Published in final edited form as:
Nat Protoc. ; 6(8): 1142–1151. doi:10.1038/nprot.2011.352.

Synthesis of magnetic resonance–, X-ray– and ultrasound-visible alginate microcapsules for immunoisolation and noninvasive imaging of cellular therapeutics

Brad P Barnett[1,4], **Aravind Arepally**[1], **Matthias Stuber**[1,6], **Dian R Arifin**[1,4], **Dara L Kraitchman**[1,5], and **Jeff W M Bulte**[1,2,3,4]

[1]Russell H. Morgan Department of Radiology, Division of Magnetic Resonance Research, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

[2]Department of Biomedical Engineering, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

[3]Department of Chemical and Biomolecular Engineering, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

[4]Cellular Imaging Section and Vascular Biology Program, Institute for Cell Engineering, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

[5]Department of Molecular and Comparative Pathobiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Abstract

Cell therapy has the potential to treat or cure a wide variety of diseases. Non-invasive cell tracking techniques are, however, necessary to translate this approach to the clinical setting. This protocol details methods to create microcapsules that are visible by X-ray, ultrasound (US) or magnetic resonance (MR) for the encapsulation and immunoisolation of cellular therapeutics. Three steps are generally used to encapsulate cellular therapeutics in an alginate matrix: (i) droplets of cell-containing liquid alginate are extruded, using an electrostatic generator, through a needle tip into a solution containing a dissolved divalent cation salt to form a solid gel; (ii) the resulting gelled spheres are coated with polycations as a cross-linker; and (iii) these complexes are then incubated in a second solution of alginate to form a semipermeable membrane composed of an inner and an outer layer of alginate. The microcapsules can be rendered visible during the first step by adding contrast agents to the primary alginate layer. Such contrast agents include superparamagnetic iron oxide for detection by ^{1}H MR imaging (MRI); the radiopaque agents barium or bismuth sulfate for detection by X-ray modalities; or perfluorocarbon emulsions for multimodal detection by ^{19}F MRI, X-ray and US imaging. The entire synthesis can be completed within 2 h.

Correspondence should be addressed to J.W.M.B. (jwmbulte@mri.jhu.edu).
[6]Present address: Department of Radiology, Centre Hospitalier Universitaire Vaudois, Center for Biomedical Imaging (CIBM) and University of Lausanne, Lausanne, Switzerland.

AUTHOR CONTRIBUTIONS B.P.B., A.A., D.R.A., M.S., D.L.K. and J.W.M.B. designed the experiments and analyzed the data. B.P.B., A.A., M.S. and D.L.K. conducted the experiments and collected data. B.P.B. and J.W.M.B. wrote the article.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests (see the HTML version of this article for details).

INTRODUCTION

Immunoisolation of cells in semipermeable microcapsules has been proposed as a means to prevent or delay their immune destruction following transplantation[1]. Immunoprotective microencapsulation is particularly attractive, as it both abrogates the need for chronic immunosuppressive therapy and opens up the possibility of immunoisolating xenogenic grafts[2,3]. To date, encapsulation has shown clinical potential for insulin[4,5] and parathyroid[6] hormone replacement therapy. Encapsulation has also shown considerable research potential for the delivery of a large variety of additional cellular therapeutics[7–9]. Nevertheless, several fundamental issues remain to be addressed before considering widespread clinical applications of this method. If microcapsules were to be monitored with noninvasive imaging modalities, questions related to the ideal transplantation site, the best means of delivery and the long-term survival of such grafts could be better addressed. We have developed new alginate-based microcapsule formulations (refs. 10–13 and D.R.A., unpublished data) that are detectable with X-ray, US and magnetic resonance imaging (MRI)—traditional imaging modalities that are clinically used and widely available. Contrast agents that can be readily incorporated include superparamagnetic iron oxide (SPIO)[11,13] and gadolinium (D.R.A., unpublished data) nanoparticles for MRI; barium sulfate, bismuth sulfate and gold particles (ref. 13 and D.R.A., unpublished data) for detection with X-ray modalities (fluoroscopy, computed tomography (CT)[10], digital subtraction angiography); and perfluorcarbons[12] as versatile agents for all three imaging modalities.

Previous methods for tracking cellular therapeutics with MRI have been limited to the use of either direct labeling of cells with contrast agents[14,15] or the use of a reporter gene[16] method. In this protocol, we describe the first method for long-term tracking of cellular therapeutics using X-ray imaging modalities. By encapsulating cells in contrast-containing alginate microcapsules, we potentially avoid many of the limitations involved with direct labeling of cells, such as contrast dilution in the case of dividing cells or loss of contrast with cell death. This latter feature is both a strength and weakness as it enables prolonged tracking of cellular therapeutics but does not report on the viability of such cells. As opposed to the majority of cellular labeling methods, which involve prolonged incubation of cells with contrast agent[14], contrast-containing capsules can be synthesized in <2 h, using clinically approved materials. In addition, an inherent limitation of intracellular labeling with contrast agents is the resulting dilution effect when cells divide. In our approach, because the contrast agent is present in the capsules and not the cells, this dilution effect does not occur even in the presence of cell proliferation within the microcapsule. It is unlikely that proliferating cells would expand to such an extent that the polymer barrier will break; there are no literature reports on this phenomenon. Microencapsulation has most widely been used for islet cells; these mixed cell spheres are growth arrested and cells do not divide. In the event that capsule integrity is maintained and the encapsulated cells are no longer viable, it is not anticipated that a change in signal from the capsules will occur, as it has been shown that capsule rupture leads to a loss of contrast[1]. However, in the event of capsule rupture, we would lose the ability to track cells when adding contrast agents. Adding a contrast agent to the capsule instead of into cells may potentially bypass toxicity issues that can result from direct cell labeling[17]. The addition of either iron oxide, barium and bismuth sulfate, or perfluorocarbons does not reduce cell viability and C-peptide secretion of islet cells[1–3]. Viability of encapsulated cells is determined mainly by the host microenvironment of the engrafted site. One of the main limiting factors for encapsulated cell survival is hypoxia resulting from a lack of direct vascularization. For this reason, transplantation of capsules intravascularly, in particular in the portal vein, is believed to be optimal but has not been unambiguously proven. In addition to providing a means of tracking, contrast-containing capsules provide immunoisolation[18]. Thus, rejection of cellular

therapeutics in an immunocompetent environment is potentially prevented or delayed when using mismatched donors or xenografts.

Other groups have explored doping nonalginate hydrogel microcapsules with radiopaque material for use as embolic agents. A number of biomaterials have been explored to deliver cellular and noncellular therapeutics[6,19–21]. The choice of material is guided by the application, with some materials providing a controlled degradation profile and other application-specific benefits[22]. Regardless of the material used, contrast agents can commonly be incorporated into the polymer matrix to enable detection with clinical imaging modalities. In addition to the previously mentioned contrast agents, other materials have been explored, including tantalum powder or tungsten powder[23], iothalamic acid and iopanoic acid[24,25]. As an alternative method, we have tried to incorporate these agents into the primary alginate layer of our microcapsules in order to make them radiopaque. We have also tested numerous other agents, which included diatrizoate sodium and diatrizoate meglumine (MD-Gastroview), iohexol (Omnipaque), diatrizoate sodium and diatrizoate meglumine (Hypaque), manganese chloride and potassium iodide. In all cases, contrast was not stably incorporated within the microcapsule but instead eluted over a period of hours (B.P.B., unpublished data). We did find that tantalum and tungsten powder could be readily encapsulated and retained within alginate microcapsules, but the radiopacity of these agents was much lower than that of barium and bismuth sulfate. Although not currently approved for parenteral use, uncoated barium sulfate is used as an oral contrast agent[26]; therefore, preparations of barium or bismuth complexed with stabilizing molecules may prove to be highly efficacious and have good relative safety compared with currently used agents. A recent report on the safety and efficacy of a novel bismuth sulfide nanoparticle CT contrast agent compared with traditional iodinated contrast agents supports this claim[27].

In the protocol that follows, contrast agents are added to the primary alginate layer. The labeling techniques described here could be used for alternative alginate microcapsule preparations. These include the use of polycations other than poly-L-lysine (PLL) (ref. 28 and D.R.A, unpublished data), or different cations in the gelation bath[29]. In addition, other compounds may be mixed with the alginate such as poly(ethylene glycol)[30]. As for using alternative cations, it has been shown that alginate gelled in barium chloride can also provide immunoisolation[31]. If the addition of contrast agent to the primary alginate layer results in retention of the material without affecting porosity, then potentially the use of polycations and an additional alginate layer may be circumvented. SPIO-containing capsules or ‘magnetocapsules’[11], radiopaque capsules or ‘X-Caps’[10], trimodal fluorocapsules[12], SPIO-Gold capsules[13] and GadoGold capsules (D.R.A, unpublished data) can, in principle, be applied to track a wide variety of cellular therapeutics by ^{1}H conventional MR, ^{19}F hot spot MR[32], X-ray and US imaging, all at a sensitivity level of a single capsule.

MATERIALS

REAGENTS

- Primary cells, cell clusters, cell lines or spheroids from the tissue of interest
- Cell culture medium (any appropriate culture medium for the cells being used)
- PRONOVA UP LVG ultrapure low-viscosity high-guluronate alginate (NovaMatrix, cat. no. 4200001)
- PRONOVA UP LVM ultrapure low-viscosity high-mannuronate alginate (NovaMatrix, cat. no. 4200201) ▲ **CRITICAL** To avoid endotoxin contamination and potential activation of the cells in the microcapsule, ensure that the source of alginate has low endotoxin levels.

- Poly-L-lysine (PLL; Sigma, cat. no. P7890)
- Barium sulfate (Sigma, cat. no. B8675)
- Bismuth sulfate (MP Biomedicals, Fisher Scientific, cat. no. ICN213705)
- Feridex® I.V. (Berlex Laboratories, Bayer, cat. no. 59338-7035; see SPIO in the REAGENT SETUP)
- PFOB ($C_8F_{17}Br$) (Apollo Scientific, cat. no. PC6167)
- Perfluoro-15-crown-5-ether ($C_{15}F_{30}O_5$) (Fluorochem, cat. no. 009312)
- Soy lecithin (Sternfine, Sternchemie)
- Safflower oil USP (ScienceLab, cat. no. SLS3622)
- Calcium chloride, anhydrous powder (Sigma, cat. no. 499609)
- Sodium citrate (Sigma, cat. no. S1804)
- HEPES (Sigma, cat. no. H6147)
- Hospira 0.9% sodium chloride solution (Fisher Scientific, cat. no. AB1583-01-01)
- Fluorescein diacetate (FDA, Sigma, cat. no. F7378)
- Propidium iodide (PI, Invitrogen, cat. no. P3566)
- Endorem (Guerbet)
- Resovist (Bayer-Schering AG, cat. no. SHU 555A)
- Ferumoxtyol (AMAG Pharmaceuticals)
- Combidex (labeled Sinerem in Europe, Guerbet)
- LIVE/DEAD viability assay kit (Molecular Probes)

EQUIPMENT

- Nano syringe pump (Cole-Parmer, cat. no. EW-74902-00)
- Ring stand (Fisher Scientific, cat. no. 14-675B)
- Three-prong extension clamps (Fisher Scientific, cat. no. 057696Q)
- High-voltage power supply (Bertran, model no. 230-30R)
- Sonicator (Misonix, model no. WU-04712-81)
- Electrical tape (3/4-inch; RadioShack, cat. no. 64-2375)
- Automotive hookup wire (10AWG; RadioShack, cat. no. 278–569)
- Kronus 4.5-inch mini diagonal wire cutters (RadioShack, cat. no. 64-2951)
- Becton-Dickinson tuberculin syringe—1 ml only with slip tip (non-safety lock; Becton-Dickinson, cat. no. 454663107)
- Blunt needles (24 gauge × 1/2-inch; Brico Medical Supplies, cat. no. BN2405, 20 gauge × 1/2-inch; Brico Medical Supplies, cat. no. BN2015)
- Three-way stopcock with swivel male Luer lock (Medex, cat. no. MX5311L)
- Conical tubes (15 and 50 ml; BD Biosciences, cat. nos. 352096 and 352070)
- Millipore filter flask (0.2 μm; Fisher Scientific, cat. no. XX1004705)

- Nylon filter (0.2 μm; Acrodisc, Pall Corporation, cat. no. 4436)
- Corning 100 × 20-mm untreated culture dish (Fisher Scientific, cat. no. 08-772-32)
- Serological pipettes (Fisher Scientific, cat. no. 13-675-3F)
- Drummond Pipet-Aid (Fisher Scientific, cat. no. 13-681-15E)
- Erlenmeyer screw cap flask (1 liter; Fisher Scientific, cat. no. CG154305)
- Ceramic stirring hot plate, 10 × 10 inches, 230V 50/60Hz (Fisher Scientific, cat. no. 11-102-100SH)
- Stir bars (Fisher Scientific, cat. no. 14-511-98)
- Vortex-Genie (Fisher Scientific, cat. no. 50212393)
- Boekel Rocker II platform rocker (Fisher Scientific, cat. no. 05-450-34)
- Centrifuge equipped for 50-ml conical tubes
- Tissue culture hood
- Gas-sterilization facilities
- Autoclave bags and gas-sterilization autoclave tape
- Philips Achieva MR scanner (Philips)
- 1.5T (CV/i, GE Medical Systems) or 3.0T (Philips Achieva) MR scanner
- Toshiba Infinix VC-i unit (Toshiba)
- XSPECT scanner (Gamma-Medica)
- 9.4T, or 11.7T Bruker horizontal bore animal scanner (Bruker)
- MHz probe on a Micromaxx US system (Sonosite)
- Imaging processing software (Amira)

REAGENT SETUP

Preparation of primary alginate—Prepare a solution of 10 mM HEPES-buffered normal saline. Sterilize the filter solution with a filter flask and pour the sterilized solution into an Erlenmeyer flask in a tissue culture hood. Add a stir bar to the flask and place it on a stirring hot plate with the heater turned off. With the stirring plate on, being careful to minimize aggregation of alginate, slowly pour Pronova UP LVG alginate to a concentration of 2% (wt/vol). Place a screw cap on the flask and allow it to rotate overnight in a tissue culture hood with the UV light turned off. As it is difficult to dissolve alginate in small volumes of liquid, it is better to prepare a larger volume and aliquot it into 15-ml conical tubes for later use. For best results, store unused aliquots at 2–4 °C for up to 1 month. Remove necessary aliquots from refrigeration the day before encapsulation and allow to return to room temperature (20–25 °C).

Preparation of secondary alginate—Prepare secondary alginate in a manner identical to that of primary alginate but instead use Pronova UP LVM at a concentration of 0.15% (wt/vol); store aliquots in 50-ml conical tubes.

Preparation of Poly-L-lysine solution—Prepare a solution of 0.05% (wt/vol) PLL in 10 mM HEPES-buffered normal saline and filter-sterilize the solution with a filter flask. Store aliquots at 2–4 °C for up to 1 year in 15-ml conical tubes.

Preparation of gelation bath—Prepare a solution of 100 mM calcium chloride in 10 mM HEPES-buffered normal saline and filter-sterilize the solution with a filter flask. Store aliquots at 2–4 °C for up to 1 year in 50-ml conical tubes.

Superparamagnetic iron oxide (SPIO)—Feridex I.V. has been commercially available in the USA but not in Europe, where it was marketed as Endorem (Guerbet); both formulations are identical. However, these SPIO formulations have recently been taken off the market and are no longer available. As alternatives to these SPIOs, other formulations may be encapsulated, such as Resovist (SHU 555A, Bayer-Schering AG), another clinically approved SPIO, or Ferumoxtyol (AMAG Pharmaceuticals). In addition, ultrasmall SPIOs (USPIOs) such as Combidex (labeled Sinerem in Europe, distributed by Guerbet) may be incorporated, although it is anticipated that the resulting magnetocapsules will be less magnetic and MR-visible because of the smaller size and magnetic moment (Bohr magnetons) of the individual USPIO particles. Currently, the commercial availability of these and other agents is rapidly changing, but regardless of the choice of SPIO, the same basic protocol applies.

PROCEDURE

Assemble electrostatic droplet generator ● TIMING 40 min plus sterilization time

1| Cut two pieces of 5-foot connection wire and strip 1 inch of insulation tubing off the distal ends of both wires. Attach one end of the wire to a suitable ground (e.g., a ring stand placed on the floor) and place the other end of the wire into a Petri dish containing the cationic gelation bath. Attach the other wire to the ground on the high-voltage generator and connect the other end of the wire to a separate ground (e.g., the inner metal wall of a tissue culture hood; see Fig. 1).

2| Pass a 20-g 1/2-inch blunt needle through the insulation wall of the wire through wire braids and out the opposite insulation wall of the output wire of the high-voltage droplet generator.

3| Gas-sterilize the setup using ethylene oxide gas (available at most medical centers); alternatively, if ethylene oxide is not available, use a UV light assembly for sterilization (available in most tissue culture hoods).

4| Place a culture dish below the needle and ensure that it is properly grounded (e.g., by running a wire from the encapsulation bath out of the tissue culture hood and onto a ring stand on the ground, ensuring that the proximal portion of the wire in the gelation bath and hood remains sterile).

5| Just before encapsulation, add 50 ml of gelation solution to the culture dish.

? TROUBLESHOOTING

Incorporation of contrast in primary alginate ● TIMING 20 min

6| Follow the steps in option A for MR contrast, B for X-ray contrast and C for trimodal (perfluorocarbon) contrast.

A. MR contrast agent

i. To create MR-visible alginate capsules, mix 20% (vol/vol) Feridex® (stock = 11.2 mg Fe per ml) with 80% (vol/vol) primary alginate.

ii. For best results, load alginate and Feridex® into two separate syringes and connect the syringes to a three-way stopcock; carefully remove any excess air.

iii. Once air is removed, move the stopcock to the closed position and mix the contents of the two syringes back and forth until a homogenous solution is created.

B. X-ray imaging agent

i. To create X-ray–visible alginate capsules, first dissolve barium or bismuth sulfate into 0.9% saline at a concentration of 50 mg ml^{-1}.

ii. Vortex this solution to breakdown any large aggregates.

iii. Mix this barium sulfate solution with primary alginate as described in Step 6A(ii,iii). As barium sulfate is in powder form, it can be added at the necessary concentration for the imaging modality used. For example, for CT imaging a concentration of 5% (wt/vol) is acceptable, whereas for detection on fluoroscopy a concentration of >10% (wt/vol) is needed.

C. Multimodal contrast agent

i. To prepare fluorine MRI-, X-ray- and US-visible capsules, first prepare a perfluorocarbon emulsion. Prepare a solution of 5% lecithin (wt/vol) and 2% safflower oil (vol/vol) in water and filter-sterilize it through a 0.2-μm nylon filter.

ii. Sonicate the lecithin/safflower oil solution at 40% power on ice until the solution is almost transparent.

iii. Add perfluorocabon (Crown Ether or PFOB) to sonicated lecithin solution at a concentration ranging from 12% perfluorocarbon (wt/vol) to 40% perfluorcarbon (vol/vol). In the case of MRI, the ratio of perfluorocarbon can be chosen on the basis of the field strength of the MR scanner being used. High-field scanners offer a higher sensitivity for single capsule detection as compared with low-field scanners. The same rationale should be applied for optimizing for X-ray imaging on CT versus fluoroscopy.

iv. Sonicate perfluorocarbon lecithin solution for 25 min on ice until a milky homogenous solution is formed. Leave fluorocarbons on ice until incorporation in the primary alginate layer.

v. Emulsion should be incorporated into the alginate layer within 1 h of emulsion preparation, as described in Step 6A(ii,iii).

? TROUBLESHOOTING

Adding cells to primary alginate/contrast solution ● TIMING 2 min

7| Pellet cells or allow clusters or spheroids of cells to settle in a 50-ml conical tube and remove all but 100 μl of culture medium. Depending on the expected growth of the cell population, total concentration of cells should be adjusted to a concentration of 1–5% (vol/vol) to that of the primary alginate layer. For growth-arrested cells, a higher number of cells can be encapsulated. For highly proliferative cells, e.g., immortalized cell lines, cells should be added at lower concentrations.

? TROUBLESHOOTING

8| Draw up primary alginate in one syringe and contrast in an alternate syringe and attach a three-way stopcock. In the case of dehydrated contrast agents such as barium sulfate, the contrast agent should be dissolved in a minimal volume of appropriate solvent (i.e., saline, double-distilled water and so on.).

9| Carefully remove all air by placing the stopcock in the open position and depressing the syringe until air is released. Close the stopcock and then gently mix the contents of the two syringes.

10| With the appropriate mixture of contrast and alginate, add the cells at the appropriate concentration to the alginate using a 50-ml conical tube. Use a 1-cc tuberculin syringe to mix the alginate/contrast solution with cells until a homogeneous mixture is achieved. Appropriate cell concentrations vary depending on cell types. For human islets, which may contain up to several thousand cells, one islet per capsule should be used. For single cells, e.g., mesenchymal stem cells, a maximum of up to 300 single cells can be encapsulated.

▲ **CRITICAL STEP** The duration of this step must be minimized, as oxygen diffusion is greatly reduced when cells are suspended in relatively large volume of unpolymerized alginate.

11| If viability of cells is reduced substantially at this step, one could supplement the primary alginate in Step 6 with glucose, amino acids, insulin, transferrin or other nutrients. Encapsulated cell viability can be assessed as described in Box 1

Box 1

ASSESSING CELL VIABILITY ● TIMING 15 MIN (STAINING AND MICROSCOPIC IMAGING) plus 120 MIN (CELL COUNTING)
Cell viability is determined through a dual staining process using FDA, which stains live cells, and PI, which stains dead cells.

1. Prepare an FDA stock by mixing 9.9 mg of FDA with 1 ml of acetone, and add 1 ml of PBS. An FDA working solution is prepared by mixing 500 μl of the stock with 500 μl of PBS. The PI stock comes as a 1 mg ml^{-1} solution; a working solution is prepared by mixing 100 μl of the stock with 900 μl of PBS. To ensure adequate sampling, ten microspheres are randomly selected from each preparation.
2. Remove culture medium and rinse capsules twice with PBS, then resuspend in 480 μl of PBS. Using both working solutions, add 10 μl of FDA and 10 μl of PI and shake gently for 2 min.
3. Remove capsules immediately and rinse twice with PBS.

4. After staining, examine the capsules with a fluorescence microscope. FDA is excited at 495 nm, with the emitted fluorescence detected at 520 nm. Propidium iodide is excited at 535 nm, with the emitted fluorescence detected at 615 nm.

5. Adjust the focus of the microscope, and capture the images of red fluorescent (PI) dead cells and green fluorescent (FDA) live cells within each capsule at one focal plane. This is repeated for ten different focal planes. As prolonged exposure of dyes may kill the cells, the staining and imaging process must be completed within 15 min.

6. Calculate the number of live and dead cells from the images. The percentage cell viability is expressed as the average of a total of 100 cell counts (i.e., ten capsules with ten focal planes per capsule).

Under certain circumstances, the microscopic detection of individual encapsulated cells may be difficult because of light refraction through the alginate layer. When such difficulty is encountered, it is recommended that the microscope focus be adjusted and the magnification be increased (×20 or ×40) until single cells can be easily visualized. If adequate data are not collected within the 15-min staining time limit, repeat the staining and imaging protocol using a fresh sample of capsules. Alternatively, depending on the type of cells, other viability assays may be used (i.e., using a LIVE/DEAD viability assay kit (Molecular Probes) according to the manufacturer's protocol).

If the viability of cells is reduced substantially at this step, supplement the primary alginate and/or the PLL solution with glucose, amino acids, insulin, transferrin or other nutrients.

? TROUBLESHOOTING

Cell encapsulation ● TIMING 6 min per 1 ml of primary alginate

12| Affix a blunt-tip needle with the wire from the high-voltage generator to the syringe containing the suspension of alginate, contrast and cells.

13| Place the syringe with the needle attached in the syringe pump and adjust the height between the needle and the cationic (calcium or barium) solution by adjusting the positioning knobs of the stereotaxic apparatus. The distance should be approximately on the order of an inch (this results in a voltage differential of ~8 kV).

14| Turn on the high-voltage generator. Adjust the current beforehand, using an alginate solution without cells, to obtain the finest stream (and thus the smallest droplets) possible.

15| Preset the microinjector to inject a volume of 1 ml at a rate of 200 μl min^{-1}.

16| Ensure that the gelation bath is properly grounded and start the syringe pump.

? TROUBLESHOOTING

17| If microcapsules are too large, as determined using a phase-contrast inverted microscope, increase the distance between the needle tip and the gelling bath or increase the voltage from the generator or lower the concentration of the primary alginate.

? TROUBLESHOOTING

18| If microcapsules begin to adhere to each other as they are collecting directly beneath the tip of the needle in the gelling bath, it may be necessary to place a sterile stir bar in the gelation bath and place it on a stir plate.

? TROUBLESHOOTING

19| Allow microcapsules to remain in the gelation bath for 5 min.

20| Use a Pipet-Aid equipped with a 10-ml serological pipette to remove microcapsules from the gelation bath; add them to a 50-ml conical tube.

21| Remove calcium chloride solution by allowing microcapsules to settle to the base of the conical tube and then aspirate the entire volume of calcium chloride solution.

Application of polycation coat ● TIMING 8 min

22| Rinse microcapsules with three changes of 25-ml 0.9% (wt/vol) sodium chloride solution. Remove the solution for each change as described in Step 21.

23| Add 40–50 ml of the PLL solution described above to microcapsules in the 50-ml conical tube.

24 Seal the conical tube and tape it horizontally on a platform rocker. Rock at medium speed for exactly 6 min.

Application of secondary alginate layer ● TIMING 4 min

25| Rinse microcapsules with three changes of 25-ml 0.9% (wt/vol) sodium chloride solution. Remove solution for each change as described above.

26| With all 0.9% (wt/vol) sodium chloride solution removed, add 40–50 ml of secondary alginate, prepared as described above, to the microcapsules in a 50-ml conical tube.

27| Seal the conical tube and tape horizontally on a platform rocker. Rock at medium speed for 3 min.

28| Rinse the microcapsules with three changes of 25-ml 0.9% (wt/vol) sodium chloride solution. Evacuate the solution for each change as described in Step 21 and immediately transplant or place in appropriate culture conditions for the encapsulated cell type.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● **TIMING—**Reagent setup, 30 min plus dissolving time

Steps 1–5, Assembly of electrostatic droplet generator: 40 min plus sterilization time

Step 6, Incorporation of contrast in primary alginate: 20 min

Steps 7–11, Adding cells to primary alginate/contrast solution: 2 min

Steps 12–21, Cell encapsulation: 6 min per 1 ml of primary alginate

Steps 22–24, Application of polycation coat: 8 min

Steps 25–28, Application of secondary alginate layer: 4 min

Box 1, Assessing cell variability: 15 min

ANTICIPATED RESULTS

Magnetocapsules have a characteristic rust color with an average diameter of ~300 μm (Fig. 2a). X-ray visible caps or X-caps have a characteristic white (Ba X-Caps) or yellow (Bi X-Caps) macroscopic appearance, with an average diameter of ~350 μm. For all capsule types, encapsulated cells are distributed randomly throughout the microcapsule matrix (Fig. 2b).

As compared with naked cells or islet cells encapsulated without contrast material, neither magnetocapsules[1], X-caps[2] nor fluorocapsules[3] is anticipated to interfere with cell viability and C-peptide secretion by islet cells. Because of their ability to function as an oxygen sink, fluorocapsules can be expected to improve cell function[3].

The iron content of magnetocapsules is sufficient to enable a clear depiction of single capsules in agarose phantoms (Fig. 2c). Using a novel 3D inversion recovery on-resonance (IRON)[33] positive-contrast imaging technique, which is very sensitive to microscopic magnetic susceptibility changes, the capsule surface of single magnetocapsules can be selectively enhanced (Fig. 2d). Thus, MR imaging not only has the spatial resolution to detect 350-μm capsules but also allows distinct visualization of the surface of intact capsules. However, liver IRON imaging *in vivo* presents additional challenges including, but not limited to, compensation for respiratory motion, the need for good shimming of the magnet and the need to account for other causes of susceptibility artifacts, such as at the lung/liver interface.

For assessment of MR contrast, MCs were suspended in 2% (wt/vol) agarose at a density of 50-capsules per ml gel. Phantom imaging was performed on a 3T Philips Achieva MR scanner with a six-element cardiac phased-array receiver coil. For 3D T2*-weighted gradient echo imaging, which provides hypointense contrast, the imaging parameters were as follows: repetition time (TR) = 7.0 ms; echo time (TE) = 2.3 ms; flip angle (FA) = 15°; field of view (FOV) = 22 cm; matrix = 512 × 512; and slice thickness = 1 mm. For fast-spin echo 3D IRON imaging, which provides positive contrast, the imaging parameters were the same except for TR = 1,300 ms; TE = 12.0 ms; IRON pulse bandwidth (BW_{IRON}) = 170 Hz; and turbo factor = 18. Microcapsules were also visualized after transplantation into the portal vein of swine with a 1.5T MR scanner (CV/i, GE Medical Systems; Fig. 2e). The imaging parameters were as follows: TR = 4.8 ms; TE = 1.4 ms; FA = 25°; BW = 31.2 kHz; FOV = 30 cm; and image matrix = 256 × 256. For the liver, magnetocapsules were more unambiguously detected with IRON imaging as compared with conventional T2-weighted imaging (Fig. 2f,g). *Ex vivo* imaging was performed on a 3T Philips Achieva MR scanner with an eight-element head phased-array receiver coil. For 3D T2*-weighted gradient echo imaging, the imaging parameters were as follows: TR = 3.9 ms; TE = 1.7 ms; FA = 20°; FOV = 14 cm; and voxel size = 0.74 × 0.75 × 0.74 mm^3. For fast-spin echo 3D IRON imaging, which provides positive contrast, the imaging parameters were the same except for TR = 858 ms; TE = 18.3 ms; IRON pulse bandwidth (BW_{IRON}) = 170 Hz; α_{SAT} = 100°; and turbo factor = 10.

The radiopaque labeling of alginate capsules enables fluoroscopic imaging and noninvasive X-ray tracking of encapsulated cells in both small and large animal models. With standard clinical-grade fluoroscopic imaging units such as the Toshiba Infinix VC-i unit (Toshiba) with imaging settings of 64 kVP (kVP = kilovolt peak), 66-ms exposure time, 112-mA tube current and 910-mm SID single Ba X-Caps and Bi X-Caps could be easily visualized *in vitro* (Fig. 3a). Individual, single Ba X-Caps can also be resolved after transplantation in the

peritoneal cavity of a mouse with CT (Fig. 3b). Using a Gamma-Medica XSPECT scanner, microcapsules were imaged in the peritoneal cavity with 1,024 projections obtained during a 360° rotation with 0.703° rotation steps and reconstructed into 1,024 × 1,024 images using filtered backprojection reconstruction supplied by the vendor. Acquisition time for each view was 1 s at 50 kVP and 600 mA. Scanning was performed in a clockwise direction with an X-ray tube to detector distance of 269 mm and an X-ray tube to center of rotation distance of 225 mm. Segmentation and 3D reconstruction was carried out using the imaging software Amira. Ba X-Caps could also be resolved after transplantation in the thigh of the New Zealand White rabbit (Fig. 3c,d), using a Toshiba Infinix VC-i unit with imaging settings of 64 kVP, 66-ms exposure time, 112-mA tube current and 910-mm image intensifier size.

In vitro ^{19}F MR imaging of 350-μm fluorocapsules showed the ability to detect single capsules at 11.7T (Fig. 4a). Using high-resolution ^{19}F MRI (Bruker 9.4T horizontal bore animal scanner), following transplantation into the peritoneal cavity of mice, fluorocapsules were identifiable, and when overlaid on anatomical ^{1}H MRI scans, capsules were easily distinguishable from soft tissue (Fig. 4b,c). Different amounts of capsules were imaged in order to determine the sensitivity of detection without signal quantitation. With micro-CT imaging, individual capsules were visible *in vitro* (Fig. 5a–c) and *in vivo* when transplanted into the peritoneal cavity of a mouse (Fig. 5d,e). In addition, individual fluorocapsules in both phantoms (Fig. 6a,b) and after transplantation into the kidney of swine (Fig. 6c) were visible under US. US imaging was performed with a L25E 13-6 MHz probe on a Micromaxx US system (Sonosite). Grayscale imaging was performed with a center probe frequency of 6.00 MHz, a dynamic range of 55 dB, and a persistence setting of 2. Grayscale gain was adjusted for baseline imaging.

Acknowledgments

The development of this synthesis protocol was supported by a Howard Hughes Medical Institute Research Training Grant (B.P.B.), NIH grants K08 EB004348 (A.A.), R01 HL073223 (D.L.K.), R01 EB007825, U54CA151838 (J.W.M.B.) and RO1 HL084186 (M.S.).

References

1. Mullen Y, Maruyama M, Smith CV. Current progress and perspectives in immunoisolated islet transplantation. J Hepatobiliary Pancreat Surg. 2000; 7:347–357. [PubMed: 11180854]
2. Schneider S, et al. Long-term graft function of adult rat and human islets encapsulated in novel alginate-based microcapsules after transplantation in immunocompetent diabetic mice. Diabetes. 2005; 54:687–693. [PubMed: 15734844]
3. Luca G, et al. Xenograft of microencapsulated sertoli cells reverses T1DM in NOD mice by inducing neogenesis of beta-cells. Transplantation. 2010; 90:1352–1357. [PubMed: 21197711]
4. Soon-Shiong P, et al. Insulin independence in a type 1 diabetic patient after encapsulated islet transplantation. Lancet. 1994; 343:950–951. [PubMed: 7909011]
5. Tuch BE, et al. Safety and viability of microencapsulated human islets transplanted into diabetic humans. Diabetes Care. 2009; 32:1887–1889. [PubMed: 19549731]
6. Tibell A, et al. Survival of macroencapsulated allogeneic parathyroid tissue one year after transplantation in nonimmunosuppressed humans. Cell Transplant. 2001; 10:591–599. [PubMed: 11714193]
7. Borlongan CV, et al. Intracerebral transplantation of porcine choroid plexus provides structural and functional neuroprotection in a rodent model of stroke. Stroke. 2004; 35:2206–2210. [PubMed: 15284450]
8. Visted T, et al. Prospects for delivery of recombinant angiostatin by cell-encapsulation therapy. Hum Gene Ther. 2003; 14:1429–1440. [PubMed: 14577923]

9. Mai G, et al. Treatment of fulminant liver failure by transplantation of microencapsulated primary or immortalized xenogeneic hepatocytes. Transplant Proc. 2005; 37:527–529. [PubMed: 15808699]
10. Barnett BP, et al. Radiopaque alginate microcapsules for X-ray visualization and immunoprotection of cellular therapeutics. Mol Pharm. 2006; 3:531–538. [PubMed: 17009852]
11. Barnett BP, et al. Magnetic resonance-guided, real-time targeted delivery and imaging of magnetocapsules immunoprotecting pancreatic islet cells. Nat Med. 2007; 13:986–991. [PubMed: 17660829]
12. Barnett BP, et al. Fluorocapsules for improved function, immunoprotection, and visualization of cellular therapeutics with MR, US, and CT imaging. Radiology. 2011; 258:182–191. [PubMed: 20971778]
13. Kim J, et al. Multifunctional capsule-in-capsules for immunoprotection and trimodal imaging. Angew Chem Int Ed Engl. 2011; 50:2317–2321. [PubMed: 21351344]
14. Bulte JW, et al. Magnetodendrimers allow endosomal magnetic labeling and *in vivo* tracking of stem cells. Nat Biotechnol. 2001; 19:1141–1147. [PubMed: 11731783]
15. Ahrens ET, Flores R, Xu H, Morel PA. *In vivo* imaging platform for tracking immunotherapeutic cells. Nat Biotechnol. 2005; 23:983–987. [PubMed: 16041364]
16. Gilad AA, et al. Artificial reporter gene providing MRI contrast based on proton exchange. Nat Biotechnol. 2007; 25:217–219. [PubMed: 17259977]
17. Kostura L, Kraitchman DL, Mackay AM, Pittenger MF, Bulte JW. Feridex labeling of mesenchymal stem cells inhibits chondrogenesis but not adipogenesis or osteogenesis. NMR Biomed. 2004; 17:513–517. [PubMed: 15526348]
18. de Vos P, Faas MM, Strand B, Calafiore R. Alginate-based microcapsules for immunoisolation of pancreatic islets. Biomaterials. 2006; 27:5603–5617. [PubMed: 16879864]
19. Kakizawa Y, et al. Controlled release of protein drugs from newly developed amphiphilic polymer-based microparticles composed of nanoparticles. J Control Release. 2010; 142:8–13. [PubMed: 19804802]
20. Meunier CF, Dandoy P, Su BL. Encapsulation of cells within silica matrixes: towards a new advance in the conception of living hybrid materials. J Colloid Interface Sci. 2010; 342:211–224. [PubMed: 19944428]
21. Zustiak SP, Leach JB. Hydrolytically degradable poly(ethylene glycol) hydrogel scaffolds with tunable degradation and mechanical properties. Biomacromolecules. 2010; 11:1348–1357. [PubMed: 20355705]
22. Orive G, Pedraz JL. Highlights and trends in cell encapsulation. Adv Exp Med Biol. 2010; 670:1–4. [PubMed: 20384213]
23. Gobin YP, Vinuela F, Vinters HV, Ji C, Chow K. Embolization with radiopaque microbeads of polyacrylonitrile hydrogel: evaluation in swine. Radiology. 2000; 214:113–119. [PubMed: 10644109]
24. Horak D, et al. Hydrogels in endovascular embolization. III. Radiopaque spherical particles, their preparation and properties. Biomaterials. 1987; 8:142–145. [PubMed: 3580473]
25. Jayakrishnan A, Thanoo BC, Rathinam K, Mohanty M. Preparation and evaluation of radiopaque hydrogel microspheres based on PHEMA/iothalamic acid and PHEMA/iopanoic acid as particulate emboli. J Biomed Mater Res. 1990; 24:993–1004. [PubMed: 2394768]
26. Megibow AJ, Bosniak MA. Dilute barium as a contrast agent for abdominal CT. AJR Am J Roentgenol. 1980; 134:1273–1274. [PubMed: 6770647]
27. Rabin O, Manuel Perez J, Grimm J, Wojtkiewicz G, Weissleder R. An X-ray computed tomography imaging agent based on long-circulating bismuth sulphide nanoparticles. Nat Mater. 2006; 5:118–122. [PubMed: 16444262]
28. Ponce S, et al. Chemistry and the biological response against immunoisolating alginate-polycation capsules of different composition. Biomaterials. 2006; 27:4831–4839. [PubMed: 16766026]
29. Morch YA, Donati I, Strand BL, Skjak-Braek G. Effect of Ca2+, Ba2+, and Sr2+ on alginate microbeads. Biomacromolecules. 2006; 7:1471–1480. [PubMed: 16677028]
30. Haque T, et al. Superior cell delivery features of poly(ethylene glycol) incorporated alginate, chitosan, and poly-L-lysine microcapsules. Mol Pharm. 2005; 2:29–36. [PubMed: 15804175]

31. Omer A, et al. Long-term normoglycemia in rats receiving transplants with encapsulated islets. Transplantation. 2005; 79:52–58. [PubMed: 15714169]

32. Bulte JW. Hot spot MRI emerges from the background. Nat Biotechnol. 2005; 23:945–946. [PubMed: 16082363]

33. Stuber M, et al. Positive contrast visualization of iron oxide-labeled stem cells using inversion-recovery with ON-resonant water suppression (IRON). Magn Reson Med. 2007; 58:1072–1077. [PubMed: 17969120]

34. Dufrane D, Goebbels RM, Saliez A, Guiot Y, Gianello P. Six-month survival of microencapsulated pig islets and alginate biocompatibility in primates: proof of concept. Transplantation. 2006; 81:1345–1353. [PubMed: 16699465]

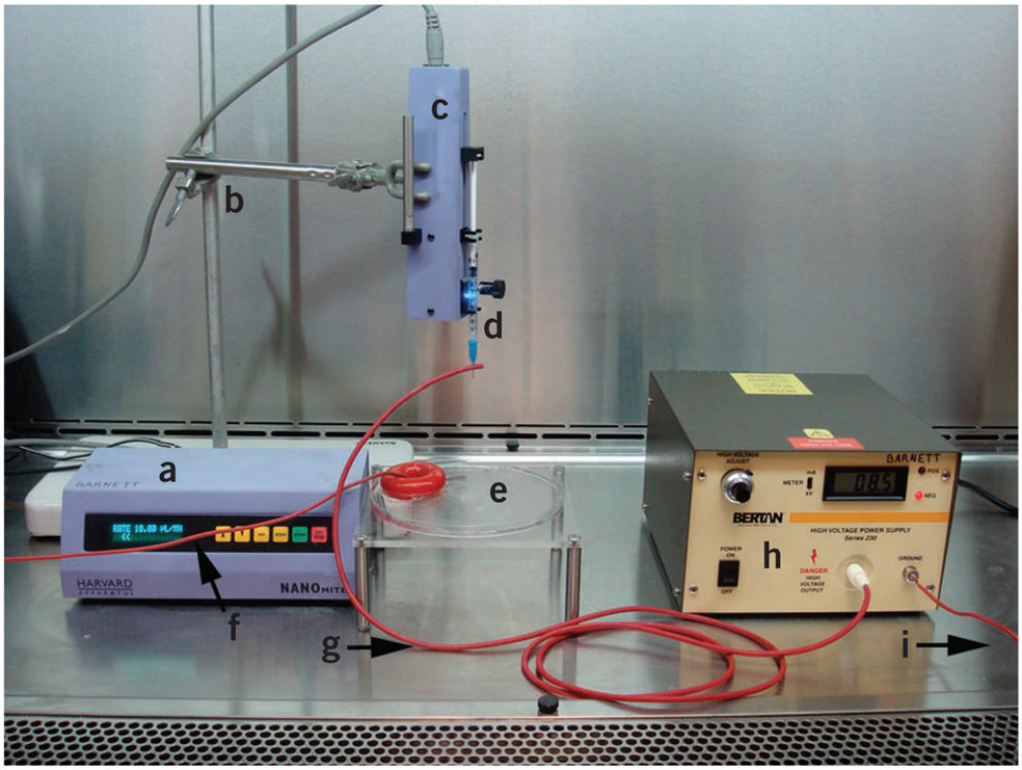

Figure 1.

Setup of capsule synthesis equipment. (**a**–**i**) Syringe pump control box (**a**), ring stand and clamp (**b**), syringe pump remote injector (**c**), syringe with blunt-tip needle through high-voltage output wire (**d**), gelation bath (**e**), gelation bath ground wire (**f**), high-voltage output wire (**g**), high-voltage generator (**h**) and high-voltage generator ground wire (**i**).

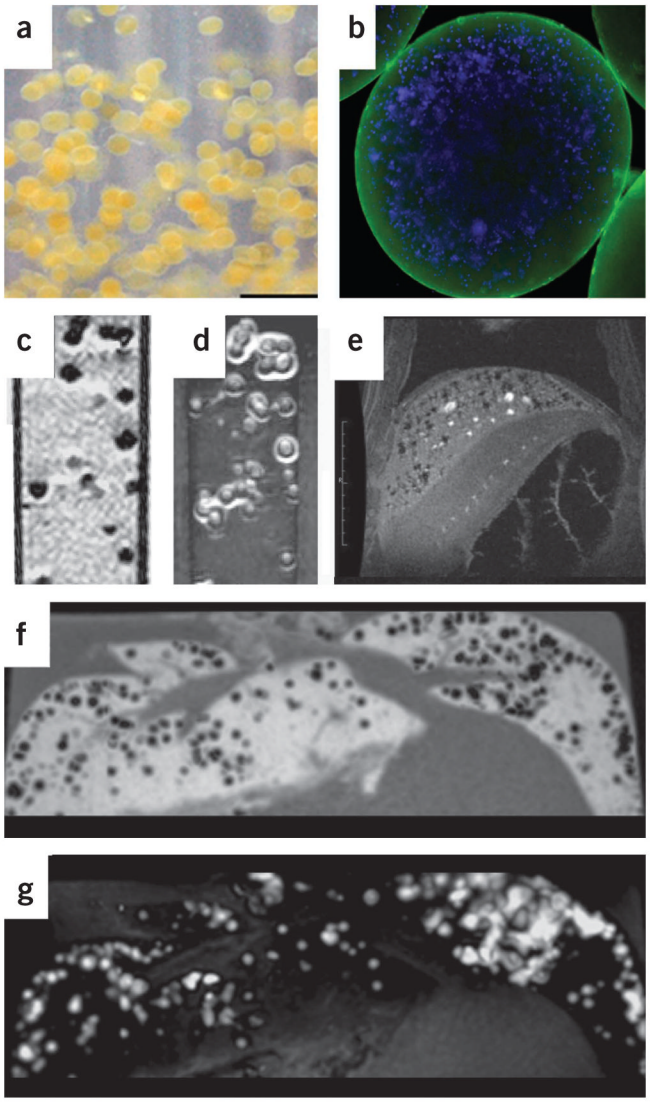

Figure 2.

Magnetocapsules. (**a**) Unstained Feridex-containing magnetocapsules show a ferric rust-like color originating from the Feridex iron oxide particles. (**b**) Single magnetocapsule containing encapsulated TC-6 cells. Dextran-specific (Feridex-specific) immunostaining, green; 4,6-diamidino-2-phenylindole (DAPI), blue. (**c**,**d**) As magnetocapsules rapidly settle in solution, they were embedded in a 2% (wt/vol) agarose phantom at a density of 50 capsules per ml of gel. By using conventional T_2*-weighted images (**c**), individual magnetocapsules can be easily identified as hypointensities. By using the IRON sequence for generating positive contrast (**d**), individual magnetocapsules appear as a bright signal with depiction of the capsule surface. (**e**) After intraportal infusion of magnetocapsules in a swine, the capsules can be seen as hypointense signal voids distributed throughout the liver. (**f**,**g**) *Ex vivo* scan of porcine liver after transplantation of 50,000 magnetocapsules with T_2*-weighted gradient echo imaging (**f**) and an IRON fast-spin echo sequence (**g**). Panels **a**–**e** are adapted with permission from reference 11.

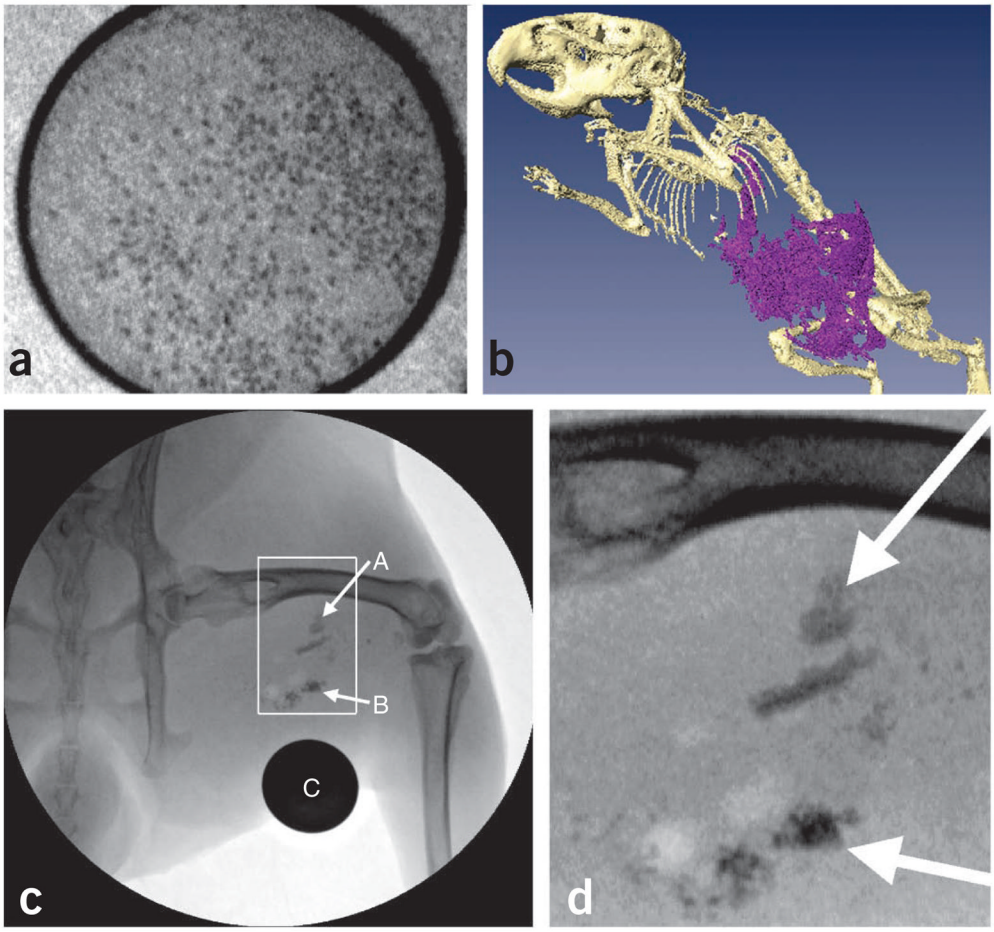

Figure 3.

Barium and bismuth X-Caps. (**a**) Fluoroscopic images of Ba X-Caps. Single capsules can be clearly identified. (**b**) *In vivo* imaging of X-Caps, as seen on high-resolution CT (XSPEC, Gamma-Medica) after transplantation in the peritoneal cavity of a mouse. (**c**) *In vivo* imaging of X-Caps immediately after intramuscular transplantation into a rabbit hind limb. A = 2,000 Ba X-Caps; B = 2,000 Bi X-Caps; C = quarter for reference of size and opacity. (**d**) Magnification of the fluoroscopic image shown in boxed area of **c**. Adapted with permission from reference 10.

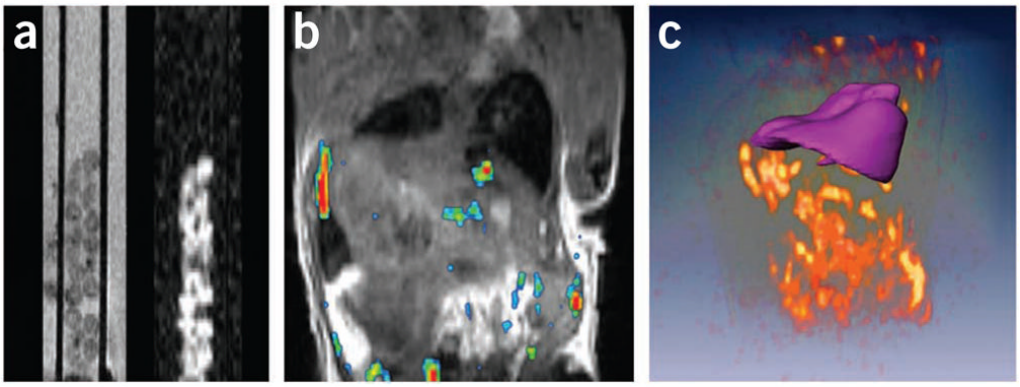

Figure 4.
^{19}F MR imaging of perfluoropolyether (PFPE) microcapsules.
(**a**) *In vitro* ^{1}H (left) and ^{19}F (right) 9.4 T MR images of an NMR tube filled with PFC fluorocapsules shows detection of individual capsule entities. (**b**) A total of 2,000 fluorocapsules were transplanted into the peritoneal cavity of a mouse. Shown is the 4.7 T ^{19}F MR image overlaid on the anatomical ^{1}H image. (**c**) The 3D reconstruction of fluorine capsules with overlay of ^{1}H image of liver. T2-weighted spin echo ^{1}H images were acquired with TR/TE = 1,500/15 ms; FOV = 3 × 3 cm, matrix = 196 × 196; slice thickness = 0.8 mm; NAV = 4. For ^{19}F imaging gradient echo images were obtained with TR/TE = 500/6 ms, FOV = 3 × 3 cm, matrix = 296 × 296 and FA = 30°. Adapted with permission from reference 12.

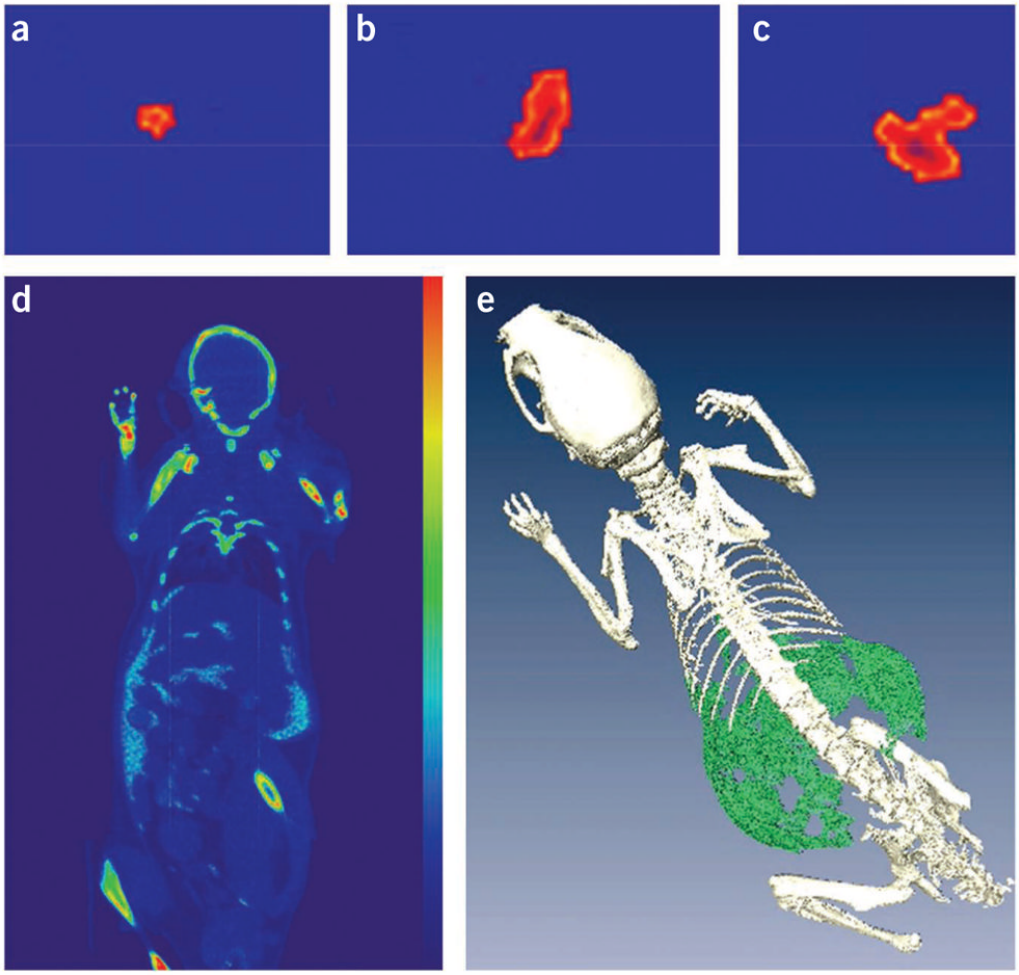

Figure 5.

CT imaging of PFOB microcapsules. (**a**–**c**) Images showing one capsule (**a**), five capsules (**b**) and ten capsules (**c**). (**d**) Single-plane high-resolution CT image of 2,000 PFOB caps transplanted in the peritoneal cavity of a mouse. (**e**) Amira software 3D reconstruction CT images from the same mouse shown in **d**. Imaging was performed with a high-resolution CT (XSPEC, Gamma-Medica). Adapted with permission from reference 12.

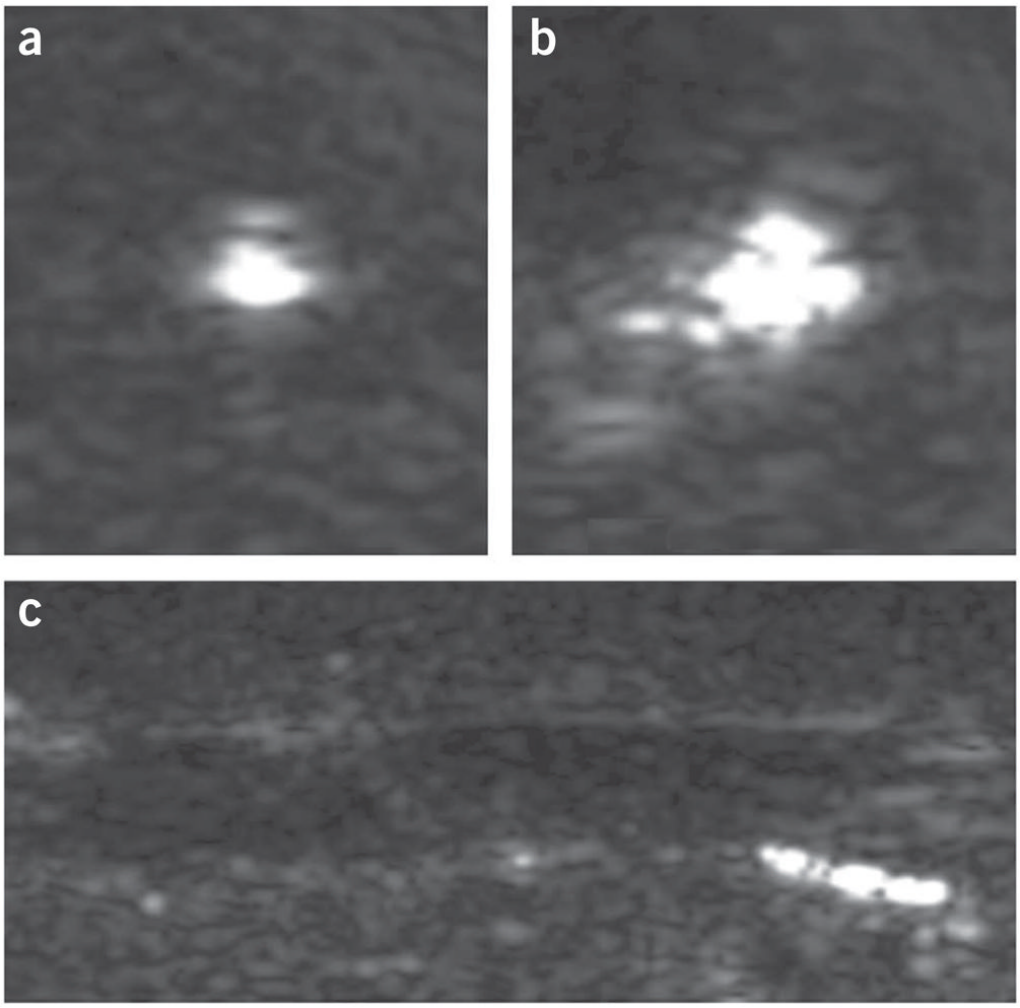

Figure 6.
Ultrasound imaging of PFOB microcapsules. (**a**–**c**) Images showing one capsule in gelatin phantom (**a**) and five capsules in gelatin phantom (**b**). (**c**) US image of transplanted PFOB microcapsules in swine kidney. Adapted with permission from reference 12.

TABLE 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
5	Microcapsule surface is irregular	Swelling in saline bath is occurring before cross-linking with PLL	Add cations (i.e., 1 mM $CaCl_2$) to saline to prevent swelling during wash[34]
6	Contrast agent leaches out of microcapsule	Size of contrast agent is too small to be entrapped or is not water soluble	Incorporate contrast agent within liposome carrier before mixing with alginate
	Bloom from signal created by magnetocapsule is too large	The concentration of SPIO in capsule is too high	For field strengths greater than 1.5 T, reduce the concentration of SPIO
7, 11	Encapsulated cells show poor survival	Cell density in alginate is too low or too high	Make microcapsules with a range of seeding densities to identify what range is best for the cell of interest
		Cells need critical matrix components in gel material to maintain viability	Optimize alginate with additives for the particular cell type
		Cells kept in alginate solution too long prior to bead creation	Use smaller aliquots of alginate-cell solution
11	Cells are difficult to see during viability staining	Refraction of light within the capsule shell	Adjust the focus and magnification of microscope to obtain a focal plane wherein single stained cells are easy to visualize
16	Microcapsules teardrop shaped	Primary alginate concentration is too low	Increase alginate concentration
	Alginate stream sputters	Primary alginate concentration is too high	Decrease alginate concentration
	Needle frequently clogs	Cell spheroids or clusters are too large for needle	Use larger-gauge blunt needle
17	Microcapsules too large	Distance between needle and gelling bath too small, electrostatic charge too low or primary alginate layer too viscous	Increase the distance between the needle tip and the gelling bath or increase the voltage from the generator, or lower the concentration of the primary alginate
18	Microcapsules adhere to each other	Microcapsules are in contact prior to complete gelation due to collecting directly beneath the tip of the needle in the gelling bath	Place a sterile stir bar in the gelation bath and place it on a stir plate.