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Trisacryl Gelatin Microspheres for Therapeutic Embolization, I: Development and In Vitro Evaluation

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PURPOSE: To develop a precisely calibrated, perfectly spherical, stainable, soft, and implantable but nonresorbable particulate embolization material. **METHODS:** Calibrated particles with a trisacryl gelatin polymer core and hydrophilic surface characteristics were obtained by reversed emulsion synthesis followed by application of a wet-sieving technique. Particles were suspended in saline, bottled, and sterilized. Quality control included analysis of particle diameters before and after sieving and of suspension sterility and apyrogenicity. Particles were subsequently tested to ascertain their compatibility with commercially available microcatheters. **RESULTS:** The resulting embolization material consisted of spherical, stainable microspheres of medical grade with diameters ranging from 130 μm to 1200 μm. Sieving the suspension produced particle groups of homogeneous size (accuracy, ± 20–100 μm). At injection, the particles showed no tendency to build aggregates or to obstruct the microcatheters. **CONCLUSION:** Precisely calibrated and easy-to-use microspheres were obtained that satisfied the biomedical requirements for implantation as an embolization material.

Index term: Interventional materials, particles and microspheres

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The purpose of the study was to develop a nonresorbable embolization material with well-calibrated particles in a defined range of sizes to be made available as a ready-to-use injectable suspension. Inherent to most of the available and commonly used particulate embolization materials are undesirable characteristics, such as irregular shape (1) or undesirable biochemical characteristics (2–4).

Other particulate embolization materials have been developed and investigated experimentally, but none has reached the stage of clinical application. Many of the embolization materials that have been investigated—for example, gelatin (5), albumin (6), polysaccharides (dextran) (7), starch (8), ethyl cellulose, (9, 10), and poly-(D,L lactide/glycoside)-copolymer (11, 12)—include a resorbable component, and are thus not suitable for permanent vascular occlusion. Best suited for use as nonresorbable embolization material are either particles of a noncompressible nature, such as glass (13), wax (14–16), silicone (17–19), and polystyrene (20), or particles of a compressible nature, such as polymethyl-methacrylate (21) and poly-(2-hydroxyethyl methacrylate) (22).

In view of the above, we determined that the following two general requirements were most essential for the development of a particulate embolization material: First, the particles must be spherical in shape to allow for accurate grading and optimal geometric vessel occlusion. Spherical particles, which have a single dimension, can be calibrated by sieving more easily than can nonspherical particles, which have more than one dimension. Moreover, microspheres block geometrically more of the vascular lumen, whereas nonspherical particles produce a more incomplete occlusion (23). Second, the particles must have a nonresorb-

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able nature with good biocompatibility; that is, they must be inert but with surface characteristics that enable cell adhesion (4).

Several factors led us to choose microspheres made from a trisacryl polymer matrix impregnated and embedded with gelatin. First, the trisacryl polymer was used previously for many years as a base material for the manufacture of a chromatography column for protein filtration, by virtue of its low porosity (exclusion limit, 5000 d) (24, 25). Local tolerance of trisacryl polymer has been demonstrated by toxicity tests in rabbits and rats (24). Second, from 1982 to 1987, trisacryl gelatin microspheres were manufactured commercially as a microcarrier for cell cultures (Micarcel G, IBF-Sepracor, Villeneuve-la-Garenne, France). The role of gelatin was to facilitate the growth and spread of cells on the microspheres (24). The biocompatibility of trisacryl gelatin microspheres has been verified with the use of various cell lines: BHK (24); HeLa Ohio and HeLa Oxford, HTC (26); rat aortic myocyte (27, 28); Vero (IBF France Product Information no. 202904); and MRC 5 and human foreskin (E. Boschetti, unpublished data, 1988). Third, we compared the trisacryl gelatin microspheres with other microspheres: trisacryl microspheres without gelatin, gelatin-coated dextran microspheres (Cytodex III, Pharmacia, Uppsala, Sweden), and dextran microspheres (Sephadex, Pharmacia, Uppsala, Sweden).

We embolized pulmonary arteries in rats through a peripheral venous injection of the various types of microspheres, then we performed a histologic examination of the lungs during the course of a 9-month period. We found that trisacryl gelatin microspheres remained unchanged for up to 9 months, and they initiated a polymorphonuclear and giant cell reaction. We observed no sign of cellular toxicity in the vicinity of the microspheres. For dextran microspheres, the results were different: the gelatin coating of the dextran dissociated from the dextran nucleus in 4 weeks; the two types of dextran microspheres were resorbed over several weeks by polymorphonuclear cells (macrophages), which invaded the dextran matrix.

However, before we could use trisacryl gelatin microspheres for embolization in humans, we needed to make several improvements related to calibration, conditioning, and coloration. In addition, it was necessary to perform in vitro evaluations and to verify the toxicity of the final material. The purpose of this article is to report on the above improvements and in vivo evaluations.

Materials and Methods

Particle Preparation

The trisacryl gelatin microspheres were obtained by applying techniques as described for the production of trisacrylic microcarriers (25) (N.E. Brown et al, French patent no. 7723223; 1977). With a pH adjusted to 6.5, an aqueous solution of *N*-acryloyl-2-amino-2-hydroxymethylpropane-1,3 diol (hydrophilic trisacryl monomer) (Aldrich, St Quentin Fallavier, France) and of *N,N′*-methylene-bis-acrylamide (MBA) (Aldrich) was heated to 57°C. Subsequently, a peroxide (ammonium persulfate) (Aldrich) and a polymerization initiator (*N,N,N′,N′*-tetramethylenediamine) (Aldrich) was added. Gelatin was then added to the aqueous solution of trisacryl monomer, after which the solution was emulsified in paraffin oil. The addition of glutaraldehyde and incubation at 25°C for 18 hours produced a chemical reaction. Neutralization was obtained by continuously incubating the solution at 25°C in the presence of sodium borohydride. The addition of a small quantity of cations to the mixture resulted in a positively charged particle surface, which reinforced the mechanism of cellular adhesion. To obtain microspheres of different sizes, we varied the physicochemical conditions of the synthesis. Two batches, 1 and 2 (Fig 1), each containing 500 cm^3 of particles, were obtained by using different monomer and reagent concentrations, shaking speed, temperature, and pH.

Calibration

Both batches were analyzed microscopically after further separation into subgroups of different sizes by means of a manually controlled wet-sieving technique to obtain particle calibration. Standardized square mesh sieves (Bioblock, Illkirch, France) were used to obtain subgroups of microspheres that ranged narrowly in size. For this purpose, a set of sieves with openings gradually decreasing in size were piled up, the largest mesh size on top. The mesh sizes used were 1250, 1000, 900, 800, 710, 630, 500, 400, 300, 200, 180, 120, and 100 μm (1 $\mu m = 10^{-6}$ m). The stack of sieves was placed on a sieving machine (Bioblock) for mechanical support rather than for the purpose of sieving itself.

Each sample (a few milliliters of the microsphere suspension to be sieved) was put on the top sieve of the stack and then soaked with demineralized water until the microspheres no longer passed through the sieve. The highest sieve, containing the correspondingly calibrated subgroup of microspheres, was then removed from the pile and wet sieving was repeated on the next sieve down with a smaller mesh size. This operation was repeated with the original sample throughout the whole height of the pile. Each excluded part of the sample was suspended in a beaker

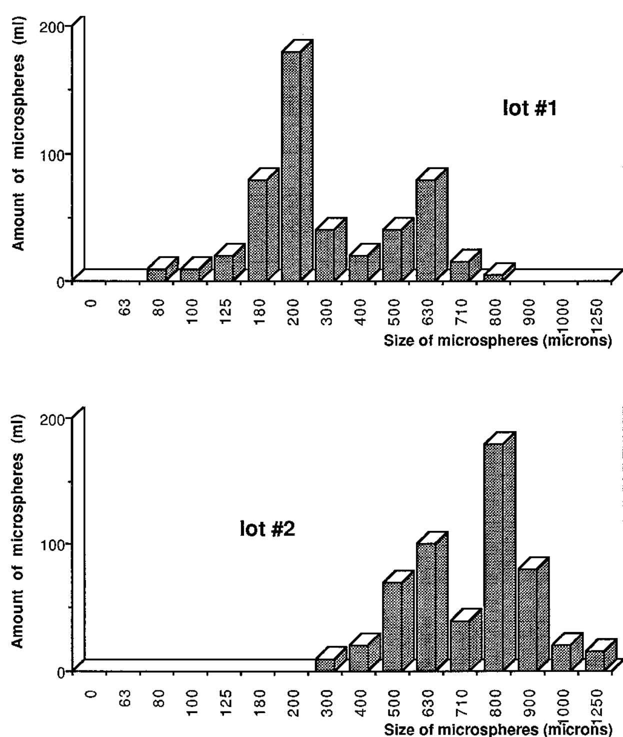

Fig 1. Graphs showing microsphere granulometric ranges. A change in the microsphere synthesis, in which different physicochemical conditions were applied during production, resulted in a group of particles with a larger than average size range. This technique was used to obtain the whole range of particles evaluated.

containing demineralized water. This operation was used to calibrate both batches of microspheres into subgroups according to the mesh sizes. Samples of each microsphere subgroup were then analyzed under a microscope. For each subgroup, a sample of 50 particles was measured to determine the distribution of microsphere diameters. Validation of this granulometric technique was assumed after a comparison of two samples taken from the same subgroup revealed no significant difference in particle sizes ($\chi^2 = 0.462$).

Conditioning (Apyrogenicity Test, Sterilization, Toxicity Test)

Each subgroup of calibrated microspheres was then put separately onto a fritted disk with red blood cell porosity (STP, Paris) and rinsed with sterile water. After two rinses, 0.4 to 0.7 cm^3 of the microspheres were put in a 5 to 6 mL suspension with sterile water, distributed by means of a sterile pipette, and bottled in clean glass containers, which were previously treated to remove pyrogenic factors. The glass containers were capped and then sterilized in an autoclave for 1 hour at 121°C at 1 atmosphere.

To test sterility and apyrogenicity of the microsphere suspension, two of the sterilized containers were randomly picked from each size range of microspheres, and a bacteriological analysis was performed to exclude contamination by aerobic and anaerobic germs and yeasts (29). The presence of pyrogen was excluded with the limulus amoebocyte lysate chromogenic test.

The toxicity of the injectable solution was evaluated by the test of abnormal toxicity to mice (30). This test consisted of injecting the solution intravenously in mice and observing any adverse effects.

Coloring

Immediately before using the microspheres, we stained the particles by means of contact color techniques. For that purpose, an aqueous solution of Patent Blue V was used (31). Three to four drops of this colorant were used to stain 0.5 cm^3 of sedimented microspheres once the solvent was removed from the suspension. The colorant was put directly onto the microspheres and the resulting mixture slightly shaken to allow for homogeneous particle staining. Excess colorant was then removed, and 0.09% saline and contrast solution was added to the stained microspheres just before using them as embolization material.

In Vitro Evaluation

We tested the compatibility of the embolization material with a variety of commercially available microcatheter systems by injecting different microsphere suspensions. The microcatheter systems evaluated included 3.6F polyethylene catheters (Cook Inc, Bloomington, Ind), 0.6 × 1.0 mm Pursil and Magic STD catheters (Balt, Montmorency, France), and Tracker 25 and 18/12 catheters (Target Therapeutics Inc, San Jose, Calif). Size ranges of the microspheres were 240 to 360, 340 to 460, 400 to 600, 550 to 750, 750 to 850, 800 to 950, 950 to 1050, 1050 to 1150, and 1150 to 1250 μm. Testing included grading the ease with which the microspheres passed through the catheter during injection and evaluating the microspheres microscopically during and after transit in the microcatheter.

Results

Particle Preparation

By applying the techniques described for the production of trisacrylic microcarriers (25) (N.E. Brown et al), we obtained two batches of microspheres, which we examined microscopically after calibrating them in order to analyze their morphologic appearance. In 99% of cases, particle morphology of both batches revealed a perfectly spherical shape with a smooth surface. The gelatin coating was highly visible and its thickness accounted for approximately 5% to 10% of the microsphere diameter (Fig 2). Only 1% of the microspheres appeared atypical, and

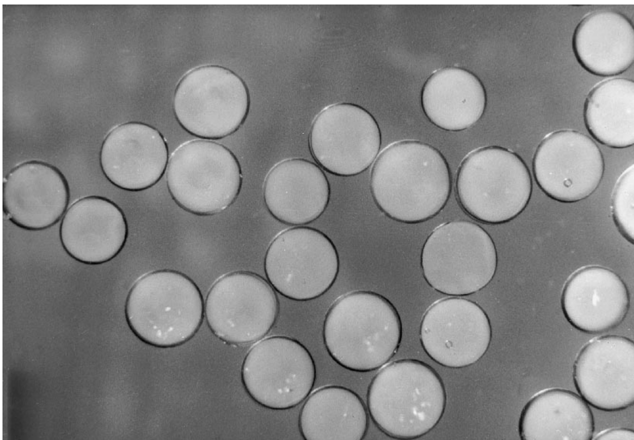

Fig 2. Photomicrograph of trisacryl gelatin microspheres shows the contour of the particles is smooth, and their size is homogeneous.

this appearance was due to coating of an irregular trisacrylic core or to irregular coating of a normal core. Coating of two associated cores together resulted in an elliptic form of the microsphere, with the smaller diameter of the ellipse similar to the average diameter of the calibrated microspheres in the corresponding subgroup. Another anomaly of the core formation occurred when a multitude of small cores formed a cluster, which then was coated. The last morphologic anomaly involved microspheres with a smaller than average core size that was covered with a thick coating, resulting in microspheres with a total diameter larger than the neighboring microspheres.

Analysis of size distribution in both batches showed a range of particle sizes with a heterogeneous distribution. In batch 1, this analysis revealed microsphere diameters ranging from 80 to 1000 μm, with a larger peak around 220 μm and a smaller peak around 600 μm. Most of the particles were smaller than 500 μm, and only a few particles were larger than 700 μm, with none larger than 1000 μm (Fig 1). The analysis of batch 2 revealed a similar pattern of size distribution, with diameters ranging from 200 to 1250 μm, with a smaller peak at 650 μm and a larger peak at 850 μm.

Calibration

Sieving of both batches allowed us to obtain subgroups of microspheres that contained particles that ranged narrowly in size. In the subgroups with large diameters, the standard deviation was 100 μm, whereas in particles with small diameters the standard deviation was 20 μm.

The standard deviation of particle sizes correlated with the mesh size of the corresponding sieves (Table 1). A comparison of the average mesh size of two adjacent sieves (mesh size of sieve n plus mesh size of sieve $n + 1$ divided by 2) and the average diameter of the collected excluded subgroup of microspheres showed a difference that progressively decreased from 150 μm for the large diameters to 20 μm for the small diameters.

Conditioning (Apyrogenicity Test, Sterilization, Toxicity Test)

All the sterility control samples tested in a culture medium (aerobic blood gels, anaerobic

TABLE 1: Difference between size of sieve opening and size range of microspheres

Range	Average Size of the Sieve Opening,* μm	Diameter of Microspheres, μm	Difference, μm
1	1125 ± 125	1200 ± 100	75†
2	950 ± 50	1100 ± 100	150
3	850 ± 50	1000 ± 100	150
4	755 ± 45	875 ± 100	120
5	670 ± 40	800 ± 100	130
6	565 ± 65	650 ± 100	85
7	450 ± 50	500 ± 100	50
8	350 ± 50	400 ± 60	50
9	250 ± 50	300 ± 60	50
10	190 ± 10	220 ± 30	30
11	152.5 ± 27.5	185 ± 35	32.5
12	112.5 ± 12.5	130 ± 20	17.5

* Average size of sieve opening (mesh size) = size of the opening of the upper sieve plus size of the opening of the lower sieve divided by 2.
† Microspheres in this range were selected manually.

TABLE 2: Test injections in Tracker 18/12 microcatheter (internal distal diameter, 530 μm)

Size Range of Microspheres, μm	Ease of Injection	Control of Sphere Shape in the Catheter	Control of Sphere Shape after Injection
240–360	Easy	Spherical	No alteration
340–460	Easy	Spherical	No alteration
400–600	Easy	Slight oval deformation	No alteration
550–750	Easy	Oval deformation	No alteration
750–850	Easy	Oval deformation	No alteration
800–950	Difficult	Marked deformation	75% equatorial split
950–1050	Difficult	Marked deformation	100% equatorial split
1050–1150	Difficult*	Marked deformation and broken spheres	100% equatorial split and spheres fragmented
1150–1250	Difficult*	Marked deformation and broken spheres	100% equatorial split and spheres fragmented

* Use of a Luer-lock syringe required.

blood gels, blood gels under CO_2) proved to be free of contamination. Results of the apyrogenicity test pertained to all samples less than 0.05 U/mL (human sensibility is 5 U/kg of body weight) (31). Results of toxicity tests on mice were negative.

Coloring

The contact coloring technique resulted in staining of the polymer core and the gelatin surface. This allowed for good visualization of the microspheres within the suspension and for control of the particles during handling of the material for embolization procedures.

In Vitro Evaluation

It has been possible to inject microspheres of all sizes (200 to 1200 μm) without difficulty through all catheters investigated, with the exception of the Tracker 18/12. This microcatheter, with a distal internal diameter of 530 μm, required the use of Luer-lock syringes, which allowed for high injection pressures for microspheres larger than 800 μm (Table 2). Particles showed no tendency to form aggregates. Microscopic observation of the microspheres during their transit through the microcatheter tip revealed significant particle deformation (Fig 3). Particles with diameters of up to 750 to 850 μm regained their original shape without signs of damage after being forced through a catheter lumen of 530 μm. For microspheres with a diameter larger than 850 μm, the forceful squeezing resulted in equatorial fissures and sometimes even complete particle fracture. The percentage of diameter reduction during forceful injection without subsequent permanent alteration due to compression was calculated as a reduction of 33% to 40% of the initial diameter (Table 3).

Discussion

Particle Preparation

Trisacryl is an entirely synthetic, hydrophilic, and nonresorbable material. In previous studies, this material proved to produce no toxic tissue reaction, thus allowing absorption and cellular adhesion (25) (N.E. Brown et al). The compact molecular model of the polymer shows a central location of the polyethylene core, with the hydroxymethyl groups oriented toward the outside, which accounts for the highly hydrophilic nature of the polymer (24). The cationic admix-

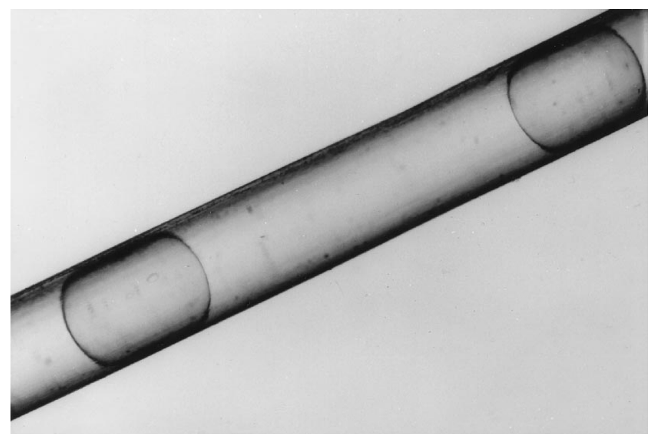

Fig 3. Photomicrograph of an 800-μm microsphere within the distal segment of a Tracker 18/12 catheter (internal diameter = 530 μm). The suppleness of the microspheres allowed injection of an 800-mm-diameter microsphere through the smaller (530 mm) distal internal diameter of the microcatheter.

TABLE 3: Microsphere deformation in test catheters

Catheters	Distal Internal Diameter of Catheter, μm	Greatest Size Range of Microspheres*, μm	Deformation of Microspheres, %†
Magic STD, Balt; Montmorency, France	300	400 ± 50	33
Tracker 18/12, Guerbet Biomédical, Target Therapeutics	530	800 ± 100	40
Pursil 0.6 × 1, Balt; Montmorency, France	600	800 ± 100	33
Tracker 25, Guerbet Biomédical, Target Therapeutics	710	1000 ± 100	35

* It was possible to inject microspheres that had a larger diameter than indicated in this table, but then some of them had equatorial fissures or complete fracture.

† Transient deformation: the size of the microsphere during its passage through the catheter compared with its size before injection.

ture during synthesis and, even more, the presence of denatured collagen on the microsphere surface support the cellular adhesion onto the material (24, 32, 33).

The mechanical properties, the porosity, and the swelling in an aqueous environment are also defined by the physicochemical properties of the material. The cross-linked copolymer appears as a tridimensional microporous network of gellike material. The grade of cross-linkage correlates with the percentage of *N,N'*-methylene-bis-acrylamide (MBA) in the matrix of the polymer. The resulting soft and deformable character of the material is evidenced by the temporary deformation of the particles when they are injected through a catheter with an inner diameter smaller than that of the particles. The amount of deformation allowed by a particle depends on its size and is proportional to the volume of the sphere. This capacity to deform also explains the progressively decreasing difference in the ratio of the average mesh size of two adjacent sieves and the average diameter of the collected subgroup of microspheres, with values from 150 μm for the large diameters to 20 μm for the small diameters.

Rare variants of the spherical shape, which we found during granulometric studies, have a rate of occurrence of 1% and are inherent to the reversed emulsion synthesis process. However, none of the particle irregularities observed have a significant impact on the homogeneity of the distribution of diameter size. Elliptic and cluster type variants have diameters that correspond to the average particle size of the corresponding subgroup (short for the elliptic shape and average for the cluster type). In the case of microspheres with thick coating, the difference in the diameter is balanced by the particles' increased compressibility, resulting from a higher volume of gelatin component.

Calibration

The importance of precise calibration of particle size has been emphasized by studies that have investigated the extent to which different types of particles penetrate the arterial bed (2, 7, 15). Carnauba wax microspheres, used in a study of animal kidney embolization (15), produced distal and homogeneous experimental occlusions within the vascular bed, and the size of the microspheres proved to correlate well with the vessel lumen. A study comparing polyvinyl alcohol particles with dextran, polystyrene, and silicone microspheres for spleen embolization in dogs found that polystyrene and dextran spheres were superior for vascular occlusion. These microspheres remained in suspension longer, showed no particle aggregation, and generated a more homogeneous and distal distribution of particles within the splenic arterial tree (2). Another study, involving animals and humans, similarly documented the ease of an embolization procedure when dextran microspheres were used, and these researchers also obtained a homogeneous and distal location of the embolization material (7).

Conditioning (Apyrogenic Preparation, Sterilization)

There is a risk of contamination during synthesis and sieving; however, the quality control study revealed satisfactory results, with good sterility and apyrogenicity of the microsphere suspension, indicating that the technique we used is appropriate for the preparation of injectable material for use in animals and humans.

Coloring

Embolization procedures are typically performed in a relatively dark environment, and the admixture of contrast materials may give a

cloudy appearance to the microsphere suspension, making a visual survey of the particle injection difficult. Yet, a good view of each microsphere may be required when the precise amount of particles to be injected is critical. Staining the microspheres improves the ability to see the particles during embolization procedures, and to recognize them in tissue that has been embolized. Although contact coloration requires an additional step in the preparation of particles, it is a simple and effective procedure. Other techniques of coloration (eg, by incorporating a colored substance into the matrix or by chemical grafting) may also be considered.

In Vitro Evaluation

The most important factor related to clogging of a catheter is the tendency of particulate material to aggregate. The clustering of several particles in the catheter hub can obstruct the catheter system at a level close to the embolization site. More distal occlusion is likely to occur with irregular particulate materials, which may lock together as a result of their polyhedral structure. The aggregation of nonspherical particles is a well-known phenomenon that can not only obstruct catheters (34) but also produce a more proximal occlusion in the arterial tree to be embolized (35).

Injection of the microspheres was always easy, and there was no obstruction in any of the tested catheters, even when the inner diameter of the microcatheters led to considerable temporary compression of the microspheres. If this compression became too great, the particles fragmented as a result of the shear forces produced by their forceful passage through a narrow catheter.

Conclusions

Trisacryl gelatin is a good material from which to produce microspheres for the purpose of arterial embolization. The preparation technique we used provided particles with diameters ranging from 130 to 1200 μm. Calibration by means of sieving techniques allowed us to separate the particles into subgroups that ranged narrowly in size ($\pm$20 to $\pm$100 μm). Tests showed the microsphere suspension to be sterile and apyrogenic. Before embolization, the microspheres may be stained by use of a contact coloring technique with Patent Blue V. Tests evaluating the ease with which this material may be injected through catheters showed these hydrophilic particles to be soft, adapting to smaller microcatheters with no tendency to clog through the formation of aggregates. The trisacryl gelatin microspheres consist of biocompatible materials that are of a nonresorbable nature and seem to have all the necessary characteristics to be used both safely and beneficially for embolization purposes in animals and humans.

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