PREPARATION AND APPLICATION OF MAGNETIC POLYMERS FOR TARGETING OF DRUGS

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1. Introduction

There has been considerable interest during the last few years in the area of drug targeting as it has become clear that in order to achieve optimal conditions for the action of pharmacological agents interaction with non-target sites should be minimized [1] Investigations to complex antitumour drugs with antibodies are noteworthy in this context [2] Similarly, the use of liposomes as carriers deserves mention as they are predominantly transported into the liver and spleen [1]

While studying the use of magnetic carriers in affinity chromatography [3] it occurred to us that magnetic material might also be used as support for drugs which could be brought to the target site with the aid of a magnetic field [4] Apart from minimizing interactions with non-target sites such a specific location would allow a high concentration of the drug to be used and would extend the time of exposure at the site

In the following we wish to report on the preparation and application of such magnetic drug-carrying materials

2. Materials and methods

2 1 *Materials*

Acid-hydrolysed starch with mol wt ~40 000 was used in all experiments The starch (Farinex PS1®) was a gift from AB Stadex, Malmo H–D-Val–Leu–Lys–pNA was obtained from Kabi Diagnostika, Stockholm

The emulgator Gafac PE-510® (an ester of phosphoric acid) as well as the carbonyl–iron powder (diam 2–3 μm containing a minimum of 99 5% iron) were a gift from AB Svenska GAF, Stockholm, (GAF, 140 West 51 Street, New York, NY 10020) Powdered magnetite in aqueous slurry (~45% solids with diam 0 05 μm, type 4100) was from Wright Industries, Brooklyn, New York

(^{125}I)Albumin, type IM-17P, with an activity of 50 μCi/ml (20 mg/ml) as well as [2-^{14}C]ethanolamine with an activity of 44 mCi/mmol were from the Radiochemical Centre, Amersham

α-Amylase with spec act 20 μKat/mg was obtained from Sigma, St Louis, MO Plasmin was a gift from NOVO A/S, Copenhagen, with spec act 3 2 NU/mg (NOVO units One unit is defined as the amount of enzyme that in 20 min at pH 7 5 and 35 5°C gives rise to the formation of perchloric acid-soluble degradation products with an A_{275nm} of 1)

2 2 *Preparation of magnetic microspheres*

In a typical procedure 10 ml water were mixed with 6 g starch and 5 g carbonyl–iron in a beaker The slurry was heated under gentle agitation and allowed to boil for a few seconds to ensure complete solubilization of the starch At >90°C, the starch–carbonyl–iron slurry was slowly poured into 100 ml stirred toluene at room temperature containing the dissolved emulgator Gafac PE-510 at 1% The speed of stirring was adjusted according to the desired size of the microspheres (e g , 6000 rev /min resulted in beads of ~3–15 μm diam) Immediately thereafter the mixture was cooled in an ice bath (still under stirring) to 10°C Recently we found that on increasing the amount of carbonyl–iron 10-fold, a high yield of beads (95%) with a narrow size distribution of 2–5 μm could be obtained

For non-magnetic starch microspheres the same amounts and procedure as for magnetic microspheres was applied except for omission of the iron particles. In the washing procedure with acetone, the microspheres were allowed to sediment and when washing with water, centrifugation was used. When the precipitate turned too hard, a quick sonication resuspended the microspheres.

The magnetic microspheres obtained were transferred to a 500 ml beaker containing 200 ml acetone and placed on an electromagnet. The supernatant was aspirated. The same procedure was repeated 4 times with equal amount of acetone followed by washing with water 6 times.

When powdered magnetite was used the same procedure was followed except that 10 g powdered magnetite was used and that after boiling the slurry was sonicated for a few seconds to ensure complete resuspension of aggregated magnetite particles.

The yield of beads in both cases was in general >90%. The microspheres were sieved through a nylon net with an av. diam. 10 μm; the amount of beads recovered from the sieving step was ~10–20%. The beads collected were then used in the subsequent experiments.

2.3. *Covalent coupling of compounds to the microspheres*

2.3.1. [2-^{14}C]Ethanolamine

Activation according to the CNBr procedure [5] was for 7 min at pH 11 using 500 mg CNBr in 15 ml water and with 1 ml magnetic microspheres (carbonyl–iron), size 2–15 μm (not sieved preparation). Coupling proceeded overnight at room temperature in 2 ml total vol. of 0.1 M $NaHCO_3$ containing 1 mg ethanolamine (55 μg [^{14}C]ethanolamine). For animal experiments 200 μl sieved magnetic microspheres (size 2–10 μm, major fraction 3–5 μm) were activated with 100 mg CNBr and 33 μg [^{14}C]ethanolamine was added for coupling. After coupling the microspheres were washed extensively in the sequence 0.1 M $NaHCO_3$, 1 mM HCl, 0.5 M NaCl and water until no radioactivity was found in the supernatants.

2.3.2. (^{125}I)Albumin

Activation following the CNBr procedure was carried out for 7 min at pH 11 using 20 mg CNBr in 15 ml water and with 200 μl magnetic microspheres (carbonyl–iron), size 2–10 μm, sieved, major fraction 3–5 μm. Coupling proceeded overnight in 10 ml 0.1 M $NaHCO_3$ after addition of 100 μl (^{125}I)albumin preparation.

2.3.3. Plasmin

Magnetic microspheres (powdered magnetite) were activated with 200 mg CNBr in 15 ml water and with 1 ml microspheres present. For coupling 3 mg plasmin were added (in 10 ml total vol.) and coupling proceeded overnight at 4°C in 0.1 M $NaHCO_3$.

2.3.4. Plasmin assay

Microspheres (0.5 ml) were suspended in 10 ml 50 mM Tris–HCl (pH 7.4) buffer containing 12 mM NaCl. Air was bubbled through the suspension to maintain mixing. The suspension was then pumped through a continuous-flow system essentially following the procedure for immunoassays in [6]. The modifications introduced include the omission of the extra inlet and the use of one pump on each side of the membrane to obtain filtration. After obtaining equilibration of the system, 0.5 ml of the synthetic peptide substrate H–D-Val–Leu–Lys–pNA was added to give 1.2 mM final conc. Hydrolysis was continuously followed at 405 nm.

2.4. *Animal experiments*

Rabbits (2–4 kg) were anaesthetized with Nembutal® through a catheter in the marginal ear vein.

Magnetic microspheres (~100 μl) suspended in 3 ml 0.154 M NaCl, were then injected through the same catheter as the Nembutal®. The distal part of the opposite ear was placed in the gap of an electromagnet (0.7 Tessla). The ear not placed in the magnet served as reference.

After 10 min, a period shown to be sufficiently long to allow 'location' of the magnetic beads, the rabbits were killed by an overdose of Nembutal. The distal part of the ear (4 cm) was then cut off and placed in a gamma counter to measure the radioactivity emanating from the (^{125}I)albumin coupled to magnetic microspheres that had been trapped in the ear. Alternatively, when injecting [^{14}C]ethanolamine carrying magnetic microspheres the part of the ear directly lying within the area of the magnetic field

(~1 cm^2) was cut out and dried Subsequently it was subjected to total combustion [7] and the radioactivity of the trapped $Ba^{14}CO_3$ was determined

2 5 *Enzymic digestion of the microspheres*

The microspheres were treated with α-amylase in 0 15 M phosphate buffer (pH 7 4) measuring reducing groups liberated by reduction of 3,5-dinitrosalicyclic acid [8] The amount of enzyme added varied from 3 nKat to 1 μKat/ml in the assay solution

3. Results and discussion

In our work we have applied as magnetic material carbonyl–iron (2–3 μm), powdered magnetite (0 05 μm) [9] or a magnetic fluid called ferrofluid [3] (In preliminary studies the latter material was kept entrapped in acrylate polymers to which β-galactosidase was bound) The predominant part of our investigation including the animal experiments was carried out with entrapped carbonyl–iron As the iron preparation oxidized on long storage, we also later employed the same entrapping technique to powdered magnetite Apart from the fact that the latter preparations did not contain as much magnetic material, the properties of the beads obtained appeared identical Further studies with the latter preparations are in progress

The polymers in which the magnetic material was entrapped consisted of starch Starch was chosen as carrier as it is biocompatible and also because the α-amylase capable of degrading starch is present in the human body The magnetic material was entrapped in the beads as the starch was retrograded The term retrogradation describes changes occurring normally in starch on ageing which is basically a crystallization process and involves hydrogen-bond formation between hydroxyl groups on adjacent starch molecules [10] With heat treatment followed by quick cooling such retrogradation can rapidly be accomplished The beads obtained are relatively stable not requiring additional crosslinking which makes their preparation easy (Beads made up of crosslinked starch have been described in [11,12] However, the addition of crosslinking agents such as epichlorhydrin makes these preparations more hazardous and the procedure more complicated, though in [13] we found that sonication of the preparation during crosslinking resulted in beads of a narrow size distribution (2–5 μm)) The magnetic properties of the beads obtained are excellent, allowing easy retrieval, employing even weak magnets The bead diameter could easily be varied by changing in particular the stirring rate

The two types of magnetic beads obtained are shown in fig 1 In the beads containing entrapped carbonyl–iron the individual iron particles (diam 2–3 μm) can be distinguished whereas in the beads containing the far smaller magnetite particles (diam 0 05 μm) the latter cannot be visualized separately and appear as a dark area because of the limited resolution of light microscopes

Fig 1 Photomicrographs showing magnetic microspheres (diam 50 μm) consisting of entrapped carbonyl–iron (left) and powdered magnetite (right) In the animal studies the major fraction of the carbonyl–iron–starch microspheres applied was much smaller, essentially made up of one single iron particle covered with a 1–2 μm layer of starch

3.1. *Covalent coupling to the magnetic starch microspheres*

As high molecular weight model substance (enzyme) we have chosen (^{125}I)albumin and as low molecular weight model substance (low molecular weight drug) [^{14}C]ethanolamine, both of which were coupled covalently to the support. No attempts were made to optimize the coupling conditions. With (^{125}I)albumin 110 000 cpm/ml microspheres were found which corresponds to a coupling yield of 2% or 0.04 mg albumin/ml microspheres. The relatively low coupling yield is most likely due to steric hindrance exerted by the starch only permitting surface binding of the relatively large albumin molecule.

In contrast, 45% of added ethanolamine was coupled, yielding 4.75 μmol/ml.

In addition we also coupled covalently an enzyme, plasmin, to the magnetic microspheres. This enzyme was chosen because of its potential therapeutic value due to its reported activity in fibrinolysis of blood clots [14]. On the average the microspheres showed ~10% of added total enzymic activity corresponding to 1 NU/ml wet gel.

3.2. *Animal experiments*

On injecting magnetic starch microspheres (carbonyl–iron) to which (^{125}I)albumin had been coupled, enrichment of radioactivity was repeatedly observed in the ear to which the magnetic field had been applied, despite the fact that injection was carried out in the other ear which served as reference. Of the total radioactivity injected, only a minor fraction (0.3%) was 'retrieved' in the 'magnetic' ear. However, considering that the major fraction (~80%) of the injected beads (2–10 μm, major fraction 3–5 μm) was trapped in the animals' lungs this is not surprising. Studies are now in progress to obtain preparations with a very narrow size distribution as well as smaller microspheres containing entrapped magnetite powder. We also injected likewise coupled [^{125}I]thyroxine and found that enrichment in the 'magnetic' ear compared to the blank ear was obtained on all occasions. However, as some leakage of the radioactive material in this particular experiment was continuously taking place no quantitative data are given here.

Finally, [^{14}C]ethanolamine-carrying magnetic microspheres (total radioactivity of the 100 μl beads injected was 6×10^6 dpm) were injected. Whereas 2515 dpm were found in the part of the ear to which a magnetic field had been applied only 370 dpm were found in the reference ear.

In all animal experiments the enrichment factor between the 'magnetic' and 'non-magnetic' ear was 4–8, in one animal an enrichment factor of 21 was obtained. Summarizing the results obtained, one can conclude that enrichment of the injected magnetic 'drug'-carrying support can be accomplished with the application of an external magnetic field.

From preliminary studies it is evident that the microspheres could be degraded by amylase. Applying a concentration of enzyme corresponding to that present in human blood, i.e., 3 nKat/ml, magnetic microspheres (not CNBr-activated) were dissolved within 2 h. In contrast, CNBr-activated microspheres were degraded only after application of far higher amylase concentrations (100-times), which is probably due to higher crosslinking of the latter preparations. This could probably be compensated for in vivo by longer exposure to the enzyme.

In the future we hope to demonstrate in in vivo studies how to locate and concentrate in a magnetic field drug-carrying magnetic microspheres, in particular plasmin for degradation of blood clots and cytotoxic drugs for tumour treatment.

It will be necessary to investigate the strength of the magnetic field required to keep the iron preparation in place. Reports in the literature indicate that it should be possible in principle to create strong enough magnetic fields to do this. Thus, studies on magnetically-controlled iron thrombosis in rabbits showed that it is possible to arrest injected iron particles in the blood stream [15]. Similarly, the demonstration of a superconducting electromagnet being able to keep ferrosilicone particles in the arterial blood stream is noteworthy in this context [16].

Regarding the removal of injected microspheres, which is of importance in particular on long-term use, the following alternatives can be envisaged. The intact microspheres including the magnetic material are collected by application of a magnetic field across an extracorporeal vascular shunt device. If the microsphere is degraded by enzymes present in the body the magnetic material could be removed out of the body depending on its size either by the above approach or by excretion. Excretion seems possible in

particular for the small magnetite particles (0 05 μm) used in this study

In general, when using carriers in drug delivery, ideally the following requirements should be met

(i) The carrier should be biocompatible, i.e , non-toxic and non-immunogenic,

(ii) after reaching the target area the drug must be allowed to exhibit its activity

This can in principle be achieved by

(1) Enzymic, chemical or physico-chemical methods leading to controlled release of either a covalently or otherwise bound, e g , entrapped or adsorbed drug

(2) In some cases the drug, in particular if it is of macromolecular size such as an enzyme, may exert its activity even in its immobilized state in which case no release mechanism is required

Starch has hitherto not been used as carrier of immobilized biomolecules except, for instance, as a pad for entrapped enzymes monitored in an enzyme electrode [7] or in an electrochemical cell [18] Compared to macroreticular structures such as agarose, the total loading capacity seems to be lower and microbial disintegration may occur However, besides applications of the type described here where biocompatible and likewise biodegradable polymers are advantageous, starch as a support may be useful also for other applications, partly because of its low cost Thus, the starch microsphere preparations described, in particular those containing magnetite, appear to represent alternatives to the described magnetic supports applied in cell separation [9], enzyme-immunoassay [19] and similar areas

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