Published in final edited form as:

Lab Chip. 2020 October 07; 20(19): 3591–3600. doi:10.1039/d0lc00098a.

Microfluidic-Prepared, Monodisperse, X-ray-Visible, Embolic Microspheres for Non-Oncological Embolization Applications

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Abstract

Embolotherapy using particle embolics is normally performed with exogenous contrast to assist in visualization. However, the exact location of the embolics cannot be identified after contrast washout. We developed a novel, pseudo-check valve-integrated microfluidic device, that partitions barium-impregnated alginate from crosslinking solution, thereby preventing nozzle failure. This enables rapid and continuous generation of inherently X-ray-visible embolic microspheres (XEMs) with uniform size. The XEMs are visible under clinical X-ray and cone beam CT both *in vitro* and *in vivo*. In particular, we demonstrated the embolization properties of these XEMs in large animals, performing direct intra- and post-procedural assessment of embolic delivery. The persistent radiopacity of these XEMs enables real-time evaluation of embolization precision and offers great promise for non-invasive follow-up examination without exogenous contrast. We also demonstrated that bariatric arterial embolization with XEMs significantly suppresses weight gain in swine, as an example of a non-oncological application of embolotherapy.

Graphical Abstract

A multi-nozzle device incorporating pseudo-check valves rapidly generates monodisperse, spherical microbeads, impregnated with X-ray contrast agents, suitable for targeted embolic therapy.

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].
See DOI: [10.1039/x0xx00000x](https://doi.org/10.1039/x0xx00000x)

Introduction

Embolization is a minimally invasive treatment, frequently using particles, to block blood flow in the targeted arteries to prevent gastrointestinal bleeding^{1, 2} or to starve tumors of oxygen and nutrients.^{3, 4} Chemoembolization involves combining chemotherapeutics with particle embolics to target high doses of anticancer drugs to tumors and, thereby, substantially reduce systemic side effects.⁵ Conventional embolization therapy is usually performed under X-ray guidance, i.e., X-ray fluoroscopy or computed tomography (CT), to guide delivery of the embolic agents to the target regions. However, most clinical formulations of embolic agents lack imaging visibility. Thus, the location of the embolics is initially inferred by the visualization of the contrast agent. Over time, the contrast either washes out or dissociates from the embolic agents, leaving the radiolucent embolics untraceable. The lack of direct intraprocedural visualization of the embolics can potentially contribute to non-target embolization (NTE) or embolic reflux into adjacent organs. While some degree of NTE or reflux may be tolerable in conventional transarterial chemoembolization (TACE),⁶ sub-optimal treatment, i.e., insufficient quantities of embolics, in non-oncological applications (e.g., uterine fibroid embolization (UFE)), is often chosen rather than risk delivery to normal, healthy tissue. A direct consequence of under-embolization is the need for repeated interventions.⁷

The ability to directly visualize the embolics could facilitate the assessment of the completeness of embolization for increased efficacy, as well as minimize the unwanted NTE for patient safety.⁸ To address the lack of direct radiologic visibility, several radiopaque microspheres have been developed using processes such as encapsulation,⁹⁻¹¹ chemical precipitation,¹²⁻¹⁴ chemical conjugation of radiopaque moieties,^{8, 15, 16} or absorption.^{17, 18} However, these preparation methods are either complicated or yield non-uniform embolics that are too large for small vessel embolization. For example, two FDA-approved smaller radiopaque beads, DC Bead LUMI™ and LC Bead LUMI™, are highly variable in size (70-150 μm and 40-90 μm, respectively). For catheter-directed transarterial embolization, precise control of the microsphere size can be particularly important, because it is a strong determinant of embolic biodistribution and penetration depth in the targeted tissue.⁸ In particular, a new emerging technique for treating obesity, called “bariatric arterial embolization” (BAE),¹⁹ must achieve delivery of embolics to the fundus of the stomach to cause ischemia, without severe ulceration, and prevent NTE to adjacent critical structures, such as the esophagus, pancreas, and spleen.

Alginate hydrogel is a biocompatible and mechanically robust material that has been widely used for biomedical applications, including embolic therapy.²⁰⁻²⁴ The use of microfluidics to prepare alginate particles on the order of tens of microns with a narrow size distribution had been previously demonstrated.²⁵⁻²⁹ The simplest method is to rapidly crosslink the droplets by introducing them into a divalent cation solution (e.g. Ca²⁺ or Ba²⁺) as they exit the microfluidic device,^{30, 31} which tends to yield inhomogeneous and misshaped beads.^{24, 31, 32} Alternatively, crosslinking can be achieved by fusing the droplets with separately-prepared crosslinker droplets,³³ direct mixing of alginate and crosslinker streams prior to droplet formation,³⁴ or by infusing the continuous phase with gelation inducers (e.g., slowly-diffusing crosslinkers or compounds that activate crosslinker precursors).^{25, 35} Droplet

fusion methods typically require fine-tuning of timing to ensure synchronization and are consequently slow and sensitive to flow velocity fluctuations, while direct mixing is difficult to control without rapid gelation and nozzle clogging. While uniform beads may be prepared by separating the droplet formation and gelation steps, and inducing gelation only after droplet formation is complete,²⁷ the approach requires further handling steps including gelation, demulsification, centrifugation, and possibly additional crosslinking to achieve strong gels. It is also unclear whether the beads may form clumps as a result of droplet-to-droplet proximity.

To address these issues, we have developed a microfluidic device containing pseudo-check valves, for production of radiopaque, uniform, alginate microspheres. The pseudo-check valve partitions the crosslinker-containing oil and alginate streams, thereby limiting the chances of inadvertent clogging of the device. Our method generates the microspheres on-chip, and complete gelation is achieved as they exit the device, reducing the chance of clumping. By impregnating the alginate with 10% w/v barium sulfate (BaSO_4), the microfluidic device enables highly scalable, robust parallel generation of X-ray-visible embolic microspheres (XEMs) with uniform sizes. Because of their high radiopacity, these XEMs provide real-time feedback of location and distribution of the embolic particles with fluoroscopy, thus enhancing the precision of transcatheter arterial embolization.³⁶ The potential of these new XEMs in non-oncological applications is shown in otherwise healthy animals to prevent weight gain as an innovative and safe method to treat obesity.

Materials and Methods

All animal studies were approved by the institutional animal care and use committee at the Johns Hopkins University School of Medicine, which is in compliance with the Animal Welfare Act regulations and Public Health Service Policy and adheres to the National Research Council's Guide to the Care and Use of Laboratory Animals. Euthanasia was performed as recommended by the American Veterinary Medical Association Guidelines for the Euthanasia of Animals. All chemicals are purchased from Sigma-Aldrich, unless otherwise stated.

Microfluidic Device Design and Fabrication

We designed a two-frolyer microfluidic device consisting of three input channels for alginate, calcified oleic acid, and cross-linking solution, driven by independent pressure regulators. The custom-made microfluidic device contains three distinctly functional regions (Figure 1, Supplementary Figure 1): pseudo-check valves; droplet generation/weak crosslinking region (using calcified oil); and phase transfer and collection region (isopropanol rich, higher calcium concentration). By varying the channel dimensions and pressures in the oil and alginate channels, XEMs with target diameters as small as 20 μm , and up to 70 μm , with narrow size distribution, were successfully synthesized. The typical channel dimension was 80 μm in depth and width, and the nozzle cross section dimension was 30 μm x 30 μm to generate 50 μm beads.

Fabrication of the polydimethylsiloxane (PDMS, Ellsworth Elastomers, Germantown, WI) microfluidic device (Figure 1) was done using conventional soft photolithography as

previously described.³⁷ Briefly, for the fluidic layer mold, a positive 30- μm photoresist layer (SPR 220-7, Microchem Corp) was spin-coated onto a hexamethyldisilazane-treated silicon wafer. Valve pads were formed by exposing the resist at 5,000 mJ/cm^2 . After development, an SU-8 3050 layer was spin-coated, exposed at 350 mJ/cm^2 , and developed to provide 80- μm -tall main fluidic channels. The valve control mold was fabricated on a separate wafer using SU-8 3050 photoresist under the same conditions.

A thin PDMS layer (Sylgard 184, Ellsworth Elastomers, 15:1 base-to-curing agent wt/wt ratio) was spun onto the fluidic mold, while a thicker layer (7:1 base-to-curing agent) was cast onto the valve control mold, and baked for 7 and 12 minutes at 80 °C, respectively. The two layers were aligned and bonded before sealing with a glass coverslip using an oxygen plasma treatment.

Microfluidic Preparation of Microbeads

The pseudo-check valve between the alginate and oleic acid channels was initially pressurized at 10 psi (P_{valve}) with deionized water for closure. Calcified oleic acid was prepared by dissolving calcium chloride (2 g) in ethanol (10 mL), and then, mixed with oleic acid (10 mL). After 48 hours, phase separation occurred, and the ethanol-rich top phase was removed and calcified oleic acid was diluted 10-fold with oleic acid to give the final working calcified oil, which was introduced into the device at 20 psi (P_{oil}). Next, alginate (1 wt%, Pronoval UP LVG, FMC Biopolymer) was prepared in phosphate buffered saline (PBS) and delivered into the device at 25 psi (P_{alg}). Lastly, the crosslinking solution containing 70% (v/v) of isopropyl alcohol (IPA) and 7% (w/v) of CaCl_2 was introduced near the outlet at 5 psi for further crosslinking and oil removal, with IPA acting as a disinfectant as well. Microbeads were rinsed extensively with PBS before use.

Calcium Diffusion into Alginate Beads

To characterize calcium diffusion across the oil-water interface into the alginate gel, a fluorescent calcium dye (10 μM Fluo-4, Invitrogen) was incorporated into the 1 wt% alginate solution. Fluorescent readings (F) were then obtained as the beads traversed the microfluidic device. To calculate the concentration of free calcium, we use the formula

$$Ca_{\text{free}} = K_d \frac{F - F_{\text{min}}}{F_{\text{max}} - F}$$

where $K_d = 345 \text{ nM}$ for Fluo-4, and F_{min} and F_{max} are the fluorescence intensity measured for 10 μM Fluo-4 in PBS with no calcium and 1 mM calcium chloride, respectively. By monitoring the fluorescence intensity, we can estimate the concentration of free calcium in the gel matrix. The dissociation constant of calcium-alginate depends on the composition of the calcium. The dissociation constant reported for the alginic acid L-guluronan component (poly-GluA) and D-mannuronan (poly-ManA) is $2 \times 10^{-4} \text{ M}$ and $1 \times 10^{-3} \text{ M}$, respectively.³⁸ For the alginate used in our preparation (Novamatrix PRONOVA UP LVG, high-GluA), we estimated the dissociation constant to be $4 \times 10^{-4} \text{ M}$. Fluorescence images were acquired during the bead-generation process (QImaging Intensified Retiga mounted on Olympus IX-71 Inverted Microscope, FITC fluorescence cube; Settings for the camera on QCapture

software were as follow: exposure = 11.4 ms; intensifier gain = 3365; CCD gain = 10.5; and offset = 477) and processed with ImageJ (NIH, Bethesda, MD).

Preparation of XEMs

To prepare XEMs, barium sulfate suspended in PBS was sonicated for 15 minutes (Microson Cell Disruptor XL) to disrupt clumps of crystals, in order to minimize device nozzle clogging. The suspension was mixed with the alginate to yield a solution of 10% (w/v) of BaSO₄ in 1% (w/v) of alginate. This barium sulfate-alginate solution was then introduced into the alginate channel on the microfluidic device, as described above.

Characterization of XEMs Stability

The morphology of XEMs was examined by an inverted optical microscope (Nikon Eclipse Ti-U; Nikon Instruments, Melville, NY) and an environmental scanning electron microscopy (ESEM, FEI Quanta ESEM 200). The average size and size distribution were determined from optical images of randomly selected XEMs using NIS-Elements BR imaging software (version 4.12; Nikon Instruments Inc.). XEMs stability was determined based on size and shape changes after 0, 1, 3, 7 and 10 days in PBS, serum, or 10% serum at 37°C or after 18 months in crosslinking solution at 4°C.

In Vitro Radiopacity of XEMs

The sensitivity of XEMs detection was determined in a 6-well plate agarose phantom containing 0.5 to 5 µl of XEMs. Digital radiographs (Axiom Artis, Siemens; 48 cm field of view (FOV), 72 kV, 62 mA) and cone beam computed tomography (CBCT, 20s DR-Head DynaCT, 20 second rotation, 0.4° increments, 217° rotation, and 543 projections) were acquired to determine XEMs visibility. The radiopacity of XEMs relative to the iodinated contrast agent, iohexol (Omnipaque, GE Healthcare), was assessed in a 96-well plate phantom consisting of serial of dilutions of XEMs with agarose and serial of dilutions of iohexol with saline by a clinical multidetector CT (MDCT, SOMATOM Definition Flash, Siemens, 0.5 mm slice thickness, 17.7 cm² field-of-view, 512 × 512 image matrix, 80 keV/211 mAs and 140 keV/109 mAs energy levels). The radiodensity of each well was calculated using uniform regions of interest using the vendor software.

In Vivo Studies

To examine the visibility and embolization effect of XEMs *in vivo*, we performed renal and bariatric arterial embolization in healthy swine (Yorkshire, ~30 kg) under general isoflurane anesthesia. Details of the protocols are available in the Supplementary Information.

Renal embolization

We first evaluated and optimized the handling characteristics and visibility of XEMs relative to conventional embolic beads (Embozene, 300-500µm, Boston Scientific) in a renal embolization study where four pigs (acute: n = 2; chronic: n = 2) were subjected to selective renal artery embolization under X-ray fluoroscopy guidance (see Electronic Supplementary Information, ESI). For acute study, the superior pole of one kidney was selectively embolized with XEMs (suspended in saline) without exogenous iodinated contrast, while

the superior pole of contralateral kidney was embolized with conventional embolic beads mixed with iohexol contrast until five beats stasis (*i.e.* no flow over five cardiac beats) was achieved. After embolization, digital subtract angiogram (DSA) and CBCT images were obtained from both kidneys to confirm the success of the embolization. Animals were humanely euthanized, and the kidneys were harvested for gross and histopathological examination. For chronic studies, only XEMs were administered, followed by a CBCT without contrast and a DSA. Repeat CBCTs without iodinated contrast injections were acquired at approximately weekly intervals for three weeks post-embolization. At the final imaging study, a CBCT and an aortic DSA were obtained to assess the persistence of XEM radiopacity and embolization effect. The animal was then humanely euthanized for post-mortem histology.

Bariatric arterial embolization (BAE)

After optimizing the handling and imaging properties of these XEMs, we examined their embolization effect on BAE, an emerging novel treatment for obesity where NTE would be unacceptable.³⁹⁻⁴¹ Ten healthy growing swine were randomized to receive either XEMs infusion (acute: $n = 3$; chronic: $n = 4$) into one to three fundal arteries or a sham procedure ($n = 3$) using a minimally invasive, percutaneous approach as previously described (see ESI).⁴¹ In chronic studies, all swine were administered 40 mg of oral omeprazole daily from 3 days before to 21 days after BAE procedure. Upper gastrointestinal endoscopy was performed at one-week post-embolization to assess the effect of XEMs on the stomach mucosa using a standard adult gastroscope (Pentax, Denver, CO). At weekly intervals, the pigs were anesthetized, weighed, and a non-contrast CBCT of the stomach was obtained. The animals were humanely euthanized 4 weeks after BAE, and the stomach, spleen, pancreas, kidney, and liver were assessed grossly and histopathologically. After fixation, the stomach was scanned with CBCT to determine the distribution of XEMs prior to sectioning. Feeding of the animals was performed by caretakers without knowledge of the treatment arm, and analysis of the data was performed in a blinded fashion.

Histopathological Analysis

Hematoxylin and eosin (H&E) and trichrome staining were performed on the embolized organs and adjacent structures, which may have received NTE, to detect the presence, location, and integrity of XEMs and/or conventional embolic beads, as well as to determine whether inflammation was present. Images were taken using an inverted microscope (Nikon Ti-U) at 100x or 200x power field.

Results

Pseudo-Check Valves Improves Device Robustness

Microbead generation on-chip is typically based on microfluidic droplet generators, which are capable of generating monodisperse droplets. However, gelation of these droplets presents unique challenges, since the process is largely irreversible on chip, and can quickly lead to device failure. For example, microfluidic devices typically need to be primed to remove air from the channels. Doing so with components such as calcium and alginate will result in gelation, and clogging of the device unless extreme care is taken. In our design, the

alginate channels were isolated from the oil stream by the pseudo-check valves, thus allowing device priming without risking alginate channel contamination (Figure 1). Moreover, the pseudo-check valves also protect the production process from exogenous pressure shock, such as when the device is moved onto a microscope stage for inspection. This improves the robustness and duration of device operation to several hours.

In addition, conventional microfluidic preparation of microbeads is typically slow^{25, 31, 34, 35} and subjected to flow velocity variations,^{11,12,25} resulting in compromised device longevity and polydisperse size distribution of the microbeads. Our pseudo-check valves impart high fluidic resistance that ameliorates the effects of the complex flow patterns, in particular, when nozzles are in close proximity. This permits the use of 40 parallel nozzles in a single device, with each nozzle operating at 20–30 Hz, $>3 \times 10^6$ or ~ 0.2 mL/hr/chip of highly uniform, 50 μ m XEMs could be generated. While the current design employed 40 parallel nozzles, further scaling-up for large volume production, including the use of multiple chips in parallel, could be easily realized.

Slow Gelation in Calcified Oil Ensures Sphericity of Microbeads

The sphericity of the microbeads is an important property for catheter-guided embolization therapy,^{32, 42, 43} since irregularly-shaped particles have a much higher tendency to become interlocked, and clog up the delivery catheter, or fail to enter the smallest vessels.^{32, 44} Moreover, spherical spheres are less likely to activate the host immune response.²³ Because the alginate droplets are generated in our T-junction by side-shearing (Supplementary Video 1), they are almost teardrop-shaped on exiting the nozzle (Figure 2). If gelation occurs too rapidly, the non-sphericity becomes fixed.

We adopted an approach using calcified oil to induce gelation,³⁵ that gives the droplets ample time to equilibrate into a spherical shape, as the calcium slowly diffuses across the oil-water interface. Since the rate of gelation at the nozzle is determined by a competition between diffusion of calcium into the alginate channel, and convective flow of the alginate out of the nozzle, the slow diffusion of the calcium across the oil/water interface also minimizes gelation in the nozzles, and contribute to device robustness. Based on fluorescence measurements, the free calcium in the gel microbeads as they approach the exit of the device was approximately 200 nM. Using the dissociation constant of alginate calculated, it is estimated that a total of 6 μ M of calcium was incorporated in the gel. Yet, the reported calcium concentration in a gel soaked in 0.8% calcium chloride solution for 5 hours is approximately 25 mM.⁴⁵ As such, the gel microbeads have only been partially crosslinked by the calcified oleic acid. This is further corroborated by the clumping of beads at the outlet of the device if further crosslinking is not performed.

To rapidly and completely crosslink the microbeads, we used a 70% IPA solution containing 7% wt/v calcium chloride to break the phase boundary between the oleic acid and microbeads. Furthermore, this solution was introduced in a sheath flow configuration to maintain the microbeads in single-file (Figure 1), thereby preventing clumps from forming. As the microbeads come into contact with the high calcium solution, they cross the phase boundary one-by-one (Figure 1, Supplementary Video 2). We note that, while the calcified oil cannot fully crosslink the beads due to the limited calcium concentration, the partially

gelled surfaces act as a shell to prevent fusion, thus stabilizing the beads for further processing. If the calcium is omitted from the oleic acid, the droplets rapidly coalesce when they come into contact, yielding beads with much higher variability in terms of diameter and morphology.

Control of Microbead Diameter

Biodistribution of embolic beads is largely determined by their diameters, with smaller beads being able to occlude more distal and smaller vessels,^{39, 42, 43, 46} and, hence, providing finer control over which branches are targeted.⁸ However, the size of commercial preparations of embolics is often achieved by filtering, resulting in a large range of diameters, which makes biodistribution less predictable. One of the main motivations for using microfluidics to prepare the beads is to achieve small diameters with a narrow range of size distribution. However, the relatively low throughput of microfluidic systems necessitated the use of multiple generators operating in parallel. This could in turn increase variability in the beads, as the flow resistance (and hence, flow rates) at different nozzles are expected to be different due to fabrication variability.

Despite the use of 40 nozzles, we found that the beads formed were very uniform in size (Figure 3). XEMs prepared on a device designed for 50 μm beads were spherical, with diameters of $48.8 \pm 4.9 \mu\text{m}$ (Figure 3A-C). Optical microscopic and environmental ESM images confirmed that BaSO_4 crystals were uniformly distributed within the alginate matrix, and the XEMs were spherical with a smooth surface (Figure 3A, 3B).

Due to space constraints, our chip design has a small difference in resistance across the nozzles of around 2% (Supplementary Figure 1). Coupled with the aforementioned fabrication variability, the use of a single pressure setting to control the alginate flow rate across the 40 nozzles thus results in differences in the flow rate of alginate. To study the effects of these differences, we varied the flow rate of alginate from around 300 pL/s to 3 nL/s, and found that the bead diameter showed a weak dependence on the flow rate (exponent ~ 0.14). This result was also corroborated with COMSOL simulations of our system (Supplementary Figure 2).^{47, 48} Consequently, despite not having fine control over flow rates at each nozzle, we do not consider that the flow rate differences contribute appreciably to the size variation. Nevertheless, parallelization using symmetric structures, such as Christmas tree-like designs,⁴⁹ may improve microbead monodispersity.

In Vitro Characterization of XEMs

In current clinical practice, reflux and NTE is prevented by erring on the side of underdosing, which can lead to incomplete treatment. Thus, the ability to visualize the beads directly may not only facilitate the embolization procedure and improve efficacy by identifying tissue at risk of undertreatment, but also have the potential to enable delivery of precise quantities of embolic beads to the target location with minimal NTE.

Using conventional fluoroscopy and CBCT, as few as 0.5 μL XEMs were readily visualized in a phantom (Figure 4A, 4B). This corresponds to a cluster of 346 beads that can be visualized in an embolized vessel. On the other hand, to study the radiopacity of dispersed XEMs, which indicate how visible the XEMs are as they are flowing through the vessels, we

suspended the beads to different concentrations in agarose. A linear relationship between radiopacity and barium-impregnated XEMs concentration was demonstrated on MDCT (Figure 4C). Based on MDCT phantom studies, a concentration of 129 XEMs/ μ l would be equivalent to the radiopacity of a 10% dilution of 350 mg iodine/mL iohexol (Omnipaque, GE Healthcare) (Figure 4C). The XEMs were stable at 37°C in serum, IPA, and PBS for at least 10 days (< 10% change in diameter) and for 18 months when stored at 4 °C in the crosslinking solution ($52.3 \pm 8.6 \mu\text{m}$) with minimal clumping (Supplementary Figure 3), suggesting that they will be suitable for sustaining long-term occlusion of the target vessels.

***In Vivo* Visibility of XEMs and Embolic Effect**

Direct visualization of renal arterial embolization using 50 μm XEMs was observed in all four pigs without the need for exogenous contrast injections (Figure 5, Supplementary Video 3), while the commercial embolic beads were not visible on X-ray images. XEMs reflux was detected in one animal (Figure 5A-F). This demonstrated the importance of real-time feedback of imaging-visible embolic beads during delivery to assess and minimize the extent of NTE. In chronic studies, the visibility of XEMs on CBCT was persistent up to three weeks after renal delivery (Figure 5G-H). Post-mortem and histopathological examination of the kidneys revealed that XEMs remained intact three weeks post-administration (Figure 5I) with no appreciable fibrosis or inflammatory infiltrates (Figure 5J). Both acute and chronic renal studies demonstrated that XEMs distributed in the distal aspects of the arterial tree toward the kidney cortex (Figure 5I).

For the BAE studies, successful embolization of two or three arteries supplying the gastric fundus was achieved with $0.7\text{--}1.5 \times 10^6$ of 50- μm XEMs in all BAE animals (Figure 6A-E) with direct visualization of XEMs during the procedure (Figure 6C). One-week post embolization, endoscopic examination showed absence of gastric ulceration in animals that received XEMs in two fundal vessels (Figure 6F). In the pig that received embolization in three fundal vessels, gastric ulceration developed with diminished food intake at one-week post embolization (Supplementary Figure 6). This is attributed to the embolization of three vessels, which resulted in more extensive ischemic damage to the gastric tissue. This highlights the importance of controlled and targeted embolization in this application. All four BAE pigs gained only 3.0 ± 0.9 kg over four weeks compared to 6.3 ± 0.9 kg in control pigs ($P = 0.03$). XEMs were detected *in vivo* by CBCT up to four weeks post-administration (Figure 7A-D), as well as in post-mortem stomach sections (Figure 7E, Supplementary Figure 7). In addition, no evidence of NTE to the pancreas, spleen, liver, or distal esophagus was noted as determined by CBCT and histopathology.

DISCUSSION

Embolic therapy is an important part of the interventional radiologist's toolkit. However, despite its widespread use, more non-oncological applications could be envisioned if the means existed to minimize adverse events, such as NTE, and to assess post-procedure embolic persistence. As a result, efforts are being made to develop imaging-visible embolics.^{8, 11, 12, 15, 50} In particular, highly radiopaque embolics (corresponding to 10 wt % BaSO₄) can enable real-time monitoring using X-ray fluoroscopy without the addition of iodinated

contrast agents but must balance handling properties that allow administration without settling and smoothness that prevents catheter clogging.^{8, 36} Zeng *et al.* recently reported a one-step electrospray method for tantalum encapsulated microsphere production, which typically has a lower size limit of 100 μm for alginate bead stability, and demonstrated the X-ray visibility of these 330 μm microspheres in renal artery embolization in rabbits.¹¹ Wang, *et al.* adapted a microfluidic device for preparing radiopaque microspheres, with radiopacity conferred by BaSO_4 nanocrystals formed *in situ* via the reaction between Na_2SO_4 and BaCl_2 , and performed embolization of rabbit kidneys with the 250 μm microspheres.¹² The gradual diffusion of barium ions from the external bath into the microspheres sets up a concentration gradient, which forms BaSO_4 nanoparticles more densely at the edges than the interior regions. At higher concentrations of barium ions in the external bath, the resulting microspheres have excessive BaSO_4 on the surface, making them brittle. This dense shell of BaSO_4 can also limit diffusion of barium ions into the center of the microspheres, resulting in under-crosslinked centers and making them susceptible to rupture, which in turn yields irregularly-shaped particles that have poor embolic performance.¹² Consequently, the BaSO_4 concentration in embolics prepared with this method is limited to around 5 wt %, which is inadequate for fluoroscopic imaging.^{12, 36} By comparison, we suspend 10 wt % BaSO_4 directly into the alginate solution, ensuring that our XEMs are visible with X-ray fluoroscopy and CT both *in vitro* and *in vivo*. Sedimentation of the BaSO_4 crystals is negligible during the synthesis time of a few hours due to the high viscosity of the alginate solution. Compared with Wang *et al.*, we also demonstrate embolization using significantly smaller beads (50 μm vs. 250 μm), which could not be produced by an electrospray method, and allowed us to achieve embolization of smaller, more distal vessels as would be needed for effective bariatric embolization.⁵¹ Coupled with the real-time imaging feedback, this enables us to perform more selective embolization, which we have found to be important to avoid NTE or overembolization, which can result in ulceration in the case of BAE (Supplementary Figure 6).

Recently, commercially available radiopaque embolics composed of iodinated polyvinyl alcohol beads have been marketed, but require suspension in iodinated contrast agent during delivery to prevent rapid sedimentation, which prevents the real-time assessment of microsphere distribution similar to conventional non-opaque embolics.⁸ Thus, a clinical need for radiopaque embolics is recognized even for oncological purposes. While our method is able to prepare microbeads with much smaller size variation than commercially-available embolic microspheres, it is still somewhat larger than the 3-4% typical of microfluidic droplet generators.²⁵ This variability is attributed to two factors. Firstly, as upstream droplets pass by a downstream nozzle, they perturb the pinch-off process at the nozzle. This can be improved by greatly increasing the flow rate of the oil phase, though practical considerations like reagent consumption and volume capacity of the on-chip microbead collection region place certain constraints. Secondly, droplet generation in the presence of barium sulfate crystals was less stable compared with pure alginate, likely due to the presence of finite-sized particles, which complicate the fluid dynamics. This effect is likely to be exacerbated when preparing smaller microbeads, which necessitate the use of smaller channels. One way to address this might be to prepare large alginate droplets with low BaSO_4 content, that are then shrunken down to achieve the desired size and BaSO_4

content.^{28, 29} This approach can be used in conjunction with our device, and has the added advantage of preventing clogging of much smaller microchannels by BaSO₄ crystals, which will lead to device failure.

In addition, we have demonstrated a potential new application of XEMs—embolic therapy as a treatment for obesity—using standard clinical imaging equipment and devices where NTE would be unacceptable in an otherwise healthy individual. Although embolics are frequently used to prevent gastric bleeding, the concept of BAE as a means to treat obesity is relatively new.^{19, 39-41, 52} Since other organs, such as the spleen, pancreas, liver, and esophagus, share a common vascular supply with the stomach, NTE could result in significant morbidity. In fact, in a canine BAE study, Bawudun et al. found significant NTE to the liver with the use of a sclerosing agent for BAE.⁵² In a swine BAE model, smaller size conventional embolic beads were able to reduce weight gain, but 40%-100% of animals developed gastric ulceration away from the embolized area suggesting NTE.^{39, 41} More recently, using conventional embolics, early clinical trials reported inadvertent pancreatitis presumably related to NTE.^{19, 53} Because of the lack of imaging visibility of these embolic agents, neither study could directly assess the full extent of NTE.

The radiopacity of the XEMs in our studies was sufficient for direct visualization during the procedure without causing significant artifacts on CBCT. We verified that the stability and biocompatibility of the microbeads was high without evidence of XEMs destruction or foreign body reaction in both the kidney and stomach at three to four weeks post-administration, respectively. In addition, the persistent appearance of XEMs on CBCT provides a means to determine whether repeated embolization may be needed in a particular individual without the need for iodinated contrast administration. Nonetheless, additional studies will be required to determine the long-term stability of XEMs *in vivo* before moving to clinical trials.

Due to the flexibility of this microsphere generation method, multiple payloads, such as other contrast agents, drugs, radiolabels, or cells, could be envisioned by premixing the material with alginate before introduction into the microfluidic device. In case of encapsulation of active payloads, the high viscosity of the alginate maintains a homogeneous suspension without sedimentation during the synthesis process. Moreover, due to the small size of the microfluidic device and high throughput production, one could envision the encapsulation of drugs or cells could be performed just prior to delivery in the X-ray angiographic suite.

Conclusions

We have demonstrated a highly uniform, small embolic particle with X-ray-visibility that can be generated rapidly, on a robust microfluidic device. The beads provide sufficiently high radiopacity to be compatible with conventional minimally invasive imaging and delivery techniques. This new embolic particle has the potential to enhance the safety of existing embolic therapies, such as TACE and UFE, as well as expand the horizon of embolic therapies to the treatment of non-oncological applications, such as obesity, drug delivery, or even regenerative medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Judy Mickey for assistance with the animal studies and Robert A Anders for histology evaluation.

Conflicts of interest

This work was supported by NIH R21/R33 HL089029, NIH R01 EB017615, AHA 16SDG30500010, and Maryland Stem Cell Research Fund 2011-MDSCRFII-0043. AA is a founder of Surefire Medical, Inc. Surefire Medical provided devices for the animal studies. CRW and DLK have received grant support from Siemens Medical Systems. CWB, THW, HQM, and DLK are inventors of a patent application covering the microfluidic technology described in the study.

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Figure 1. Schematic illustration of the pseudo-check valve integrated microfluidic device. The pseudo-check valve (aqua)-integrated microfluidic device has three separated channels: the continuous phase channel (purple), discrete phase channel (red), and crosslinking solution channel (green). The insets are higher magnification of pseudo-check valve and crosslinking regions.

Figure 2. Diffusion of calcium into microbeads. (A) Alginate loaded with Fluo-4 fluoresces in the presence of calcium. As the alginate exits the nozzle, it is almost teardrop-shaped. After pinching off, the droplet attains a spherical morphology. As the droplets move away from the nozzle, the calcium gradually diffuses in, resulting in gelation. Higher intensity in the middle of the beads is due to longer imaging path length. (B) The average fluorescence signal of microbeads at different distances from the nozzle approaches a plateau as it nears the end of the microfluidic device. The estimated free calcium concentration is around 200 nM, corresponding to around 6 μ M total calcium, which is insufficient to form stable microbeads.

Figure 3. XEMs characterization. (A) Optical microscopic image showing the uniformity of generated XEMs. (B) Environmental SEM image of XEMs shows that the BaSO₄ crystals are fully encapsulated within the gel matrix. (C) Histogram demonstrating that the average XEM diameter, as determined from the optical images, is highly uniform ($48.8 \pm 4.9 \mu\text{m}$). Bars = 50 μm . (D)

Figure 4.

In vitro radiopacity of XEMs. (A) Anterior-posterior radiograph of a phantom containing 0.5-5 µL XEMs showing as few as 0.5 µL XEMs (*i.e.* 346 XEMs) could be detected. (B) Multiplanar reformat of cone beam CT (CBCT) of the same phantom as (a) showing the detectability of as few as 0.5 µL XEMs. (C) The plot of signal intensity (in Hounsfield units) versus iodine concentration or the number of XEMs calculated from the multidetector CT image of a phantom with a linear regression fit.

Figure 5.

Kidney embolization with XEMs. (A-F): Individual frames from a digital subtraction angiogram during selective renal artery injection of XEMs (without iodinated contrast agent) demonstrating the ability to visualize XEM injection (open arrows) and non-target embolization (closed arrows) due to reflux of the XEMs. (G, H) DSA-CBCT acquired immediately (G) and three weeks (H) after embolization of a selective artery in the superior pole of the kidney. The mask image of the DSA-CBCT is displayed in red to highlight the XEMs (arrows) relative to the reduced kidney perfusion from the subtracted CBCT shown in grey scale. (I) XEMs are grossly visible in the vasculature of the anterior pole of the kidney (arrows). (J) Individual XEMs (arrows) appear intact on trichrome-stained photomicrographs with little evidence of a foreign body reaction or increased fibrosis (insert) relative to anticipated changes from renal infarction.

Figure 6.

Bariatric arterial embolization with XEMs. (A) Pre-embolization celiac digital subtraction angiograms (DSA) of the stomach showing the vasculatures that supply the fundus. (B) Subselective angiogram of the left gastroepiploic artery (LGEA). (C) DSA during infusion of XEMs demonstrating the radiopacity of the embolic particles (without contrast) on X-ray fluoroscopy; inset is magnified view of XEM stasis in the vessel. (D) Post-embolization celiac DSA after XEM delivery demonstrating truncation of the vessel (open arrow). (E) Celiac DSA at 4 weeks post XEM administration showing persistent embolization of LGEA. (F) Endoscopic view of stomach showing no ulceration or gastritis

Figure 7.

(A-D) Axial view of cone beam CT (CBCT) images of the stomach immediately after embolization of the gastric fundus (A), one week (B), two weeks (C), and four weeks (D) after embolization showing persistent visualization of XEMs in the stomach (arrows). (E) Representative coronal reconstruction from CBCT of a paraffin-embedded fundal tissue at 4 weeks post-embolization with XEMs demonstrating the radiopacity of XEMs ex vivo.