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● *Original Contribution*

ADVANCED CHARACTERIZATION AND REFINEMENT OF POLY N-BUTYL CYANOACRYLATE MICROBUBBLES FOR ULTRASOUND IMAGING

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Abstract—We aimed to develop and characterize poly n-butylcyanoacrylate (PBCA) microbubbles (MBs) with a narrow size distribution. MBs were synthesized by established emulsion polymerization techniques, size-isolated by centrifugation and functionalized for molecular imaging by coating their surface with streptavidin. The physical and acoustic properties of the parent solution, different-size isolated populations and functionalized MBs were measured and compared. As expected from negative zeta potentials at pH 7, cryo scanning electron microscopy showed no aggregates. In phantoms MBs were destructible at high mechanical indices and showed a frequency-dependent attenuation and backscattering. The MBs were stable in solution for more than 14 weeks and could be lyophilized without major damage. However, for injection, small needle diameters and high injection rates are shown to be critical because both lead to MB destruction. In summary, when being handled correctly, size-isolated PBCA MBs are promising candidates for preclinical functional and molecular ultrasound imaging. (E-mail: jgaetjens@ukaachen.de) © **2011 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Contrast agents, Flow cytometry, Emulsion polymerization, Microbubbles, Poly n-butyl cyanoacrylate, Molecular imaging, Streptavidin, Bioconjugation.**

INTRODUCTION

Ultrasound contrast enhancement by gas microbubbles (MBs) has been known since 1968 (Gramiak and Shah 1968). Contrast enhancement by these MBs is caused by high acoustic backscattering resulting from the acoustic impedance difference between the gas in the MBs and the tissue. The compressibility of MBs also causes nonlinear backscattering, which can be used for contrast-specific imaging. Over the years, engineering of gas MBs has led to their stabilization by shells made of proteins, phospholipids or polymers.

The shell serves the purposes of:

1. Optimizing their pharmacokinetic properties, *e.g.*, increasing the circulation time
2. Preventing the MBs from coalescing, which could lead to undesired physiological side effects (*e.g.*, embolisms)
3. Presenting a site for modification and conjugation of target specific ligands
4. Optimizing the acoustic response of the MBs for contrast specific imaging

In a bid to further increase the *in vivo* stability, low-solubility gases, mostly perfluorocarbons, have been used in place of air as the MB core (Cui et al. 2005).

Gas-encapsulated MBs can be synthesized by either mechanical agitation, sonication or the use of microfluidic devices. Although mechanical agitation and sonication are the preferred methods to generate gas-encapsulated MBs, they generate highly polydisperse populations. MBs with a narrow size distribution have been mostly produced by microfluidic techniques (T-junction) (Pancholi et al. 2008), electrodynamic atomization (Farook et al. 2009), flow focussing (Talu et al. 2008a) and ink jet printing (Böhmer et al. 2006). Although these techniques produce MBs with a very narrow size distribution, they are more expensive and produce much less MBs per unit time compared with mechanical agitation and sonication. Alternatively, the isolation of a narrow size distribution of MBs from polydispersed parent solutions has been achieved by flotation (Kvale et al. 1996) and by differential centrifugation (Feshitan et al. 2009). Imaging with size-isolated MBs

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is advantageous because the size influences important parameters such as their resonance frequency, backscatter intensities, pharmacokinetics, and biodistribution.

Depending on their shell properties, MBs have been classified into hard- (mostly protein and polymer) and soft- (mostly lipid) shelled MBs (Kiessling et al. 2009). The stiffness of the shell is important because it defines the behaviour of the MBs in the ultrasound field and thus the contrast enhancement effect by the MBs. For example, a soft-shelled MB made of phospholipids will oscillate more to ultrasound pressure waves than a hard-shelled MB. This will lead to more nonlinear ultrasound backscattering by the soft-shelled MB compared with their hard-shelled counterparts. On the other hand, hard-shelled MBs are in principle easier to destroy by high mechanical index ultrasound waves, making them better suited for destructive imaging than soft-shelled MBs. Furthermore, they often have a higher *in vivo* (intravasal) stability, which is important for their use as molecular imaging agents.

In this study, hard-shelled (poly n-bultylcyanoacrylate [PBCA]) MBs with an air core were used. They have been reported to be excellent candidates for preclinical contrast-enhanced ultrasound perfusion imaging for cardiovascular applications and in cancer diagnosis (Puls et al. 2000; Palmowski et al. 2008a, 2008b). These MBs are biocompatible and *in vitro* experiments showed their degradation depends on pH value and temperature of the buffer solution in which they are stored. Enhanced degradation was also found in serum, most probably caused by enzymatic cleavage (Stein and Hamacher 1992; Olbrich et al. 2006).

PBCA MBs carrying streptavidin on their surface, to which biotinylated antibodies can be attached, have also been used for molecular imaging of rodents (Joseph et al. 2005; Palmowski et al. 2008a). Injection of these target-specific MBs *in vivo* leads to their accumulation in areas where the target markers are expressed. Quantification of the amount of bound MBs in a region gives a measure of the marker expression (Reinhardt et al. 2005; Siepmann et al. 2010).

However, PBCA MBs like every other MB synthesized by mechanical agitation or sonication, are polydisperse. Because of this, only a small amount of MBs will show suitable acoustic characteristics such as the optimal resonance frequency. For example, Hoff et al. (2000) reported an increase in resonance frequency with decreasing MB diameter for polymer-shelled MBs. Because MBs give a maximum backscatter signal at their resonance frequency, MBs with a resonance frequency around that of the transducer are desired. Furthermore, the pharmacokinetics, gas release profile, induced bioeffects, dose of injected MB to contrast enhancement ratio, as well as the biodistribution after intravenous injection of the MBs also depend on their size, thus necessitating the use of size-isolated MBs for biomedical applications (Talu et al. 2007).

Apart from the size distribution, a detailed (chemical, physical and acoustic) characterization of PBCA MBs has never been reported, which is important to improve their contrast enhancement effect and to optimize their binding kinetics, which is particularly crucial for molecular imaging.

Therefore, it was the aim of this study to develop and characterize PBCA MBs with a narrow size distribution for preclinical functional and molecular ultrasound imaging. After synthesis, they were size-isolated by centrifugation. The effects of centrifugation on the MBs' size distribution and on their acoustic properties (resonance frequency and backscattering) were investigated. The amount of streptavidin molecules on the surface of MBs functionalized for molecular imaging was also investigated by quantitative flow cytometry. If the development of MBs for clinical use is intended, their storability becomes an important quality characteristic, which is often neglected. The stability of PBCA MBs in solution as well as the effects of freeze-drying (for storage purposes) on the MBs were therefore analyzed. Their persistence during insonication and their stability when being injected through cannulae with different inner diameters at different velocities were also studied.

MATERIALS AND METHODS

Materials

n-butylcyanoacrylate (BCA) was purchased from Sichel Werke (Henkel Sichel Werke GmbH, Hannover, Germany), Triton X-100 and Polyvinylpyrrolidon (PVP) from Sigma-Aldrich (Munich, Germany), gelatine from Merck (Darmstadt, Germany) and Dulbecco's phosphate-buffered saline (PBS) was obtained from Lonza (Walkersville, MD, USA). Deionized water was used for all experiments and all chemicals were used without any further purification.

Microbubble synthesis

PBCA-stabilized air-filled MBs were prepared by established emulsion polymerization techniques as described in Palmowski et al. (2008b). After synthesis, the MBs were purified by flotation (which usually takes 6 d) and their size distribution measured using a Multisizer 3 (Beckmann Coulter GmbH, Krefeld, Germany).

Target-specific MBs for molecular imaging were prepared by coupling streptavidin to the surface of preformed PBCA MBs as reported in Joseph et al. (2005). In short, a 10% hydrolysis of PBCA MBs was performed using 0.1 N NaOH solution. 5×10^8 of the hydrolysed MBs were diluted in 15 mL sodium acetate buffer