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Characterization and Extracorporeal Application of a New Phosphate-Binding Agent

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Summary: A new phosphate-binding agent which does not cause any severe side effects in vivo was developed by modifying a crosslinked dextran with polynuclear iron(III)oxide-hydroxide. Its particle size ranges from 150 to 300 µm, and the iron content was about 18% by dry weight. The oxidation state of iron was characterized by ESCA and *Mössbauer* spectroscopy. The maximum phosphate binding capacity of the iron(III)oxide-hydroxide-modified dextran was determined with respect to aqueous phosphate solutions, human serum and whole blood. The effects on whole blood count, haemolysis, protein concentration and enzyme activities were examined. In addition, the influence of phosphate concentration, pH and temperature on the phosphate uptake of the material was determined. The results show that this new adsorbent might provide an alternative to conventional phosphate-binding agents. This paper also describes the first experiments on the therapeutic application of the material in an extracorporeal blood perfusion system for the treatment of hyperphosphataemia during haemodialysis.

Introduction

In chronic renal failure phosphorus retention and hyperphosphataemia play a major role in the development and maintenance of secondary hyperparathyroidism and osteodystrophy (1–3). Neither an adequate diet nor efficient dialysis are usually sufficient to prevent pathologic phosphate concentrations in the blood (4). Antacids such as $Al(OH)_3$ or $CaCO_3$ possess the capacity to adsorb phosphate. However, antacids presently used for that purpose are quite inefficient in binding phosphate in vivo, although aluminum- or calcium-containing compounds are orally administered in large amounts (5, 6). The inefficiency of commonly-used phosphate binders creates a clinical dilemma, since the control of hyperphosphataemia requires increased doses, which result in a higher risk of toxicity. This includes bone disease, aluminum dementia from aluminum containing antacids (7, 8), and hypercalcaemia, as well as soft tissue calcification from antacids containing calcium (9). The increased tissue content of aluminum appears to be an important factor in the pathogenesis of dialysis-related encephalopathy and osteomalacia. The use of calcium compounds is associated with gastrointestinal problems such as diarrhoea or mild constipation and a high risk of hypercalcaemia. The dosage of magnesium hydroxide as an alternative phosphate binder is limited by the serum magnesium concentration. In addition, this agent alone has proved inadequate for the exact control of serum phosphate (10). Current therapies include reduction of dietary phosphate intake, reduction of phosphate absorption in the intestine by phosphate binding agents, and enhanced removal of phosphate from the body through more efficient dialysis techniques. The recommended intake of phosphate should not exceed 32 mmol (1000 mg P) per day in adults and should be reduced for children according to their age. With highly specialized diets, the intake of phosphorus can be reduced to less than 16 mmol (500 mg P) per day (11). In patients with mildly increased serum phosphate concentrations, haemodialysis removes about 8 mmol (252 mg P) of phosphate per day with three treatments per week. Continuous ambulatory peritoneal dialysis even removes about 10 mmol

(324 mg P) per day (12). These rates of removal, however, are still insufficient. Hence, most patients with end-stage renal failure require phosphate binders.

Recently we developed a novel phosphate-binding agent by coupling polynuclear iron(III) oxide-hydroxide to crosslinked dextran (13). In addition to its oral application, this material has the potential to serve as a phosphate adsorbent in extracorporeal perfusion systems for the treatment of hyperphosphataemia.

Materials and Methods

Preparation of phosphate adsorbent

Starting material for the preparation of an insoluble iron(III)oxide-hydroxide porous support was the crosslinked dextran Dormagel N 25 C™ (Pfeifer and Langen, Dormagen, Germany). Dormagel™ is a spherical, neutral, soft gel cross-linked by epichlorhydrin. The molecular cut-off of the unmodified material corresponds to M_r = 6000, and the particle size is 150–300 µm. The pH stability ranges from 2 to 12, and the swelling capacity is 4–6 ml/g.

For chemical modification according to l. c. (13) the material was suspended in a 50% solution of $FeCl_3 \cdot 6\ H_2O$. This mixture was then added under vigorous stirring to 1 mol/l sodium hydroxide solution. The modified gel beads were collected by filtration and washed with water to neutrality. Sterilization was carried out at a temperature of 121 °C according to $F_0$15 conditions using an autoclave (type GETING GEV 112) (14).

Test solutions

Standardized phosphate solution: 10 mmol/l NaCl, 4 mmol/l KCl, 0.5 mmol/l Na_2SO_4, 1.6 mmol/l $Na_2HPO_4 \cdot 2\ H_2O$, 1.6 mmol/l $NaH_2PO_4 \cdot 2\ H_2O$ (pH 7.4).

Standardized calcium solution: 50 mmol/l Tris, 2.5 mmol/l $CaCl_2 \cdot 2\ H_2O$, 10 mmol/l NaCl, 4 mmol/l KCl, 0.5 mmol/l Na_2SO_4 (pH 7,4).

Blood and plasma: Fresh blood from pigs was obtained from the slaughter house in Melsungen and human blood was provided by the DRK blood bank, Kassel. For stabilization, heparin (6000 IU/l) was added. The blood was centrifuged for 10 min at 4500 min^{-1} to separate blood cells from plasma.

Determination of enzymes and electrolytes

Enzyme activities of glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, lactate dehydrogenase, and alkaline phosphatase were determined according to standard methods, described by *Rick* (15). Phosphate, calcium, magnesium, iron, and protein concentrations in serum, plasma or aqueous solutions were photometrically determined according to standard protocols (16–18).

The iron content of the adsorbent was determined by atomic absorption (AAS) (19), and its phosphate concentration by inductively coupled plasma spectroscopy (ICP) (20).

Physical characterization

Scanning electron microscopy of the adsorbent was performed on a SEM XS 40 (ABT, Japan) at 20 kV. The magnification for the uncoated and the modified dextran beads was in the range of 100–3000-fold. Electron micrographs were taken with an Asanuma Camera (Mechanical Laboratory & Co, Japan) using a Polaroid Film Type 52 (400 ASA/27 DIN). ESCA (electron spectroscopy for chemical analysis), *Mössbauer* and magnetic measurements were kindly performed by Drs. *J. Knecht* and *J. Pebler*, Fachbereich Chemie, University of Marburg.

In vitro perfusion experiments

In vitro tests with standard solutions, plasma, or blood were carried out as follows. Glass columns (Bio-Rad, 120 × 10 mm) were packed with 3 ml of the adsorbent and equilibrated with Tris buffer pH 7.4. Standard phosphate solution or plasma was pumped through the columns by means of a roller pump (Infusomat, B. Braun Melsungen AG) at a flow rate of 1 ml/min. After collection of 4 ml pre-eluate, samples were drawn from the eluate at various time intervals. Phosphate, iron, calcium, glucose, heparin, and enzymes were determined in the samples. For the analysis of plasma the reservoir was kept in a shaking bath at 37 °C.

To determine the maximal binding capacity, aqueous phosphate solution, blood or plasma was circulated for 18 h at a rate of 1 ml/min through a column containing 3 ml adsorbent. The material was then washed with 100 ml distilled water to remove unbound phosphate, dried at 60 °C, and analysed for phosphate as described above.

For adsorption experiments with blood, a cylindrical Makrolon™ cartridge (volume: 250 ml) was used. The column inlet and outlet were closed by sieves with a mesh size of 94 µm. EDTA-stabilized blood (500 ml) was circulated through the cartridge at a flow rate of 100 ml/min. The blood reservoir was kept in a shaking bath at 37 °C.

In vivo perfusion tests

In vivo experiments were carried out with locally anaesthetized female sheep in the laboratory of the Experimental Surgery department (B. Braun Melsungen AG) according to an officially licensed protocol. Cartridges (250 ml) were slurry-packed with adsorbent and integrated in a dialysis circulation unit (HD secura, B. Braun Melsungen AG). Equilibration of the adsorbent and prewashing of the tubing system was carried out by circulating with 9 g/l sodium chloride solution. A conventional dialysis solution (acetate concentrate, 35 mmol/l) was used. To prevent fibrin clotting during therapy, heparin (10 000 IU/h) was administered to the sheep.

Results

Characterization of the adsorbent

Reaction of the insoluble, crosslinked dextran matrix with a concentrated $FeCl_3$ solution at high pH led to an iron uptake of 160–210 g/kg under our standard reaction conditions. A stable product was obtained after washing with deionized water to neutrality. Drying, heat sterilization, prolonged passage of phosphate solutions, or contact with blood did not result in any leaching or loss of iron from the material. Although the uniform surface of the untreated gel beads became rough and vaulted in the iron-coated spheres (fig. 1) the material could without difficulty be handled in flow-through columns or cartridges.

X-ray diffraction indicated the complete absence of crystalline structures in the solid. The binding energies of iron and oxygen in the compound, determined by X-

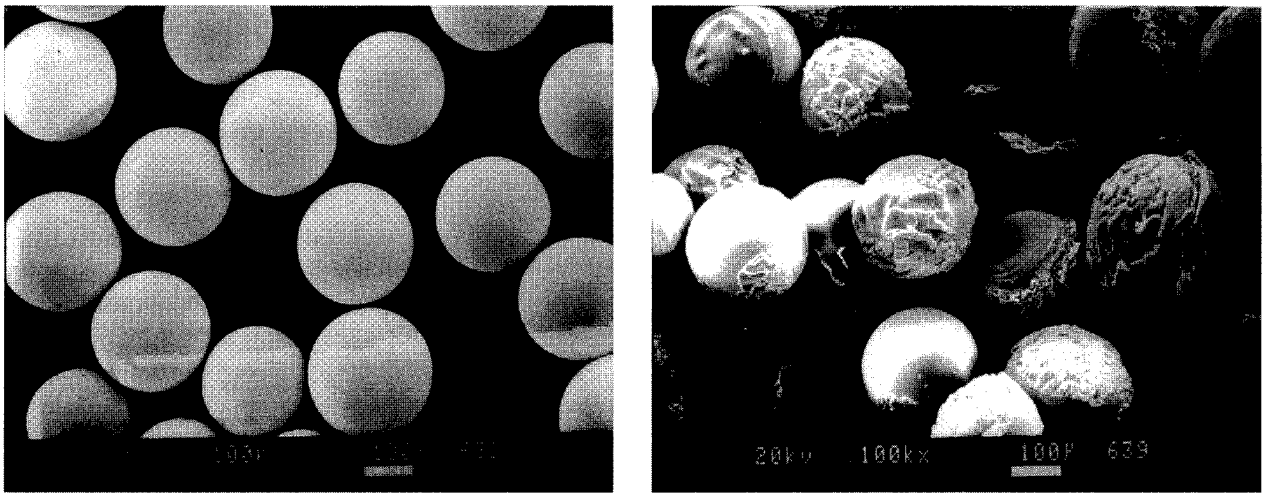

Fig. 1 Scanning electron micrographs of dextran (Dormagel N25C) adsorbent. Left: Unmodified. Right: Iron(III)oxide-hydroxide-modified (Fe: 188 g/kg). Magnification, 100-fold.

ray photoelectron spectroscopy (ESCA), were 710.5 eV ($Fe_{2P3/2}$) and 535.4 eV (O_{1S}), respectively, in close agreement with the values found in $Fe(OH)_3$ and other reference compounds (21). *Mössbauer* and magnetic susceptibility measurements revealed the presence of high-spin Fe^{3+} ions in a slightly distorted environment, and an effective magnetic moment of $\mu_{eff} = 5.9\ \mu_B$. All these data confirm that the oxidation state of the metal in the iron-modified dextran is exclusively + III.

Phosphate adsorption characteristics

Uptake of phosphate from a standard solution is shown in figure 2. Iron-dextran complexes with an iron content between 190 and 210 g/kg revealed the highest phosphate binding capacity (tab. 1). Higher amounts of iron, produced by repeated treatment of the adsorbent in $FeCl_3$ solution, did not raise the phosphate binding capacity. In equilibrium binding experiments, sterile adsorbents coated with 210 g/kg iron typically bound 250 mmol (7.7 g) P_i per kg or 90 mmol (2.6 g) P_i per litre adsorbent. These values were obtained by equilibrating 3 ml of sterilized adsorbent in a column for 18 h with 500 ml circulating phosphate standard solution, followed by removal of unbound inorganic phosphate by washing the column with 50 ml distilled water. Absorption of phosphate from an aqueous solution onto the iron-dextran complex could also be monitored by ^{31}P-NMR (not shown).

Tab. 1 Iron content and phosphate adsorption capacity of iron-dextran complexes.

Product No.	Preparation conditions	Iron content (g/kg)	Phosphate adsorption (mmol/l)	Phosphorus content (g/kg)
4	reaction at pH 6	84	56	4.0
5	standard	157	71	5.1
3	standard	188	74	5.3
8	standard	199	84	6.0
2	standard	203	88	6.3
1	standard	211	89	6.4
6	$FeCl_3$ repeated	255	85	6.1
7	$FeCl_3$ repeated	293	85	6.1

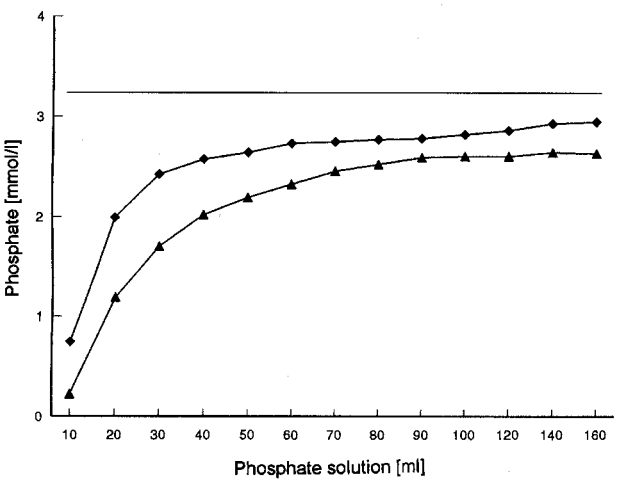

Fig. 2 Removal of phosphate from a standard solution. Columns packed with 3 ml of iron-dextran adsorbent (iron: 188 g/kg) were perfused at 1 ml · min^{-1} flow rate and the phosphate content of the eluate was determined. The horizontal line represents the initial concentration (P_i, 100 mg/l). ▲: Dried adsorbent, not sterilized. ●: Dried material sterilized at 121 °C.

The phosphate adsorption capacity was generally reduced about 30% after heat sterilization (fig. 2). On the other hand, heat sterilization appears to lead to a stabilized iron(III) oxide-hydroxide modified dextran at acidic pH values. Thus, the sterilized adsorbent does not liberate iron even around pH 2.

Perfusion of 3 ml adsorbent (950 mg dry weight, iron content 157 g/kg) with 100 ml human plasma resulted in the binding of 2.04 g phosphorus per litre gel which corresponds to a 74% reduction of the phosphate content (fig. 3). No alterations of the protein and iron(III) concentration, or of distinct enzyme activities were observed during the treatment. The profile of the elimination curve shows that additional phosphate can be adsorbed when higher blood phosphate concentrations are present.

Investigation of the haemocompatibility of the adsorbent in a 250 ml-cartridge showed that the phosphate concentration of circulating whole blood decreased from 1.1 mmol/l to 0.09 mmol/l (3.4 mg/dl to 0.3 mg/dl P_i). At a flow rate of 100 ml/min, a pressure of 80 mbar was built up. Even after 25 circulations the material did not induce haemolytic reactions, and there were no significant alterations in common blood quantities as shown in table 2.

Elimination of calcium ions

The behaviour towards calcium ions was determined by perfusing the adsorbent in a 3 ml column with 100 ml of human serum, heparinized plasma or standardized calcium solution (fig. 4). Calcium was only bound to the adsorbent in the presence of phosphate but not in phosphate-free solution. A molar ratio of calcium and phosphate elimination of approximately 1 : 1 was reached when both concentrations were varied.

In vivo experiments

The iron(III)oxide-hydroxide dextran adsorbent was tested in extracorporeal apheresis experiments with sheep. Laboratory data are reported in table 3. The aim of these tests was to investigate functional aspects such as handling, flow rate, pressure, and the influence on important quantities such as haemocompatibility, whole blood count, and the concentrations of phosphate, calcium and iron. The application of the adsorbent caused an efficient and significant decrease of the post-cartridge phosphate concentration from 1.93 mmol/l (6.0 mg/dl) to 0.06 mmol/l (0.2 mg/dl) P_i at the end of treatment (1 h dialysis). There was neither an increase in the iron nor a decrease in the calcium concentration of the blood during treatment with conventional dialysis solutions containing calcium. By appropriate dosage of heparin, the flow rate and pressure could be kept at normal levels without inducing haemolysis or significant alterations of the blood cells. Only thrombocytes were reduced during treatment. This was possibly due to some fibrin clotting which was not observed in in vitro experiments.

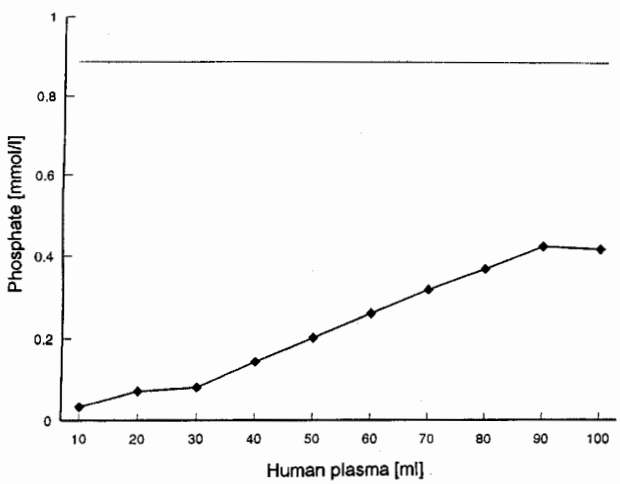

Fig. 3 Elimination of phosphate from 100 ml human plasma (initial P content, 2.8 mg). Conditions as in Fig. 2.

Discussion

Patients with kidney diseases, especially those with chronic renal failure, who have been on haemodialysis for a long time, often have symptoms associated with highly elevated concentrations of inorganic phosphate in the blood. Large amounts of phosphate above normal levels (0.6−1.3 mmol/l) result in a decrease of calcium ion concentration which in turn induces the parathyroid to secrete an excess amount of parathyroid hormone. The hormone increase and the inability of the diseased kidney to hydroxylate 25-hydroxycholecalciferol to the active forms of vitamin D are the principal biochemical factors underlying most of the related symptoms (22, 23). In the case of chronic renal failure the filtration rate of glomeruli is less than 8−10 ml/min. Pathological phosphate concentrations ranging between 2.25 and 3 mmol/l normally decrease to 1.3−1.6 mmol/l after haemodialysis (24). In this context, it has recently been shown that extracorporeal elimination of phosphate is more effective using standard dialysers than high-flux-dialysers (*T. Eisenhauer, C. Ronco*, unpublished). This effect is due to the limited phosphate concentration in the blood. Thus, the intravascular phosphate concentration rather than the clearance seems to be rate-limiting. The phosphate clearance, for example of the Diacap capillary dialyser (B. Braun, Melsungen, Germany) lies between 93 and 150 ml/min when pumping blood at 200 ml/min and dialysis solution at 500 ml/min.

The phosphate intake of a patient is 32 mmol per day or about 224 mmol per week. During one dialysis session about 32 mmol phosphate are normally eliminated (4). These rates of removal are insufficient to prevent hyperparathyroidism. The surplus of about 130 mmol phosphate per week has to be additionally eliminated. Hence,

Tab. 2 Blood count and erythrocyte characteristics during perfusion of iron-dextran phosphate adsorbent with human blood.

Quantity	Number of passages										
	0	2	4	6	8	10	12	16	20	24	25
Leukocytes (10^9/l)	4.9	5.1	5.0	5.0	4.9	5.2	4.9	5.2	4.9	4.7	4.9
Erythrocytes (10^{12}/l)	4.4	4.5	4.4	4.4	4.5	4.4	4.5	4.3	4.3	4.3	4.5
Haemoglobin (g/l)	125	126	126	126	127	126	127	124	124	125	124
Haematocrit (fraction)	0.392	0.399	0.395	0.391	0.395	0.390	0.390	0.375	0.374	0.373	0.371
MCV (fl)	89.7	89.0	88.9	88.4	88.3	87.8	87.4	86.9	86.7	86.8	86.5
Hb_E (pg)	28.5	38.0	26.3	28.5	28.5	28.4	28.5	28.8	28.7	26.7	29.0
MCHC (g/l)	318	315	318	322	324	324	324	331	331	330	335
Thrombocytes (10^9/l)	264	273	270	257	269	269	267	272	266	256	270
Lymphocytes (%)	29	30	32	33	30	30	35	27	27	31	30
Monocytes (%)	3	–	1	3	5	5	4	4	4	5	6
Segm. granulocytes (%)	62	66	63	59	63	63	53	61	65	60	62
Eosinophiles (%)	3	4	2	1	1	1	3	8	4	4	2
Basophiles (%)	1	–	–	1	1	1	–	–	–	–	–

A cartridge containing 250 ml phosphate adsorbent was perfused at 100 ml · min^{-1} under recirculating conditions with 500 ml human blood containing 7 mmol/l EDTA.

MCV = Mean corpuscular volume
Hb_E = Haemoglobin content per erythrocyte
MCHC = Mean corpuscular haemoglobin concentration

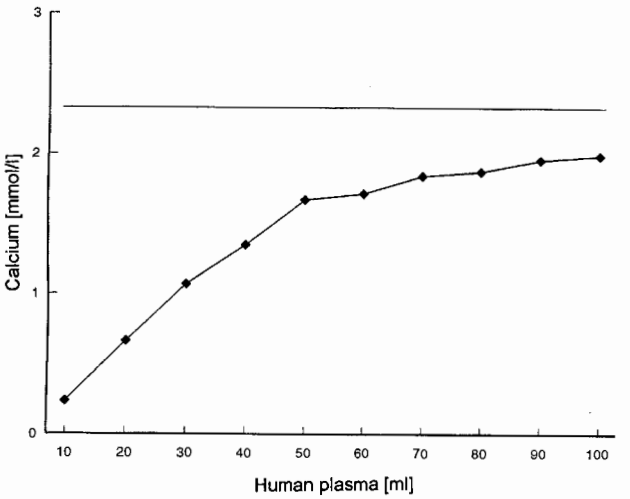

Fig. 4 Elimination of calcium from 100 ml human plasma (initial Ca content, 9.35 mg) concomitant with phosphate removal (cf. fig. 3).

Tab. 3 Electrolyte concentrations in sheep blood during an extracorporeal treatment with iron-dextran complex.

Electrolyte	Time (min)					
	0	15	30	45	60	75
P (mmol/l)	1.94	0.03	0.03	0.13	0.06	0.06
Fe (µmol/l)	2.65	2.63	2.75	2.79	2.40	2.72
Ca (mmol/l)	2.52	2.87	2.54	2.64	3.29	2.85

Blood was passed through a cartridge containing 250 ml of phosphate adsorbent; flow rate, 70–90 ml/min.

most patients with end-stage renal failure and many with moderate to marked renal insufficiency require phosphate binders. Their application is necessary to avoid skeletal pain, changes in bone mineralization, spontaneous fractures, and calcium phosphate-like deposits in soft tissues and blood vessels (25, 26). In recent years it has been reported that the most seriously intoxicated patients had been treated with dialysing fluid containing high concentrations of aluminum and phosphate binders containing aluminum (27–29). The dialysis dementia syndrome was related to the increased concentration of aluminum in the plasma and in the brain tissue (30, 31). For that reason many authors propagated the oral application of aluminum-free compounds such as calcium carbonate or calcium acetate (32, 33). However, medication with the latter is associated with gastro-intestinal problems and a high risk of hypercalcaemia (10).

From these considerations it follows that a more effective and safer treatment of hyperphosphataemia can be achieved by extending the total dialysis time and by the optional application of aluminum-free phosphate binders. Prolonging dialysis time, however, is not applicable as it additionally stresses the patient. Instead, the desired systemic reduction of phosphate should be achieved by using, in addition to and simultaneously with conventional haemodialysis, an adsorbent which specifically removes the excess phosphate. To be clinically useful, such an adsorbent has to be integrated into the extracorporeal perfusion system and must be capable of eliminating phosphate directly from whole blood. Extracorporeal removal of excess inorganic phosphate is especially of interest for patients who refuse phosphate-lowering medication because of personal discomfort. For a potentially chronic application such a procedure must be free of even minor side effects. In addition, it should permit a blood flow of at least 100 ml/min.

We therefore recently developed a new phosphate binding agent (13). A porous, highly crosslinked and insoluble dextran was complexed with polynuclear iron oxide-hydroxide (FeOOH) centres. With the exception of a non-porous agarose chromatography support described by *Hjerten* (34), only soluble complexes between iron hydroxide and mono- and polysaccharides have previously been prepared (35, 36). Such complexes, e. g. with polymaltose (Ferrum-Hausmann™), are used for the therapeutic treatment of iron deficiency anaemias in humans and animals (37, 38). Structural studies on soluble iron oxide-hydroxide complexes showed that they form chainlike, polynuclear condensation products (39) and that iron is present as tetragonal β-FeOOH (40).

Structure characterization of the newly synthesized, amorphous, insoluble iron-dextran complexes is less complete. Nevertheless, the ESCA and *Mössbauer* spectra confirm that only high-spin ferric irons with oxygen ligands (sugar OH groups, water, hydroxide ions, μ-oxo bridges) are present. The most typical iron mass proportion of about 200 g/kg found in the dry material suggests that each iron(III) centre is statistically coordinated to one glucose moiety (glucose·$Fe(OH)_3$, Fe = 195 g/kg). In fact, the magnetic susceptibility and its temperature dependence, measured in the range from 4 to 300 K, are very similar to a soluble, stoichiometric Fe-glucose complex (35).

A few reports describe the interaction of phosphate with iron-containing compounds (34, 37, 41, 42). Addition of phosphate, even in small amounts, may lead to strong effects, for example increased viscosity in iron-polymaltose solutions (37). FeOOH-modified non-porous agarose has been saturated with phosphate and used in this form as ion exchanger for the chromatography of proteins (34).

The adsorbent described here is, to our knowledge, the first one that specifically removes inorganic phosphate and which fulfils the criteria for an application in an extracorporeal blood perfusion system. The high binding capacity of the adsorbent guarantees an efficient elimination of phosphate during haemodialysis. The pathological excess of about 130 mmol of phosphate per week in patients with end-stage renal failure, for example, would be eliminated during the common three dialysis sessions by an additional, integrated phosphate adsorption apheresis using a cartridge packed with 450 ml of the new adsorbent.

On-line apheresis has the extra advantage that it can be precisely controlled by adjusting the cartridge size (i. e. total phosphate binding capacity) to the blood volume. Material costs can be significantly reduced by repeated regeneration and re-use of the adsorbent. In this context it was shown that regeneration by a simple treatment with NaOH did not cause a decrease of the phosphate binding capacity. The described in vivo experiments with sheep revealed no side effects. Concomitant calcium depletion can be compensated by dialysis. Ideally, it may be expected that oral phosphate binders can be avoided and no other measures have to be taken.

In conclusion, the newly developed iron(III) oxide-hydroxide modified dextran is suitable for the selective elimination of inorganic phosphate from whole blood. The excellent phosphate elimination capacity and blood compatibility make it attractive for the extracorporeal treatment of hyperphosphataemia in acute and chronic renal failure.

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