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Synthesis and Characterization of Image-able Polyvinyl Alcohol Microspheres for Image-Guided Chemoembolization

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Abstract

Therapeutic embolization of blood vessels is a minimally invasive, catheter-based procedure performed with solid or liquid emboli to treat bleeding, vascular malformations, and vascular tumors. Hepatocellular carcinoma (HCC) affects about half a million people per year. When unresectable, HCC is treated with embolization and local drug therapy by transarterial chemoembolization (TACE). For TACE, drug eluting beads (DC Bead®) may be used to occlude or reduce arterial blood supply and deliver chemotherapeutics locally to the tumor. Although this treatment has been shown to be safe and to improve patient survival, the procedure lacks imaging feedback regarding the location of embolic agent and drug coverage. To address this shortcoming, herein we report the synthesis and characterization of image-able drug eluting beads (iBeads) from the commercial DC Bead® product. Two different radiopaque beads were synthesized. In one approach, embolic beads were conjugated with 2,3,5-triiodobenzyl alcohol in the presence of 1,1'-carbonyldiimidazol (CDI) to give iBead I. iBead II was synthesized with a similar approach but instead using a trimethylenediamine spacer and 2,3,5-triiodobenzoic acid. Doxorubicin was loaded into the iBeads II using a previously reported method. Size and shape of iBeads were evaluated using an upright microscope and their conspicuity assessed using a clinical CT and micro-CT. Bland and Dox-loaded iBeads II visualized with both clinical CT and microCT. Under microCT, individual bland and Dox loaded beads had a mean attenuation of 7904±804 HU and

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Conflict of interest

Ayele H. Negussie, Carmen Gacchina Johnson, Gert Storm, Karun V. Sharma and Bradford J. Wood do not have any forms of conflicts of interest. The mention of commercial products, their source, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products by the National Institutes of Health. The authors alone are responsible for the content and opinions of the paper. NIH and Biocompatibles UK Ltd, a BTG International group company have a cooperative research and development agreement.

11873.96±706.12 HU, respectively. These iBeads have the potential to enhance image-guided TACE procedures by providing localization of embolic-particle and drug.

Keywords

Image-able Beads; TACE; HCC; DC/LC Bead; CT; Image-guided drug delivery

Introduction

Embolization is a minimally invasive, catheter-based procedure used to mechanically occlude blood vessels feeding a tumor or other target tissue to obtain therapeutic benefit [1, 2]. There are numerous pathologies that can be treated with embolization therapy, including bleeding [3–6], vascular malformations [7–12], and renal and hepatic tumors (both benign and malignant) [13–15]. Embolizing agents rely on mechanical obstruction to occlude vessels and the choice of a given therapy depends on the material properties and the desired clinical outcomes.

The first embolic agent in clinical use was autologous blood clots which were readily available and biocompatible [16], however, quick lysis of the clot limited the durability of the occlusion and utility. There are currently numerous types of embolic agents available including coils (pushable, injectable or detachable), solid synthetic polymers in the form of particles and microspheres (e.g., PVA hydrogels, trisacryl gelatin (TGA), poly(d-l-lactide), polymethylmethacrylate-based (PMMA) hydrogels), natural polymers (e.g., gelatin sponge, wax, starch, alginate and albumin microspheres) and liquids (e.g., cyanoacrylate glue, alcohol, ethylene vinyl acetate copolymer solutions) [16–25]. PVA, TGA, and PMMA microspheres are symmetrical, smooth and available in precisely calibrated size ranges [12, 19, 26–29]. PVA microspheres with sulfonate groups (DC Bead®) or sodium acrylate groups (HepaSphere® and Tandem®), respectively, are commonly used as drug carriers for chemoembolization to treat patients with hepatocellular carcinoma (HCC) [30–32] and generically known as drug-eluting beads (DEBs).

HCC affects approximately 500,000 people worldwide, every year [33]. It is the second most common cause of cancer mortality [34]. The curative options that are currently available in clinics depend upon the stage of the disease. For example, surgical resections, liver transplant, radiofrequency ablation (RFA), and vascular occlusion followed by RFA are options for early stage tumors and yield improved survival [33, 35–42]. When these options fail, transarterial embolization (TAE), transarterial chemoembolization (TACE; i.e., infusion of a chemotherapeutic followed by embolization), or the use of drug-eluting bead transarterial chemoembolization (DEB-TACE) [35, 43–46] are typical choices for palliative treatment [33]. Since the early 1970s, TAE and later TACE have become common clinical practice [1, 27, 33, 44, 47–62] and have improved the survival of patients with unresectable HCC [44, 53–55]. For instance, Yamada, *et al.* reported TAE with gelatin foam treatment for 120 unresectable hepatoma patients resulted in a cumulative one-year survival rate of 44% compared to 7% with conventional chemotherapy alone [63]. Similarly, Konno *et al.*, in the early 1980s, introduced anticancer drug (SMANCS) in combination with Ethiodol® as

image-guided TACE, which resulted in 10–99% tumor size reduction in 90% of patients with unresectable HCC [64]. Recently, uniformly sized microspheres with high drug loading capacity have emerged in the clinic as DEBs. These microspheres can be used in bland embolotherapy or more commonly loaded with chemotherapeutics to create a drug delivery device [31, 32, 59, 65–69]. For example, DEBs can simultaneously occlude arterial blood supply and deliver chemotherapeutics locally to the tumor (DEB-TACE) [56, 59, 61, 67, 68, 70, 71] providing theoretical benefit in limiting recurrence or time to tumor progression. One major drawback, however, with the current therapies is the lack of imaging feedback regarding the embolic bead location and drug coverage. Such information could inform and optimize therapy. To address this shortcoming, herein we report the synthesis and characterization of image-able drug eluting beads (iBeads) for real-time localization of embolic particles and drug during DEB-TACE.

The embolic particles used in this work are based upon sulfonate-modified PVA microspheres (DC Bead[®]) which have been used clinically as embolization devices for the treatment of hypervascular lesions and vascular malformations [57]. Chemically, these PVA microspheres contain abundant chemically modifiable and reactive alcohol groups. Some of the free alcohol groups of the PVA chains have been modified to attach sulfonic acid bearing moieties and are used to sequester positively charged drugs (e.g., doxorubicin, irinotecan, topotecan) [58, 72–74]. In this study, a portion of the hydroxyl groups of the PVA chain was modified with a radio-dense species to make the microsphere detectable by X-ray.

Materials and Methods

Materials

DC Bead[®] intermediate (Acrylamido Polyvinyl Alcohol-co-Acrylamido-2-methylpropane sulfonate microspheres, dried to a free-flowing powder and untainted) was obtained from Biocompatibles UK Ltd, a BTG International group company, Camberley, UK. Anhydrous dimethyl sulfoxide (DMSO), 1,1'-Carbonyldiimidazole (CDI), 2,3,5-Triiodobenzyl alcohol, 2,3,5-Triiodobenzoic acid, N,N'-Diisopropylcarbodiimide (DIC), 1-Hydroxybenzotriazole hydrate (HOBt), 4-(Dimethylamino)pyridine (DMAP), Trimethylenediamine, Triethylamine (Et₃N), and anhydrous Dichloromethane (DCM) were purchased from Sigma Aldrich (Milwaukee, WI). Doxorubicin (Dox) was obtained from Bedford Laboratories[®] (Bedford, OH). De-ionized water (DI water) was obtained from in house filtration system (Hydro Inc. Rockville, MD).

General methods

Evaluation of iBeads with microscopy—The size and appearance of beads during various steps of synthesis and Dox loading were examined and imaged in a chamber slide (Electron Microscopy Sciences; ~150 ul bead and DI water suspension). Bright field images were acquired with a 5× objective on an upright microscope (Zeiss Axioimager M1, Thornwood, NY) equipped with a color CCD camera (AxioVision, Zeiss).

Phantom preparation—In order to assess the radiopacity, beads were suspended in an agarose matrix at bead concentrations (sediment bead volume percent) that is relevant for *in*

vivo applications. Bead containing agarose phantoms (0.5% w/v) with various concentrations (sedimented bead volume percent ranging from 0, 3.1, 6.2 and 12.5%) were prepared by adding a 1% agarose (w/v, in DI water) solution (at 55 °C) to an equal volume of bead suspension in DI water. The solutions were mixed while allowing the agarose to slowly gel (over ice), resulting in a homogeneous distribution of beads. [Note: The bead volume percent is sedimented bead volume due to gravity alone and does not account for aqueous solution between the packed beads or altered bead packing efficiency].

***In vitro* evaluation of radiopacity of iBeads with clinical and Micro CT**

Clinical CT—Clinical CT imaging and analysis of iBead containing phantoms was performed on a 256 Slice CT (Philips, Andover, MA) with the following settings to determine the attenuation: 465 mAs tube current, 80 keV tube voltage, 1 mm slice thickness, 0.5mm overlap. The average attenuation of an 80 mm² rectangular region in the middle slice of a given phantom was measured using OsiriX Software (V.5.02 64-bit).

MicroCT—MicroCT imaging and analysis of iBead containing phantoms was performed with a SkyScan 1172 high-resolution micro-CT (SkyScan, Konitch, BE) to evaluate the radiopacity of each individual bead, as well as, intra-bead distribution of iodine. The radiopaque microspheres were imaged at 5 µm resolution, 78 kV, 127 micro-Amps, using a 0.5 mm Aluminum filter. The average attenuation of individual beads was measured and reported as the mean and standard error (n=10).

Doxorubicin loading into iBeads and elution—iBeads were loaded with Doxorubicin (Dox) according to previously reported method [57, 58]. Briefly, 250 µl of thoroughly washed iBeads was immersed into 0.5 ml of Dox (20 mg/ml DI water) solution and shaken for 3 hours at room temperature. The Dox-loaded iBeads were used for subsequent experiments. Dox elution kinetics from iodinated beads (100–300µm and 300–500µm) were measured with a previously reported technique [58].

Experimental procedures

Method I: Conjugation of 2,3,5-Triiodobenzyl alcohol on to DC Beads (iBead I)

After washing the beads (**1**) (200 mg) with DMSO (3X 5 ml), the beads were allowed to swell in DMSO (20 ml) for 30 minutes at 50 °C. A portion of the beads hydroxyl group were activated by stirring the suspended beads with CDI (800 mg) (CDI: OH ≈1.2:1) in the presence of catalytic amount of triethylamine (0.12 equivalent) at 50 °C for 24 hours. The reaction mixture was cooled to room temperature and washed quickly with a cocktail of DMSO and DCM (1:1) and finally with DMSO alone. The washed beads were immediately transferred into a reaction flask containing a solution of 2,3,5-triiodobenzyl alcohol (971.7 mg) in DMSO (10 ml) and stirred for 24 hours at 50 °C (Scheme 1). The resulting product cooled to room temperature and was washed thoroughly with DMSO: DCM (1:1), followed by DMSO alone. Finally, the DMSO was exchanged with DI water (under continuous agitation) and the image-able beads thoroughly washed with saline and DI water consecutively. The clean beads (**iBead I**) were suspended in DI water until further analysis.

Method II: Conjugation of 2,3,5-triiodobenzoic acid on to DC Beads through trimethylenediamine spacer

Methods II a: Grafting a spacer on to the DC Beads (4)—The beads were activated as described in method I above (200 mg of beads and 1.3 g of CDI) and reacted with excess 1,3-diaminopropane at 50 °C for 24 hours (Scheme 2). The reaction mixture was cooled and washed thoroughly as mentioned in method I to give the intermediate (4). Conjugation of the trimethylenediamine was confirmed by the Kaiser test [75].

Methods II b: Conjugation of 2,3,5-triiodobenzoic acid with trimethylenediamine grafted beads (iBead II)—A solution of 2,3,5-triiodobenzoic acid (2.4 g) in DMSO (20 ml) was activated with DIC (604.5 mg), HOBt (634.2 mg) and DMAP (586.4 mg) for 30 minutes at room temperature and the trimethylenediamine grafted beads (4) were added (Scheme 3). The resulting reaction mixture was stirred at 40 °C for 24 hrs. After cooling, the beads (**iBead II**) were thoroughly washed as described in method I. Alternatively, **iBead II** was synthesized first by activating the 2,3,5-triiodobenzoic acid (TIBA) with CDI as follows: 40 g of 2,3,5-triiodobenzoic acid (TIBA) was dissolved in 100 ml of anhydrous DMSO. The compound was activated by adding 13 g of CDI powder under magnetic stirring. The solution gradually became turbid and viscous after about 30 to 60 min. The mixture was then added into the trimethylenediamine grafted bead suspension in 100 ml of DMSO. The suspension was stirred at 50 °C, over 24 hr and was protected from light. The beads were washed with diethyl ether and DMSO mixture, then by deionized water.

Results

Synthesis and radiopacity

Method I—The beads were successfully activated with CDI under very mild conditions and conjugated with 2,3,5-triiodobenzyl alcohol (Scheme 1). Microscopic image comparison of raw and 2,3,5-triiodobenzyl alcohol conjugated beads (iBeads) revealed that the bead size was slightly reduced (Figure 1).

The 2,3,5-triiodobenzyl alcohol conjugated bead radiopacity was assessed both in clinical and microCT. With clinical CT, visualization is based on radiopacity of the beads in an agarose phantom per a given bead volume. A 3.1% packed bead volume showed a mean attenuation of 26 ± 15 HU and increased to 41 ± 16 HU and 74 ± 25 HU as the packed bead volume increased to 6.2 and 12.5 %, respectively. With micro CT, individual beads had a mean attenuation of 619.96 ± 605.08 HU (n= 10).

Method II a—Trimethylenediamine was successfully grafted onto PVA beads as confirmed with Kaiser Test.

Method II b—The 2,3,5-triiodobenzoic acid was conjugated on the beads (**iBead II**) as confirmed by the disappearance of the bluish color upon performing the Kaiser Test. Alternatively, the reaction progress was monitored with HPLC by measuring the residual diamine in the reaction mixture after converting into N-(3-aminopropyl)-2-phenylacetamide

with benzoyl chloride. Trimethylenediamine concentration gradually decreased over a period of 15 hours.

Clinical and microCT—The radiopacity was assessed with both clinical and micro CT. With the clinical CT, visualization is based on radiopacity of the beads per a given bead volume. A 3.1% packed bead volume showed a mean radiopacity of 129 ± 33 HU and increased to 269 ± 53 HU and 444 ± 83 HU as the (**iBead II**) packed bead volume increased to 6.2 and 12.5 %, respectively. Using micro CT, individual bland and Dox loaded beads showed a mean attenuation of 7903 ± 804 HU and 11873.96 ± 706.12 , respectively ($n = 10$). Different bead size showed different iodine content and attenuation under microCT (Table 1).

Microscopic comparison of raw beads (Figure 1A) and beads yielded from Method II b (Figure 3A) and thereafter Dox-loaded (Figure 3B) suggests that the conjugated hydrophobic moiety (phenyl group) results in a decreased bead size.

Doxorubicin loading and elution—The bright red colored Dox solution was depleted within 3 hours of incubating with the radiopaque beads giving reddish beads with a Dox concentration of 40–80 mg/ml of beads similar to previously published for the raw beads [57, 58]. Dox elution profiles for iodinated beads with two different size populations (100–300 μ and 300–500 μ) and the smaller sized beads (100–300 μ m) exhibited slightly more release as previously reported (Figure 6) [58].

Discussion

In this study, we present a successful methodology for the synthesis of radiopaque PVA based microspheres (iBeads). The radiopacity of the microspheres was confirmed both by clinical CT and MicroCT. The hydroxyl functional group of the PVA microspheres were mildly activated with CDI and coupled with either 2,3,5-triiodobenzyl alcohol or trimethylenediamine. Diamine conjugated beads were iodinated with 2,3,5-triiodobenzoic acid (**iBead II**, Scheme 2) under both DIC and CDI conditions. The diamine was used as spacer between the PVA bead hydroxyl group and the conjugating 2,3,5-triiodobenzoic acid. The completeness of the conjugation was monitored by the Ninhydrin test as well as by elemental analysis for iodine content. Under both conditions, on average the iodine content of iodinated beads achieved 45.6% in dried state according to elemental analysis results. Direct conjugation of the triiodobenzyl alcohol on the activated beads resulted in low conspicuity compared to the triiodobenzoic acid which is conjugated through a trimethylenediamine spacer. The spacer may provide less steric hindrance between the bulky activating reagent (carbonylimidazole) and the triiodobenzoic acid so that more trimethylenediamine spacer reacts with the activated bead thereby providing a suitable environment for more triiodobenzoic acid to conjugate to the beads. Therefore, the individual radiopaque beads made of triiodobenzoic acid through a trimethylenediamine spacer had ~13 times greater attenuation under microCT than radiopaque beads made from the corresponding benzyl alcohol.

To examine the effect of bead size on iodine content and attenuation, the beads were sieved into different sizes (Table 1 & Figure 5) and were tested for solid content, iodine content and microCT attenuation, respectively. Although the iodine content in dry beads decreased with decreasing bead size, the attenuation was greater due to high density of the smaller bead size. Overall, the size of the iBead was smaller than the raw beads due to the hydrophobicity of the triiodobenzoic moiety which reduces the interaction of water molecules with the iBead [76].

Ideally, a chemoembolization agent is capable of providing occlusion of target vessels, high capacity drug loading, selective delivery of the drug to the tumor, and real-time imaging feedback of the embolic particle and drug location with 2D and 3D X-ray imaging [77, 78]. Since all clinically available embolic agents are radiolucent, complications like non-target embolization may occur undetected with the embolic agent alone. For this reason, chemoembolization procedures currently rely on the injection of a mixture of the embolizing agent with X-ray contrast medium [79, 80]. However, due to inadequate dispersion of the embolic material in the contrast media, the liquid media can traverse to the distal blood vessels more so than the embolic materials and as a result the fluoroscopic feedback this soluble contrast material provides misleading information about the location of the embolic material [81]. Instead, if the chemoembolic material has X-ray contrast property, the localization of embolic during and post embolization can be monitored and assessed in real-time with X-ray techniques. Such radiopaque embolic materials have been reported in the literature [6, 77, 78, 82–88], however, usually without drug loading capability. Recently, radiopaque beads and radiopaque Dox-eluting beads by mixing iodinated oil (Lipiodol®) with lyophilized PVA hydrogel beads were reported by our team and their use to chemoembolize swine liver and kidney was demonstrated [56, 70]. However, due to the lack of off-the-shelf availability, this approach might have limited clinical use.

In summary, we report the synthesis of iBeads based on a commercial embolization microsphere (DC Bead) after mild activation of the bead with CDI and conjugating the activated bead with 2,3,5-triiodobenzoic acid using 1,3-diaminopropane as a spacer to alleviate steric hindrance. This radiopaque bead may be visualized under clinical CT and can load a similar level of Dox as the bland beads. It may be noted however, that the Dox loading capability is highly compromised when this radiopaque bead is subjected to high sterilization temperature. We speculate that the high sterilization temperature may cause the hydrophobic moiety to orient itself in such a way that to obscure the sulfonate group responsible for driving Dox into the bead. Therefore, alternative chemistry or sterilization technique is required to solve this problem.

Conclusion

Radiopaque drug-eluting beads were successfully synthesized by direct chemical conjugation of a radio-dense triiodobenzyl species on the commercial bead. These imageable embolic beads are visible under clinical imaging conditions which enable real-time feedback of bead location and drug localization during and after DEB-TACE.

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Figure 1. Size and appearance comparison of (A) raw bead and (B) iBead II under light microscopy. Note: conjugation of 2,3,5-triiodobenzyl acid reduces the size and changes the appearance of the beads.

Figure 2.
Clinical CT (A) and Micro CT images (B) of iBead I

Figure 3.
Size comparison of raw (A) and Dox loaded (B) iBead II under Light microscopy. Note: Dox loading reduces the size of the beads.

Figure 4.

A) Clinical CT image of bland iBeads II (the green rectangle shows the measured area, 0.8 cm²), B) Micro CT images of bland and Dox loaded (C) **iBeads II**. For micro CT, 3.1%, 6.2% and 12.5% (v/v) iBeads II pack phantom was used.

Figure 5.
Comparison of iodinated bead size with their attenuation. The 70–150 μm beads have high attenuation than all other bead sizes ($p < 0.05$)

Figure 6.
Doxorubicin elution from iodinated beads (100–300µm and 300–500µm).

Scheme 1.

Mild activation of beads (**1**) with CDI and conjugation with 2,3,5-Triiodobenzyl alcohol. a)
DMSO and DIEA, $t=50\text{ }^{\circ}\text{C}$

Scheme 2.

Grafting the trimethylenediamine onto CDI activated beads (2) and conjugation of 2,3,5-triiodobenzoic acid, a) DMSO and DIEA, $t=50^\circ\text{C}$, b) DMSO, DIC, HOBt and DMAP or c) DMSO and CDI.

Table 1Solid content, iodine content and μ CT attenuation of **iBeads II** with different sizes

Bead size (μm)	Solid content of hydrated beads (%)	Iodine content of dry beads (%)	MicroCT Attenuation (HU)
70–150	27.2	41.9	9758 ± 1476
100–300	24.8	44.3	8037 ± 1142
300–500	23.7	45.1	8243 ± 1240
500–700	23.0	45.6	7326 ± 773