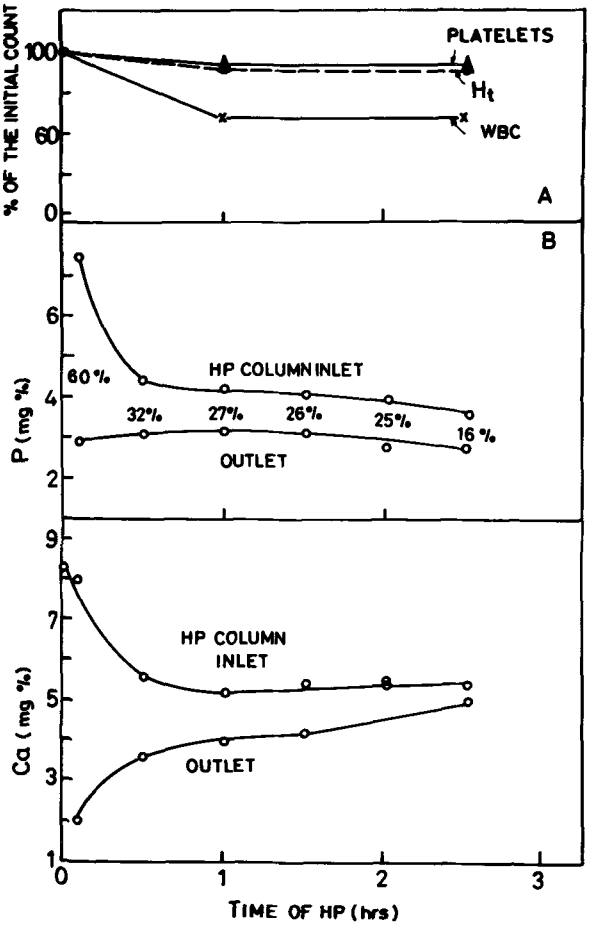

Fig. 7. Hemoperfusion over 40 mL HZO–agarose composite sorbent beads. HP initiated 50 h after injecting 10 mL glycerol/kg bw. Blood flow: 20 mL/min.

first hour of the treatment and could be corrected, in subsequent experiments with cats, by intravenous administration of calcium gluconate.

It is emphasized at this point that, since the HP column will in practice be usually connected in series before a hemodialysis unit, the Ca removal by the beads will automatically be corrected for by the dialysis process since the dialysate contains calcium ions.

Removal of LDL-Cholesterol

Lupien et al. (*26, 27*) removed LDL-cholesterol from whole blood by using batch affinity chromatography with heparin–agarose beads. This procedure is, however, impractical for clinical treatment and for processing large quantities of blood. Removal of LDL-cholesterol by heparin–agarose beads in an HP column (*33, 34, 37*) was inhibited by flow difficulties associated with the very small size of the agarose beads

(30–300 μm) used as column packing. Thus, only plasma after filtration could be treated in a continuous manner. Here, we report the development of a practical HP column for LDL removal from whole blood, utilizing crosslinked, 2% agarose beads (0.85–1.4 mm diameter), coupled with heparin and/or ethanolamine. With these materials, blood flow rates of up to 300 cc/min can be reached.

The penetration of LDL into the agarose matrix was assessed by comparing the breakthrough curves of LDL in plasma that was passed through columns packed with unmodified agarose beads. Figure 8 demonstrates that LDL penetrates the 2% agarose beads better than the 4% beads. Glass beads of a similar dimension were used as inert controls.

As shown in Figure 9, the amount of LDL removed in 3 h from a normal human plasma by the 2% agarose beads strongly depends on the beads' size. Beads in the range of 0.85–1.4 mm showed no blood flow problems and were selected for future studies. It is noteworthy in Table 5 that the agarose–heparin (H-agarose) and agarose–ethanolamine (EA–agarose) beads seem to perform very similarly. As can be seen, except for some dilution effect particularly noted in the control experiments with the unreactive agarose beads, no significant changes in plasma composition take place. Figure 10 shows the LDL is removed more effectively from hypercholesterolemic plasma than from normal plasma. This most

TABLE 4
Chemical and Biochemical Components of Plasma Before and After 3-h Contact with HZO-Agarose Composite Beads[a]

	Experiment 1		Experiment 2	
Plasma component[b]	Before	After	Before	After
K, meq/L	3.8	3.3	3.7	3.3
Na, meq/L	139	138	137	140
Cl, meq/L	105	103	106	102
Ca (total), mg%	9.1	5.8	9.5	low
P, mg%	7.2	2.6	6.3	2.3
Glucose, mg%	84	75	80	76
BUN, mg%	12	12	12	12
Creatinine, mg%	0.6	0.6	0.6	0.6
TG, mg%	109	110	115	112
Total protein, g%	6.5	7.1	6.5	6.9
Albumin, g%	4.6	4.9	ND[c]	ND[c]
Transaminase, Units/mL	9.5	8	10.5	11.5
Alk. Phos., Units/mL	104	101	130	119
LDH, Units/mL	141	104	132	147
CPK, Units/mL	18	14	25	21.5

[a]Batch-type operation, at 37°C.
[b]For abbreviations, see footnote (*b*) of Table 3.
[c]ND, not determined.

TABLE 5
Changes (%) in Plasma Composition After 3-h Contact with Agarose-Based Biosorbent Beads[a–c]

Plasma component[d]	Control[e,f]	H-agarose[g]	EA-agarose[h]
Na	+0.4 ± 2	+1.0 ± 2	+1.2 ± 2
K	−27 ± 2	−27 ± 4	−27 ± 3
Cl	+8.4 ± 6	+10 ± 8	+12 ± 8
Ca	−16 ± 5	−21 ± 3	−17 ± 8
P	−26 ± 6	−27 ± 8	−35 ± 5
Total protein	−24 ± 3	−27 ± 4	−28 ± 3
Albumin	−26 ± 3	−27 ± 3	−27 ± 3
Urea	−26 ± 4	−26 ± 4	−30 ± 4
SGPT	−25 ± 5	−30 ± 17	−27 ± 28
Alkaline phosphatase	−24 ± 10	−21 ± 12	−22 ± 10
Total cholesterol	−19 ± 7	−50 ± 4	−48 ± 3
LDL	−25 ± 7	−65 ± 4	−58 ± 7
HDL	−16 ± 13	−15 ± 13	−21 ± 12
Triglycerides	−17 ± 3	−42 ± 5	−39 ± 2

[a]Batch operation, 37°C.
[b]All beads were 0.85–1.4 mm in diameter, and contained 2% agarose.
[c]Positive and negative entries indicate increase and decrease of concentration, respectively. The results are averages of five experiments.
[d]SGPT, serum glutamic–pyruvic transaminase; LDL, low density lipoproteins; HDL, high density lipoproteins.
[e]Agarose beads to which no ligand was attached.
[f]The decrease of about 25%, observed in the control experiments for most plasma components, results from dilution by the interstitial saline present in the beads prior to the contact with the plasma.
[g]Heparin bound to agarose.
[h]Ethanolamine bound to agarose.

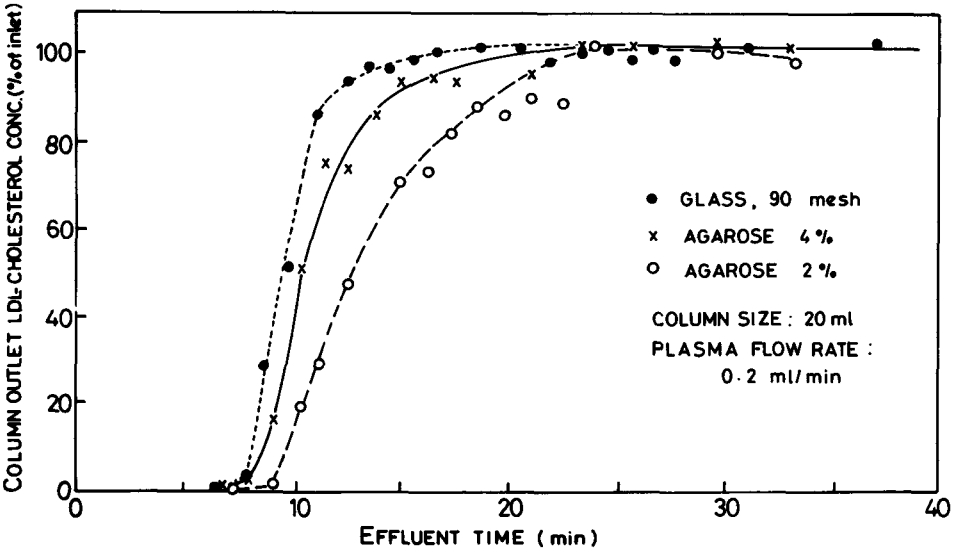

Fig. 8. Breakthrough curves of plasma LDL-cholesterol when using columns packed with nonreactive beads.

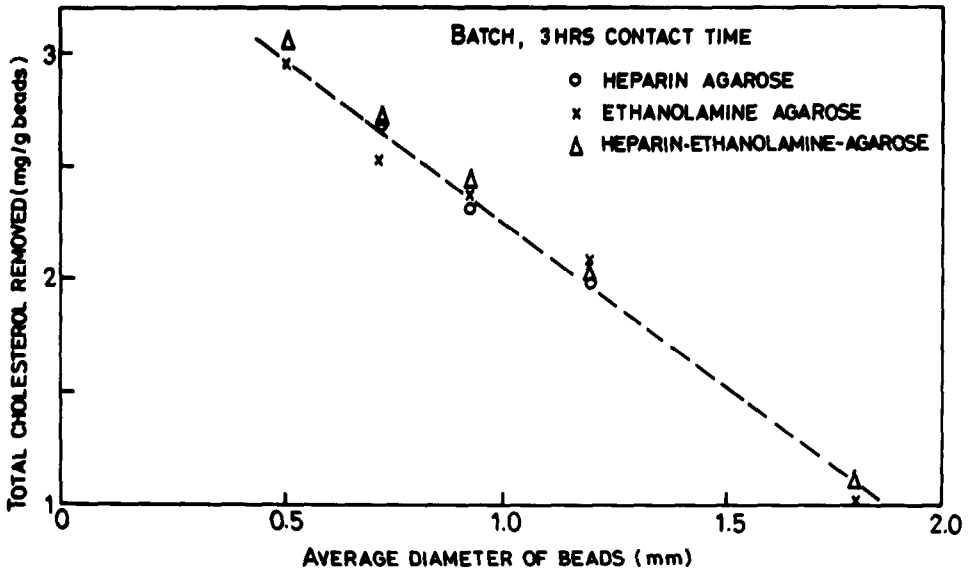

Fig. 9. Efficiency of agarose-based sorbents for removing cholesterol from a normal human plasma. Beads contain 2% agarose. Actual size ranges were (0.42–0.60 mm); (0.60–0.85 mm); (0.85–1.0 mm); (1.0–1.4 mm), and (1.4–2.0 mm).

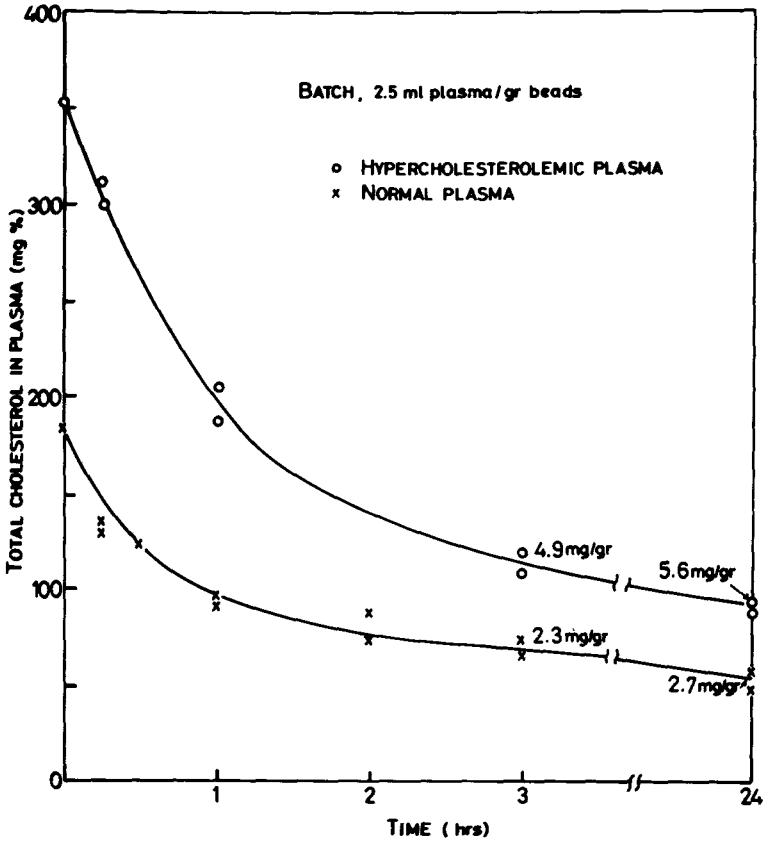

Fig. 10. Removal of cholesterol from normal and hypercholesterolemic human plasma on 2% agarose–heparin beads (1.0–1.4 mm diameter). The figures on the curves show the amount of cholesterol removed (mg/g beads) during the contact time.

probably results from the difference in the composition of the LDL in the hypercholesterolemic and normal plasma (rather than from a concentration-dependent phenomenon). About 5 mg LDL are removed per 1 mL beads, indicating that an HP column of some 600 mL will lower the cholesterol plasma level in hypercholesterolemic patients from 500 mg% LDL to a normal level by a single 2-h treatment. Preliminary in vivo tests with hypercholesterolemic rabbits (*35*) confirm this conclusion.

CONCLUSIONS

Specific, agarose-based, composite sorbents and reactive beads can now be tailor-made to efficiently remove or modify toxins, poisonous metabolites, antibodies, and other undesirable materials from blood and/or plasma. This is achieved either by encapsulating the specific sorbent power in a bood-compatible hydrogel or else by binding a reactive group to a suitable biocompatible solid carrier. Crosslinking of the agarose network in the beads gives them increased mechanical strength and temperature stability. The applicability of these procedures was demonstrated by entrapping Fuller's Earth and zirconia powders, and by attaching heparin to the crosslinked agarose matrix.

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