REVIEW

Stable Small Composite Microbubbles Decorated with Magnetite Nanoparticles – A Synergistic Effect between Surfactant Molecules and Nanoparticles

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Abstract: Three approaches to preparing iron oxide nanoparticle-decorated microbubbles (NP-*deco*MBs) have been investigated. The size and stability characteristics of these microbubbles (MBs) were investigated by optical microscopy, laser light scattering and an acoustical method, and compared with those of non-decorated MBs. First, magnetite nanoparticles (Fe₃O₄NPs) grafted with dimyristoylphosphatidylcholine (DMPC) were synthesized and used to prepare MBs by brief sonication under an atmosphere of air saturated with perfluorohexane. These MBs had a rather large mean radius ($r \sim 12 \mu\text{m}$), and a moderate volume of encapsulated gas. Remarkably, a second approach that consisted of dispersing unbound DMPC molecules in the aqueous phase along with DMPC-grafted Fe₃O₄NPs prior to sonication was found to drastically change the situation, allowing the obtaining of monomodal populations of much smaller ($r \sim 0.6 \mu\text{m}$) NP-*deco*MBs. The latter were echogenic and stable for at least 10 days at room temperature, without significant variation of their size characteristics. In a third approach, NP-*deco*MBs were directly prepared from dispersions of naked Fe₃O₄NPs in the presence of DMPC. The resulting NP-*deco*MBs suspensions consisted of broadly distributed bubble populations mostly containing two populations (with $r \sim 5$ and $\sim 15 \mu\text{m}$). Control microbubbles made of DMPC only were small ($r \sim 1.3 \mu\text{m}$), although not as small as those formed from DMPC-grafted Fe₃O₄NPs in the presence of free DMPC, and were less stable, with a room temperature half-life of only ~ 1 day. These observations imply that there is a synergy between the Fe₃O₄NPs and the DMPC molecules in the air/water interfacial film stabilization process.

Key words: phospholipid, magnetite, iron oxide, fluorocarbon, diagnostic, drug delivery, ultrasound

1 INTRODUCTION

Microbubbles (MBs) have extensive applications in the food industry, in microfluidic technologies and in medicine for both diagnostic and therapy. Carbon dioxide MBs are generated in beverages to stimulate sensations on the tongue^{1, 2)}. Oscillating MBs facilitate the mixing of two fluids by producing microstreaming³⁾ and lead to higher efficiency of directional flow transport in microfluidic devices⁴⁾. In medical diagnosis, MBs are used as effective contrast agents for ultrasound imaging^{5–11)} and have broadened the applications of ultrasound. For example, recent ultrasound molecular imaging relies on MB agents for the purpose of detecting intravascular targets^{12–15)}. Diverse therapeutic applications of MBs are also being de-

veloped. Therapeutic agents can be incorporated into MBs for the purpose of drug delivery and gene therapy^{16–21)}. Moreover, the unique sonoporation phenomenon resulting from MB resonance creates transient pores in cell membranes, allowing efficient transportation of therapeutic agents into tissues^{17, 18, 21)}.

The size and stability characteristics of MBs play a crucial role in their applications^{18–21)}. Size control is essential for both diagnostic and therapy applications¹⁸⁾. There is also an imperative need for improving the *in vivo* stability of MBs for drug delivery: in the bloodstream, MBs need to survive long enough to allow effective amounts of drug to reach the targeted area¹⁹⁾.

With the development of nanotechnologies, nanoparti-

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cles (NPs) offer the possibility of better adjusting and controlling the characteristics of MBs. Recently, some work was aimed at stabilizing MBs using NPs^{22–31}. Although relatively stable MBs have been reported, the sizes of these MBs were in the tens of microns, a size that needed to be significantly reduced in order to achieve the targeted medical applications.

In the present work, we investigate several approaches to stabilizing MBs using Fe_3O_4 iron oxide nanoparticles (Fe_3O_4 NPs) and dimyristoylphosphatidylcholine (DMPC). The MBs were prepared by brief sonication under an atmosphere of air saturated with perfluorohexane (*F*-hexane) and were characterized by optical microscopy, static light scattering and an acoustical attenuation method^{32, 33}. Fluorocarbon gases have the capacity for enhancing microbubble stability considerably through osmotic and co-surfactant effects^{6–8, 11, 13, 30, 32–35}. Further potential uses of fluorocarbons and fluorinated amphiphiles in the biomedical area are, for example, reported in Ref.^{36–39}. The nanoparticles used being magnetic, dual mode diagnostic (ultrasound and nuclear magnetic resonance) and other applications can be envisaged^{30, 31}.

2 EXPERIMENTAL

2.1 Materials

Dimyristoylphosphatidylcholine (DMPC, dry powder) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). *F*-hexane (C_6F_{14} ; purity >99%) and Pluronic F68 (polyoxyethylene-polyoxypropylene triblock copolymer; MW ~ 8300; purity >99%) were bought from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and used as received. Deionized water (electrical resistivity, 18 M Ω cm at 25°C) was produced by a Milli-Q gradient system (Millipore, Molsheim, France).

2.2 Preparation protocols for iron oxide nanoparticles grafted with DMPC

Magnetic iron oxide (Fe_3O_4) NPs (diameter ~ 10 nm) were prepared according to Ref.⁴⁰. They were washed with deionized water and centrifuged (Sigma 3K20, Sigma Laborzentrifugen GmbH, Osterode, Germany; 16000 rpm; 2 h). This procedure was repeated twice. The concentration of the final NP dispersions was 5 mg mL⁻¹. DMPC dispersions (50 mM) were prepared by sonicating the phospholipid (5 min at 30°C) in water. 1 mL of the NP dispersion was admixed with 2 mL of the DMPC dispersion. The pH was adjusted to 1.6 with an aqueous HCl solution (50% vol.). The resulting dispersions were placed into an ultrasonic bath (Bandelin Sonorex Digitec, Bandelin electronic GmbH & Co. KG, Berlin, Germany) at 60°C for 1 h, and then cooled to room temperature. The resulting nanoparticles were filtered, washed and centrifuged twice under the conditions mentioned above in order to remove non-grafted DMPC molecules. The effective grafting of DMPC onto the surface of the NPs was controlled using a Mid Infrared Spectrometer (Bruker Vertex 70, Ettlingen, Germany) equipped with accessory ATR diamond DuraSamPLIR II (Sensir Technologies, Danbury, USA). The DMPC-grafted NPs' size distribution in the aqueous dispersions was determined by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK).

2.3 Preparation of microbubbles

Four types of samples of microbubbles were prepared in aqueous solutions containing a final concentration of 5 mM of Pluronic F-68, the latter in order to facilitate dispersion and bubble formation (Scheme 1).

A first type of MB samples (Scheme 1A) was obtained by using aqueous dispersions of DMPC-grafted NPs. These dispersions were prepared by mixing 0.6 mL of a dispersion of DMPC-grafted NPs (NP conc. = 1.7 mg mL⁻¹) and 0.6 mL of deionized water.

In a second sample series (B), DMPC (150 mM) was dis-

Scheme 1 The nanoparticle-decorated microbubble (NP-decoMB) preparation routes investigated in this work.

persed in the aqueous solution prior to bubble preparation from the dispersions of DMPC-grafted NPs prepared as described above. A third series of samples (C) was prepared from naked iron oxide NPs, prepared as described above, and dispersed in an aqueous solution containing DMPC (150 mM). Finally, control microbubbles (D) were prepared from a dispersion of DMPC (same final concentration as A, B, and C), without NPs present.

The microbubble samples were prepared by applying the same sonication procedure to all the above dispersions (A to D). The sonicator (Vibracell sonicator, Bioblock Scientific, Illkirch, France) was equipped with a 3 mm titanium probe and operated at 20 kHz. 1.2 mL of the dispersion was placed into a glass tube (diameter 2 cm). The sonicator probe was positioned slightly above the bottom of the tube. The tube was maintained in a water bath at 25°C. The dispersion was pre-sonicated using power setting 5, duty cycle 40% for 30 s in ambient conditions. Dry N₂ was saturated with *F*-hexane by bubbling through three vials containing *F*-hexane (a liquid at room temperature), and the atmosphere above the dispersion in the sample tubes was flushed with *F*-hexane-saturated N₂ for 5 min. The sample was then sonicated for 15 s (power setting 5, duty cycle 40%). Each sample was immediately diluted with 10 mL of deionized water to obtain the aqueous MB test dispersion.

2.4 Microbubble characterization

Three to four droplets of bubble dispersion were placed into a concave glass slide, covered with a glass slide and observed with a Nikon Eclipse 90i microscope, one minute after preparation. The average diameters of the microbubbles were determined by optical microscopy (on 200 bubbles at least) by fitting the size distribution with a Log Normal function. Bubble size was acquired by analyzing optical micrographs with the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The size distribution of MBs was also characterized by optical light scattering with a laser diffraction particle size analyzer LS 13320 MW equipped with a universal liquid module (Beckman Coulter, Inc. Brea, CA, USA). Acoustical determination of the microbubble size distribution was also performed. The method is based on the attenuation of an acoustical pulse that propagates through the aqueous bubble dispersion. This method fits standard simple-harmonic resonator curves to measured attenuations to infer the size of the bubbles^{11, 32, 33}. A low-power emitter was used that avoids alteration of the bubble characteristics and stability. To determine the standard deviation (SD) and polydispersity index (PI) for the size distributions of bubble dispersions, Gaussian curves were fitted to the size versus volume graphs. The standard deviation is by definition half of the width ω of the curve at half of the mean value ($SD = \omega/2$). The polydispersity index is the standard deviation divided by the mean diameter $2r$ multiplied by 100 ($PI =$

$SD/2r \times 100$). Each measurement has been repeated three times on three to five different bubble preparations.

2.5 Microbubble stability

The stability of the microbubbles stored at 25°C has been monitored by optical microscopy, static light scattering and acoustical attenuation measurement.

3 RESULTS AND DISCUSSION

3.1 Characterization of the DMPC-grafted nanoparticles

The infrared absorption spectrum of the DMPC-grafted NPs (Fig. 1) shows clear differences with the absorption spectra of both naked Fe₃O₄NPs and free DMPC molecules.

The spectrum of naked NPs is relatively smooth and flat with a strong absorption peak around 890 cm⁻¹; the DMPC spectrum displays several characteristic vibration bands (*sym* and *asym* CH₂ : ~2850 and 2920 cm⁻¹; C=O : ~1730 cm⁻¹, and *sym* and *asym* PO₂⁻ : 1246 and 1075 cm⁻¹). The absorption spectrum of DMPC-grafted NPs shows absorption characters of both components. Most importantly, it has an additional absorption vibration at 803 cm⁻¹ that results from the N–O bond. This indicates that in the aqueous solution at pH 1.6, the charged N⁺ on DMPC forms a chemical bond with the charged oxygens on the NP's surface. The IR data show that the DMPC molecules have indeed been grafted on the NPs.

The mean radius of the DMPC-grafted NPs measured just after preparation by dynamic light scattering (DLS) is $r \sim 18 \pm 11.2$ nm (PI 31%), indicating that only little aggregation has occurred (Fig. 2a). After 6 h, the nanoparticle size distribution has only undergone little change ($r \sim 24 \pm 16.3$ nm (34%); Fig. 2b), with a shift, however, toward the larger radius values. It is noteworthy that, in the absence of graft-

Fig. 1 ATR/IR spectra of a) naked Fe₃O₄NPs, b) DMPC-grafted Fe₃O₄NPs and c) DMPC.

Fig. 2 Particle size distribution of DMPC-grafted $\text{Fe}_3\text{O}_4\text{NPs}$, as determined by DLS, when measured immediately after preparation (squares), and b) after 6 h (triangles).

ing, irreversible and more extensive aggregation occurs due to magnetic attraction, as demonstrated by DLS⁽³⁰⁾. These results indicate that the DMPC-grafted NPs remain dispersed in the aqueous solution and that they are stable enough to be used for the preparation of microbubbles.

3.2 Characterization of the microbubble samples immediately after preparation

The microbubbles (MBs) prepared from aqueous dispersions of DMPC-grafted $\text{Fe}_3\text{O}_4\text{NPs}$ (Approach A) are shown in Fig. 3. Their size (optical microscopy) is quite large for the intended medical applications, with a mean radius r of $12.5 \pm 5.3 \mu\text{m}$ and a polydispersity index PI of 21%. These results were confirmed by static light scattering (SLS; $12.0 \pm 9.2 \mu\text{m}$). These data agree well with earlier reports on composite microbubbles obtained from surfactant-grafted particles^(22–25, 27). Attempts to reduce bubble size, for example by increasing the energy supplied to the system, were unsuccessful. In addition, the number of MBs produced (i.e., the volume of encapsulated gas) is small.

This originates from the fact that, when the bubbles are formed, the NPs diffuse only slowly to the interface due to their relatively large size (20 nm), which is much larger than that of the small phospholipid molecules.

By contrast, the dispersions containing both the DMPC-grafted $\text{Fe}_3\text{O}_4\text{NPs}$ and free DMPC (Approach B) produced a large amount of MBs (Fig. 4a). The size of these NP-*deco*MBs is very small, with a mean radius r of $0.55 \pm 0.40 \mu\text{m}$ (PI 36%; Fig. 4b). These exceptional small size characteristics were confirmed by SLS ($0.46 \pm 0.28 \mu\text{m}$; PI 30%; Fig. 4c). The size characteristics of these MBs were further confirmed by a third method based on the measurement of the attenuation coefficient of an ultrasound wave that propagates in aqueous dispersions of microbubbles ($0.67 \pm 0.46 \mu\text{m}$; PI 34%; Fig. 4d). The latter methodology also established that the new NP-*deco*MBs are echogenic.

It is noteworthy that the size of these MBs is much lower than that of MBs prepared with the DMPC-grafted NPs alone (Samples A, i.e. in the absence of free DMPC). The ability of the DMPC-grafted NPs and free DMPC combination to enable the formation of submicron bubbles reveals a synergy between the two components. The small DMPC molecules are indeed expected to diffuse rapidly in the aqueous phase and to stabilize the air/water interface immediately after its creation, allowing time for the larger grafted NPs to reach the interface.

Next, we investigated the effect of grafting DMPC onto the NPs on bubble characteristics. Therefore, naked $\text{Fe}_3\text{O}_4\text{NPs}$ dispersed in an aqueous dispersion of DMPC were used to produce microbubbles (Approach C). The resulting bubble distribution was more polydisperse than for those prepared from DMPC-grafted NPs, and their mean radius is larger (Fig. 5). In addition, many bubbles were seen to form clusters (not shown), likely due to aggregation of unprotected NPs.

Naked $\text{Fe}_3\text{O}_4\text{NPs}$ were not able to stabilize the interface efficiently enough so that microbubbles can be formed, likely because they tend to aggregate quickly in aqueous solutions, which would hinder their adsorption on the bubble's surface, since attractive forces among particles

Fig. 3 a) Optical micrograph of MBs prepared from dispersions of DMPC-grafted $\text{Fe}_3\text{O}_4\text{NPs}$; b) particle size histogram as determined from micrographs such as a); and c) static light scattering (SLS) profile.

Fig. 4 Microbubbles prepared from dispersions containing both DMPC-grafted $\text{Fe}_3\text{O}_4\text{NPs}$ and free DMPC. a) Optical micrograph; b) particle size histogram determined from micrographs such as a); c) SLS profile, and d) acoustical size determination.

Fig. 5 Microbubbles prepared from dispersions containing naked $\text{Fe}_3\text{O}_4\text{NPs}$ and free DMPC. a) Optical micrograph; b) particle size histogram determined from micrographs such as a).

are larger than those that drive them towards the bubble's surface^{41, 42}.

Finally, we compared the above NP-decorated MBs with MBs prepared from DMPC only (i.e. in the absence of any nanoparticles). The microbubbles that were obtained had a mean radius of $1.32 \pm 0.40 \mu\text{m}$ (PI 15%) and a relatively narrow distribution, in good agreement with the SLS data ($1.40 \pm 0.45 \mu\text{m}$; PI 16%; Fig. 6).

3.3 Microbubble stability

Size monitoring over time showed that the microbubbles prepared from DMPC-grafted $\text{Fe}_3\text{O}_4\text{NPs}$ (Approach A) and

from naked $\text{Fe}_3\text{O}_4\text{NPs}$ (Approach C) disappeared within 1-2 h. By contrast, those obtained from the DMPC-grafted $\text{Fe}_3\text{O}_4\text{NPs}$ /DMPC combination (Approach B) were considerably more stable. The evolution of their size was monitored by optical microscopy and SLS, and compared to that of microbubbles made from DMPC only.

After 1 day of storage at room temperature, DMPC-grafted $\text{Fe}_3\text{O}_4\text{NP}$ suspensions containing free DMPC (Approach B) maintained a constant density of bubbles (Fig. 7), with an unchanged mean radius ($r \sim 0.6 \mu\text{m}$). After ten days at room temperature, there was only a limited increase in bubble mean radius ($r \sim 1.10 \pm 0.86 \mu\text{m}$; PI: 39%), in agreement with SLS ($r \sim 0.90 \pm 0.57 \mu\text{m}$; PI: 31%).

The samples D that contain DMPC-only MBs experienced a marked decrease in bubble number after one day at room temperature. However, one should note that the bubble's mean radius remained essentially unchanged at $1.38 \pm 0.44 \mu\text{m}$ (PI 16%; Fig. 8). It is likely that the microbubbles shrink slowly down to a critical radius for which the DMPC coating can no longer adjust and is then expelled into the aqueous phase.

Taken together, these results indicate that using DMPC-grafted $\text{Fe}_3\text{O}_4\text{NPs}$ in the presence of free DMPC (Samples B) notably reduces the mean size of the NP-decoMBs, making them compatible with intravascular use. The stability of these MBs is also greatly improved, as compared with DMPC-only based MBs (Samples D).

Fig. 6 Optical micrograph of microbubbles prepared from dispersions of DMPC alone. a) Optical micrograph; b) particle size histogram determined from micrographs such as in a); and c) SLS profile.

Fig. 7 Optical micrograph of microbubbles prepared from dispersions of DMPC-grafted Fe_3O_4 NPs (samples B) after ten days at room temperature. a) Optical micrograph; b) particle size histogram as determined from micrographs such as in a); and c) SLS profile.

Fig. 8 Optical micrograph of microbubbles prepared from dispersions of DMPC only (Approach D) after one day at room temperature. a) Optical micrograph; b) particle size histogram determined from micrographs such as in a); and c) SLS profile.

The mechanisms that underlie the increased stability of grafted Fe_3O_4 NP-*deco*MBs (Samples B) include several aspects. First, the NPs attached on the bubble's surface hinder the diffusion of gases toward the aqueous phase²⁵⁾. Second, when two bubbles come in contact, the presence of NPs slow down the drainage of the liquid film between the two bubbles by increasing its viscosity⁴²⁾. This opposes the coalescence of NP-decorated MBs with other MBs. Third, the Laplace pressure within MBs ($P_L = 2\gamma/R$ (γ , surface tension of the liquid-gas interface; R , bubble

radius) increases as bubble size decreases. Nanoparticle coating mechanically improves the resistance of bubbles to the gas pressure that contrives MBs to dissolve in the surrounding liquid^{30, 43)}. Most importantly, bubble stabilization by particle adsorption can be more efficient than that obtained by surfactant adsorption because the energy of particles attached on a bubble's surface can be up to several thousand kT per particle (k , Boltzmann constant; T , absolute temperature), whereas the energy is only of a few kT per molecule for surfactants. Therefore, particle anchoring

on a bubble's surface is generally considered as being a quasi-irreversible adsorption⁴⁴. This is consistent with the experimental results obtained in the present work: the stability of NP-*deco*MBs notably surpasses that of surfactant-only coated MBs.

4 CONCLUSIONS

DMPC-grafted NPs have been prepared. NP-*deco*MBs prepared from these DMPC-grafted NPs along with free DMPC are small and stable for at least 10 days at room temperature. Their echogenic character is established. On the other hand, NP-*deco*MBs prepared from DMPC-grafted NPs alone are much larger in size and unstable. MBs prepared from naked NPs and free DMPC were broadly dispersed and unstable. Importantly, the size of the composite NP-*deco*MBs was significantly reduced when compared with surfactant-only based bubbles and with previously reported NP-stabilized bubbles. Lastly, the stability of the composite MBs was very significantly improved by NPs when compared with surfactant-based MBs. Contrary to the stability of less than 1 day of DMPC-only MBs, the tiny composite bubbles survived for at least 10 days without undergoing significant changes in size, coalescence or collapse.

In summary, combined use of DMPC-grafted NPs and free DMPC notably reduces the size of echogenic magnetite-decorated MBs, and strongly augments their stability. The outstanding properties of these composite MBs offer potential for wide use in medical applications.

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