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Preparation and preclinical evaluation of bioresorbable hydroxyethylstarch microspheres for transient arterial embolization

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Abstract

Hydroxyethylstarch microspheres were prepared using an emulsion-heat denaturation technique. In order to achieve short-term embolization of arteries, several sizes of microparticles were needed and could be adjusted, within the range 25–400 μm, by the speed of agitation during the emulsion step. In vitro, degradation of microspheres in aqueous physiologic medium was preceded by swelling and occurred at rates inversely proportional to the temperature of preparation. In vivo in rabbits, the duration of embolization of the renal artery after selective catheterism also depended on the temperature of microsphere preparation, and could be easily adjusted within the range 5–45 min.

Key words: Bioresorbable starch microsphere; Heat denaturation; Selective catheterism; Arterial embolization

1. Introduction

During the last few years considerable interest has been focussed on the use of microparticles for both embolization and chemoembolization of human blood vessels, with particular emphasis on the treatment of cancerous tumors and brain vascular malformations (Tomlinson, 1983; Tournade et al., 1987; Fox et al., 1990; Gupta, 1990). Among the various materials used to perform arterial embolization, albumin and starch microspheres have attracted considerable attention and have been used in various animal and human studies (Scheffel et al., 1972; Russell, 1983; Ensminger et al., 1985; Taguchi et al., 1992; Laccourreye et al., 1993). Methods for preparing these microspheres are well established and are essentially based on interfacial chemical crosslinking of albumin or starch derivatives, using polymerization agents such as *N,N,N′,N′*-tetramethylethylenediamine (TEMED), epichlorohydrin, or glutaraldehyde (Yapel, 1985; Jones et al., 1989; Torrado et al., 1989; Lévy and Andry, 1990; Natsume et al., 1990).

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Moreover, interest in embolization has been largely emphasized as advanced techniques now allow hyperselective catheterization of almost all regions within the body. These techniques include wire-directable microcatheters or perforated balloons mounted on silicone catheters, combined with monitoring by real-time digital subtraction angiography (Tournade et al., 1987; Wilensky et al., 1991).

However, accurate prediction of the extent of embolization performed often remains a difficult task. This is especially the case for arteriovenous malformations, which generally consist of complex networks of entangled vessels. There is thereby a risk that particles either do not reach the arteriovenous nidus, or, in contrast, pass through the nidus and occlude healthy territories. Moreover, the embolization process itself can cause variations in arterial flow patterns, such as arterial spasm during catheterization, or non-laminar flow when catheters are placed proximal to the bifurcation of a vessel.

A similar situation is found in most solid cancerous tumors, where neoangiogenesis leads to anarchistic development of vessels, including arteriovenous shunts. Considering the high toxicity of most anticancer agents incorporated or co-administered with embolization particles, the risk of delivering these agents in healthy tissues around the tumor could limit this form of drug delivery.

Thus, an accurate knowledge of arterial catheter position and of the extent of regional perfusion is needed for optimization of embolization.

Several methods based on radionuclides have been described for evaluating catheter position and perfusion patterns. These methods generally use ^{99m}Tc macroaggregated albumin monitored by arteriographic examination (Ziessman et al., 1983; Bledin et al., 1984).

An alternative method is the use of rapidly biodegradable microspheres able to create transient embolization during a period sufficiently long to visualize the territory effectively embolized, but short enough to avoid possible damage to healthy tissues. These particles thus serve as a guideline in the evaluation of the risk associated with a definitive embolization with non-degradable particles. Moreover, they can direct the choice of the best size of non-resorbable particles to be administered in the final step.

Degradable starch microspheres (Spherex) have been widely tested (Laccourreye et al., 1993). These microspheres are made of chemically reticulated starch, and their in vitro degradation rate can be varied from several minutes to hours according to the extent of starch crosslinking, microsphere size, and the amount of amylase in the dissolution medium. Although clinical trials conducted with microspheres prepared by chemical crosslinking methods did not reveal major toxicities, a risk may remain associated with the possibility of highly reactive and toxic chemicals remaining in the final products, which could lead to unwanted side-effect reactions. Moreover, since starch microspheres prepared by chemical crosslinking are degraded in vivo by host amylases, variations in plasma amylase concentration or alterations in the enzymatic process may significantly modify the degradation rate.

The aim of this paper is to present a non-chemical method of preparation of biodegradable starch derivative microspheres as an alternative for short-time embolization. The first part of the study is devoted to the determination of the experimental parameters able to modify the size of the microspheres and their delay before in vitro resorption. The second part concerns the in vivo behavior of these microspheres in the model of the kidney in the rabbit.

2. Materials and methods

2.1. Materials

Hydroxyethylstarch (HES) derivatives were specially synthesized by Roquette Frères (Lestrem, France). Olive oil was purchased from Sigma Chimie (France). Methylene chloride (analytical grade, Prolabo, France) was used without further purification. Diethyl ether (analytical grade, Prolabo, France) was dehydrated using 4 Å molecular sieves.

Equipment for preparation of microspheres consisted of a Heidolph RZR 2101 stirring motor

equipped with a three-blade glass stirrer and either a jacketed baffled or non-baffled 1 l mixing cell (Vasse Industries, France). Heating was performed using a Julabo HC5 oil bath controlled by a Julabo PRG3 programmer.

Microspheres were sized using stainless-steel sieves with different apertures ranging from 25 to 400 μm (Bioblock, France).

Animal experiments were performed in New Zealand rabbits purchased from Iffa Credo (France). Selective catheterism was achieved using a 3F microcatheter (1 mm external diameter). X-ray examinations were performed using real-time digital subtraction angiography.

2.2. Methods

Preparation of HES microspheres

HES microspheres were prepared by adapting a heat denaturation technique previously described for the preparation of albumin microspheres.

Known amounts of HES were dissolved in distilled water (100 or 125 mg/ml). The aqueous solution was then emulsified in 500 ml of olive oil pre-heated to 50°C and stirred at various speeds (600–800 rpm) in either a baffled or non-baffled reaction vessel. After 5 min, the emulsion was gradually heated for 30 min up to a maximum ranging from 110 to 180°C (temperature of denaturation) and allowed to stand at this temperature for a period of 30 min. The mixture was then allowed to cool down room temperature (22°C). Microspheres were washed several times with methylene chloride and then suspended in dehydrated diethyl ether and sized between microsieves with different mesh apertures. Individual fractions of microspheres (25–75, 75–150, 150–300 and 300–400 μm) were collected, dried under vacuum and weighed. All batches of microspheres were stored at room temperature under vacuum.

Characterization of microsphere preparations

For each batch of microspheres, the global yield of production ($Y\%$) was calculated as the ratio of the total amount of microspheres obtained (diameter between 25 and 400 μm) relative to the initial amount of HES introduced in the reaction vessel.

Each batch of microspheres was also characterized by a mean particle size value (D), evaluated according to the following equation:

$$D(\mu\text{m}) = \left(\sum_{i=1}^{n} y\%_i \cdot d_i \right) / 100$$

with $y\%_i$ being the relative production yield calculated for each of the n size fractions as the ratio of the amount of microspheres in the size fraction relative to the total amount of microspheres obtained, and d_i denoting the central value of the diameter in the fraction considered (e.g., 50 for the 25–75 μm fraction).

In vitro evaluation of microspheres

Microsphere sphericity and size were checked by observation under an optical stereomicroscope equipped with a micrometric objective. In order to observe their behavior in aqueous medium, microspheres were then dispersed in physiological saline. Possible degradation or size changes were noted and, as microspheres inflated, the duration and extent of the phenomenon were measured. Each batch of microspheres was thus characterized by a swelling time and a swelling factor. The swelling time was measured from the instant of dispersion in physiological saline to that when the microsphere diameter remained constant. The swelling factor was estimated based on the ratio (particle diameter after completion of swelling/particle diameter in dried form).

Pre-clinical experiments in rabbits

Evaluation of the embolization properties of the HES microspheres was performed in 2–3 kg New Zealand rabbits. After anesthesia with urethane, the inguinal area was shaved and a 2–3 cm incision was performed along the path of the femoral pulse. The femoral artery was then denuded and a 3F catheter (1 mm external diameter) was introduced. Under X-ray control, the catheter was selectively advanced into the renal artery. After angiographic control of the catheter position, HES microspheres (200–300 mg) were dispersed in physiological saline (10–15 ml) and

immediately injected. Angiographies were performed at regular times in order to monitor the embolization process. The duration of embolization was measured from the instant of complete kidney embolization to that when circulation was completely restored, giving an angiographic picture identical to that obtained before embolization.

Statistical analysis

The relative effect of experimental preparation parameters on microsphere size was evaluated by multiple regression statistical analysis. The effect of the temperature of microsphere denaturation on embolization duration was tested by both least-squares linear regression analysis and non-parametric Spearman rank correlation coefficient. Tests were performed using Statgraphics software (Statistical Graphics Corp., U.S.A.).

3. Results

3.1. Preparation of HES microspheres

46 experiments were conducted in order to determine the influence of preparation parameters on both the yield of microsphere production and the size of microspheres obtained. Four preparation parameters were studied: concentration of HES, speed of agitation, maximal temperature of heat denaturation, and presence or absence of baffles in the mixing cell.

For the 46 preparations performed, the mean global yield of microsphere production was 65 ± 9% (mean ± SD). The size of microspheres ranged between 25 and 400 μm. No particles above 400 μm and very few below 25 μm were obtained. However, whereas the presence or absence of baffles had no effect on the production yield, the preparations performed with a baffled mixing cell were not reproducible in terms of particle size distribution. Therefore, all the following results concern experiments ($n = 32$) performed with the non-baffled mixing cell.

After drying, all batches of microspheres appeared as free-flowing powders. When observed under the stereomicroscope, microspheres were translucent and spherical in shape. No agglomeration of particles could be observed in every batch produced.

Table 1
Production yields (Y%) and mean particle sizes (D) of HES microspheres prepared under different conditions (see section 2 for definitions of Y% and D)

Speed of agitation (rpm)	HES concentration (mg/ml)	Temperature of (°C)	Production yield Y%	Mean particle size D (μm)
600	100	110	61	156
600	100	120	68	144
600	125	110	70	156
600	125	120	67	153
600	125	130	63	166
600	125	140	60	174
600	125	150	57	161
600	125	180	70	142
700	100	110	67	148
700	100	120	70	122
700	125	110	76	141
700	125	120	77	149
700	125	130	69	152
700	125	140	74	144
700	125	150	77	135
800	100	110	42	105
800	100	120	37	91
800	100	130	64	87
800	100	140	47	112
800	100	150	74	94
800	125	110	73	114
800	125	120	64	109
800	125	130	73	94
800	125	140	68	106
800	125	150	71	91

The reproducibility of the preparation technique (in a non-baffled mixing cell) was evaluated by performing duplicate or triplicate preparations under identical sets of experimental conditions. For each set of conditions, the difference between extrema was less than 10% for both the production yield and the mean particle size.

Table 1 summarizes the results obtained by varying the values of each of the three remaining experimental parameters (HES concentration, speed of agitation, temperature of heat denaturation). In order to determine the effect of each of these variables on both the yield of microsphere production and the mean particle size, a multiple regression analysis was carried out. This analysis

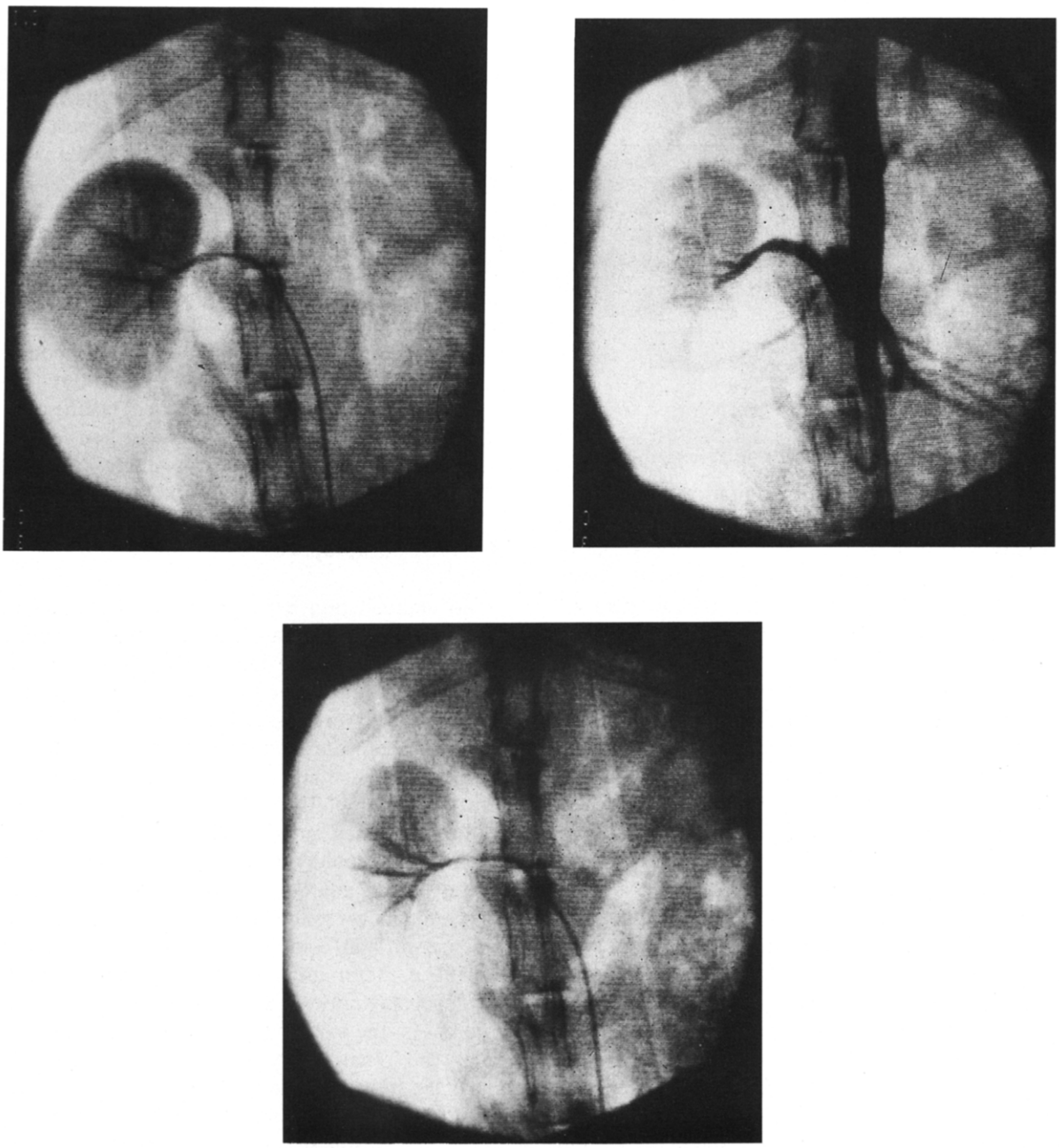

Fig. 1. Selective catheterization of the left renal artery in rabbit and embolization with rapidly degradable HES microspheres. Angiograms were recorded after injection of 3–4 ml of contrast medium. (a) Before embolization; (b) during transient embolization (embolization performed with 200 mg of 75–150 μm microspheres prepared at a denaturation temperature of 130°C; complete embolization was immediately achieved and lasted 8.5 min). Note the complete stopping of flow through the distal renal artery, with massive reflux in the aorta. (c) After embolization: angiogram recorded 10 min after the beginning of embolization is similar to that before embolization.

Table 2
Influence of denaturation temperature and particle size on the in vitro swelling time and swelling factor of HES microspheres in physiological saline at room temperature (see section 2 for definitions of the swelling parameters)

Particle size (μm)	Denaturation temperature (°C)	Swelling time (min)	Swelling factor
75–150	110	1.5–2.0	2.5–3.0
	120	2.5–3.0	2.5
	130	3.0–4.0	2.5–3.0
	140	4.0	2.5–3.0
	150	6.0	2.0
150–300	110	3.0	3.0
	120	3.5–4.0	3.0
	130	5.0–5.5	3.0
	140	6.5–7.5	3.0
	150	8.5–9.0	2.25

indicated that the speed of agitation during the emulsion step was the main parameter controlling the microsphere size ($p < 10^{-4}$), whereas the influence of HES concentration was at the limit of significance ($p = 0.04$). The temperature of heat denaturation had no effect on the size of microspheres.

Concerning the yield of microsphere production, the sole significant result of multiple regression analysis was a slight but significant decrease in this yield with HES concentration ($p = 0.02$).

3.2. Behavior of microspheres in aqueous medium in vitro

When dispersed in physiological saline and observed under the optical stereomicroscope, microspheres inflated while retaining their sphericity. The duration and extent of this phenomenon depended both on microsphere size and on the maximal denaturation temperature set for their preparation (Table 2). On increasing the temperature of denaturation (from 110 to 150°C), the swelling time increased from 1.5–2.0 to 6.0 min with the smallest microspheres (75–150 μm), and from 3.0 to 8.5–9.0 min with the largest (150–300 μm). Thus, small microspheres inflated more rapidly than large ones. In contrast, the swelling factor was influenced by neither the microsphere size nor the denaturation temperature, and corresponded approximately to a tripling in particle diameter.

Fully swollen microspheres still presented a spherical aspect but their consistency was strongly modified. While microspheres were hard and tough in the dry form, they became soft and fragile upon swelling, and were easily by the point of a needle.

3.3. Stability of HES microspheres

Several batches of microspheres corresponding to various sets of size and denaturation temperature were stored under vacuum for up to 6 months in amber-colored flasks at room temperature. Microspheres of all batches remained macroscopically unaltered after this period, and their in vitro behavior (swelling time and swelling factor in physiological saline) was not modified.

3.4. Pre-clinical experiments in rabbits

30 embolization experiments were performed on 16 rabbits, each animal undergoing a maximum of two or three injections (200–300 mg of microspheres per injection). Different batches of microspheres were tested in order to evaluate the influence of the parameters of microsphere preparation (size and temperature of heat denaturation) on embolization.

In four cases, no embolization was observed. This failure could be explained on the basis of either animal anatomy or poor injection. In fact, two animals presented an abnormally large arterial network in comparison with others, so that the 3F catheter used for microsphere injections was probably not exactly appropriate. In the other two cases, the existence of an upper polar artery near the renal artery might have diverted the particles from their destination.

In four other cases, a partial transient embolization was achieved, resulting only in slowing down of the arterial flow. These cases occurred when less than 200 mg of microspheres could be effectively administered.

In 22 cases, complete embolization of the kidney was observed, followed by the complete

Table 3
Influence of denaturation temperature and particle size of HES microspheres on the duration of renal artery embolization in rabbit

Denaturation temperature (°C)	Particle size (μm)	Number of experiments	Duration of embolization (min)
110	75–150	4	6.5–9.5
110	150–300	2	5.0–9.0
120	75–150	3	8.5–10.5
120	150–300	2	8.0
130	75–150	4	8.5–16.5
130	150–300	2	14.0–19.0
140	75–150	1	25.0
150	75–150	3	21.5–35.0
180	75–150	1	46.0

restoration of arterial flow after a period of time varying from 5 to 46 min (Fig. 1 and Table 3). In approximately one half of the cases, such embolization was obtained with 200 mg of microspheres. In other cases, an additional 100 mg dose was necessary (400 mg in one case).

From Table 3, it can be observed that, for a given temperature of denaturation, the size of microspheres (75–150 μm vs 150–300 μm) had no marked effect on the duration of embolization, although the small number of data did not allow statistical confirmation. On the other hand, the temperature of heat denaturation appeared to determine the duration of embolization, which increased from 5–9.5 min at a preparation temperature of 110°C, to 46 min at 180°C. This result was statistically confirmed both by least-squares linear regression analysis ($p < 10^{-5}$) and by the non-parametric Spearman rank correlation coefficient ($p < 10^{-4}$).

4. Discussion

A method of producing starch derivative microspheres by heat denaturation has been developed, based on previously published studies on the preparation of albumin microspheres (Gallo et al., 1984; Yapel, 1985; Torrado et al., 1989). To the best of our knowledge, such a non-chemical method has not previously been applied to starch products.

The starch derivative used was hydroxyethylstarch (HES), which was chosen since its wide clinical use as a synthetic colloid plasma volume expander ensured its safety (Thompson, 1981; Yacobi et al., 1982). Moreover, a range of HES derivatives with various properties had been synthesized by Roquette, so that the most appropriate compound could be selected from preliminary studies.

In comparison with chemical crosslinking techniques, the heat denaturation method offers at least two advantages. First, it avoids the possibility of an adverse combination of chemical crosslinking agents which might lead to toxic products if they are not completely removed from the preparation mixture. Second, their splitting in soluble starch is not dependent on the amylase activity of the host.

Globally, the parameters affecting HES microsphere preparation by the technique of emulsion-heat denaturation are similar to those previously described by others in the case of albumin.

Concerning particle size, the most useful experimental parameter in our experiments was the speed of agitation, which facilitated the directing of production towards either small or large microspheres in the desired range (25–400 μm). These microspheres also behaved as free-flowing powders, and exhibited good stability on storage in their dried form.

As in the case of albumin, the resistance of HES microspheres to dissolution in aqueous medium depended on the maximal temperature of denaturation during the preparation process. In vitro, microspheres dispersed in physiological saline swelled at rates inversely proportional to the temperature at which they had been prepared. For example, in the case of 75–150 μm microspheres, the time required for completion of swelling varied from about 2 min at a preparation temperature of 110°C, to 6 min at 150°C. This inflation time was slightly longer for 150–300 μm microspheres (3–9 min). Moreover, as microspheres progressively inflated in water, they became more and more soft and fragile. Thus, both

the temperature of denaturation and the size of microspheres determine their lifetime. These results confirm that degradation of microspheres did not occur via an enzymatic process (amylase), as was the case with chemically crosslinked degradable starch microspheres (Russell, 1983; Ensminger et al., 1985; Laccourreye et al., 1993).

The in vivo behavior of HES microspheres was examined in rabbit kidney, since the renal artery could be selectively catheterized from a femoral artery using a small preformed catheter (3F, 1 mm external diameter) under fluoroscopic guidance. Under these conditions, 22 of the 30 embolization experiments were successfully performed, independently of the microspheres' characteristics (size, temperature of heat denaturation).

As expected from in vitro experiments on swelling in aqueous medium, the in vivo duration of rabbit kidney embolization was proportional to the temperature of denaturation of HES microspheres, varying from about 5–9 to 46 min over the range of temperature tested. After this period, renal arterial flow was re-established at the initial conditions. No short-term secondary effects of transient embolization could be macroscopically detected.

In conclusion, the preparation of HES microspheres by heat denaturation allows one to achieve embolization for a short period of time. This type of rapidly bioresorbable microspheres might thus be of interest as a preliminary test before the realization of definitive embolization with non-bioresorbable microparticles. Potential clinical applications of this technique are currently under evaluation.

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