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Production approaches for microbubbles loaded with nanoparticles

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***Abstract*—A new production approach to make microbubbles (MBs) loaded with nanoparticles (NPs) (Protocol 1) was evaluated and compared with the more common procedure that was based on covalent linking of functionalized NPs (f-NPs) to MBs (Protocol 2). MBs were produced by sonicating bovine serum albumin (BSA) and dextrose solution. NPs consisted of Cy5-PLA conjugate. Protocol 1 involved incorporating the NPs into the BSA-dextrose solution before the sonication step. Protocol 2 involved mixing MBs and f-NPs, resulting from mixing the initial Cy5-PLA and PLA-PEG-COOH conjugates. For each protocol, unloaded MBs were produced as a reference. Two parameters were quantitatively analyzed using both analysis of variance (ANOVA) and t-test: diameter was estimated using a circle detection routine based on the Hough transform while the number density was estimated using a hemocytometer. Both parameters were evaluated for the NP-loaded MBs and unloaded MBs at 5 time points (2 hours to 5 days post MB fabrication). Protocols 1 and 2 resulted in significantly different loaded MBs: the more common approach of linking functionalized NPs to the MB surface (Protocol 2) was much more efficient than directly embedding NPs in the MB shell.**

Keywords—Microbubbles; Nanoparticles; Functionalized nanoparticles; Temporal stability

I. Introduction

Ultrasound contrast agents are microbubbles (MBs) exhibiting diameters that range between 0.1 and 10 μm, making them suitable for purely intravascular circulation. Such MBs consist of a gaseous core stabilized by a shell comprised of lipids, proteins or polymers [1-4]. Currently, one intensely researched MB application is that of targeted vehicles. Ultrasound-mediated drug delivery faces major drawbacks, one of which is the poor MB loading capacity that results in an inefficient therapeutic delivery vehicle [5]. To address this limitation, several studies have been focusing on MBs loaded with nanoparticles (NPs). The most common protocol involves the use of biotin-avidin interactions to load NPs into the MB shell which is, however, unsuitable for clinical purposes as avidin can cause an immunogenic response [6]. To overcome this issue, functionalized NPs have been attached to the MB surface using carbomide chemistry that requires, however, extra care as it has to be done under mild reaction conditions to avoid MB destruction during the chemical coupling.

Another new approach would be to directly embed NPs into the MB shell [7]. Such a method has already been applied to load drugs into the MB shell but to our knowledge, it has not been investigated for NP loading. If successful, then the proposed approach would allow a straightforward, inexpensive and easy way to make NP-loaded MBs.

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II. Materials and Methods

A. NP-loaded MBs

Protocol 1 involved sonicating a solution of MBs and NPs to directly embed NPs into the MB shell. Briefly, the NP solution consisted of Cy5-polylactide (Cy5-PLA) NPs prepared by using the Cy5 as the initiator for the polymerization of lactide in the presence of (BDI-EI)ZnN(TMS)2. The initial MB solution consisted of 5% BSA (Sigma-Aldrich Co., St Louis, MO, USA) and 15% dextrose (Fisher Chemical, Fair Lawn, NJ, USA)[1]. NP and MB solutions were mixed, saturated with perfluorobutane gas (FluoroMed LP, Round Rock, TX, USA) and sonicated with a 20-kHz Fisher 500 sonic dismembrator (ThermoFisher Scientific, Walthman, MA, USA) using a 1.1-cm-diameter sonic horn for 70 s at 450 W (Fig. 1A, Protocol 1). Following this sonication protocol, we aimed to produce stable one-micron diameter MBs [1].

Protocol 2 involved mixing one-micron diameter MBs and functionalized NPs (f-NPs) for several hours to attach NPs onto the MB surface through a covalent link. Briefly, Cy5-PLA conjugate and polylactide poly(ethylene glycol)-COOH (PLA-PEG-COOH) conjugate were mixed and added dropwise to nanopure water. The resulting NP suspension was collected by ultrafiltration and washed with water. Cy5-PLA/PLA-PEG-COOH NPs were incubated in an aquous solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) for 15 min at room temperature. Separately, one-micron diameter MBs were prepared by saturating a mixture of 5% BSA and 15% dextrose with perfluorobutane gas. The solution was then sonicating following the same protocol as for Protocol 1. Thus, MBs and f-NPs were gently mixed for several hours to allow the covalent linking (Fig. 1B, Protocol 2).

For each protocol, a separate batch of unloaded MBs (u-MBs) was prepared following the same procedure as Protocol 2. Evaluation and analysis of loaded MBs were performed by quantitatively analyzing two parameters at 5 time points (hour(H)2, H6, H24, H48 and day(D)5): MB size and MB number density.

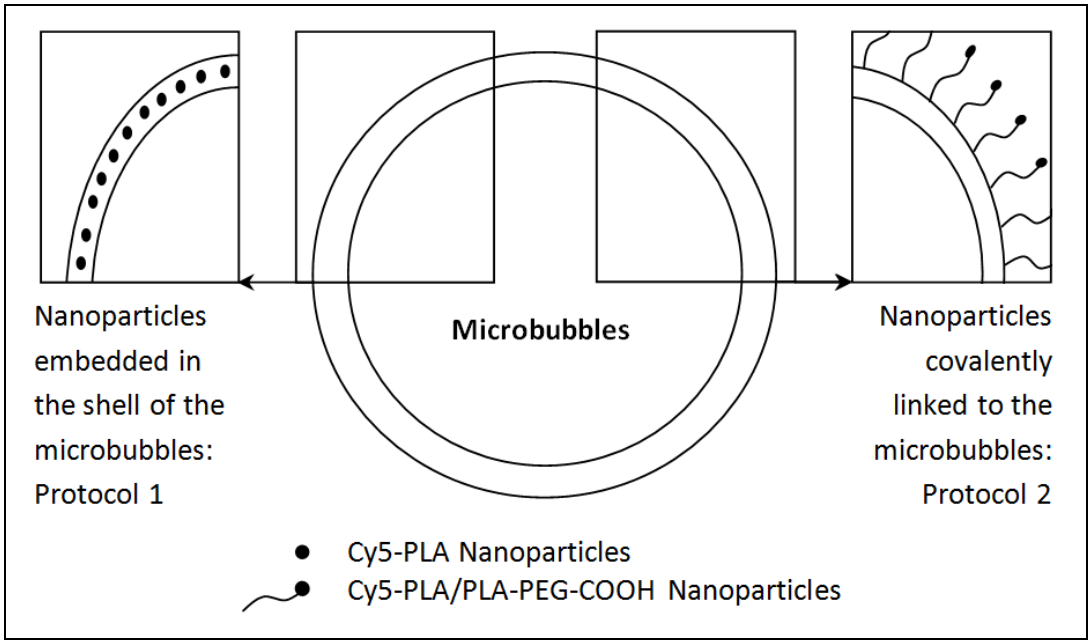

Fig. 1: Schematic representation of NPs-loaded MBs. NPs can be embedded in the MB shell (A) according to Protocol 1 or covalently linked to MBs (B) following Protocol 2.

B. Size evaluation

For each protocol, 10 optical microscope images (Olympus BX51, Tokyo, Japan) of the loaded MBs and u-MBs were acquired at each time point and 10 MBs were randomly selected from each image. MB size evaluation was performed using a circle detection routine based on the Hough transform from the 100 randomly selected MBs.

For each protocol, an analysis of variance (ANOVA) was performed on the size of the loaded MBs and u-MBs to assess temporal stability. p-values > 0.05 would suggest that the MB size was stable over time.

Also, for each protocol, MBs were assessed using a t-test performed on the loaded MB size versus the u-MBs at each time point. For p-values > 0.05, loaded and u-MBs were assumed to exhibit similar mean size at each time point.

C. Number density evaluation

For each protocol and for each group (loaded MBs and u-MBs), mean number density and its 95% confidence interval were evaluated using a hemocytometer (Hausser Scientific, Buffalo, NY, USA). The analysis was based on four separate count realizations.

For each protocol, an ANOVA was performed on the number density of the loaded MBs and u-MBs to assess temporal stability: p-values > 0.05 would suggest that the MB number density was stable over time.

In addition, as for the MB size evaluation, for each protocol, MBs were assessed using a t-test performed on the loaded MB size versus the u-MBs at each time point. For p-values > 0.05, loaded and u-MBs were assumed to exhibit similar mean number density at each time point.

III. Results

A. Size

Figure 2 shows plots of the loaded MBs and u-MBs mean diameters (A & C) for each protocol. Protocols 1 and 2 exhibited significant results: MB diameter was not stable over time when the 5 time groups were considered. However, when H2 was excluded, Protocols 1 and 2 MB sizes were not significant: MB diameter was thus assumed to be temporally stable from H6 (Fig. 2A & 2C).

In addition, for both protocols, the loaded MB size versus the u-MB one at each time point were not significant: loaded and u-MBs were assumed to exhibit similar mean size at each time point.

B. Number density

Figure 2 shows plots of the loaded MBs and u-MBs mean number density (B & D) for each protocol. Protocols 1 and 2 exhibited not significant results: independent from the protocol, loaded and u-MBs exhibited stable number densities over time (Fig. 2B & 2D).

In addition, for Protocol 1, the loaded MB size versus the u-MBs one at each time point were significant: u-MBs number densities were about 10-fold higher than loaded MBs (Fig. 2B).

For Protocol 2, the loaded MB size versus the u-MB one at each time point were not significant: loaded and u-MBs were assumed to exhibit similar number densities at each time point (Fig. 2D).

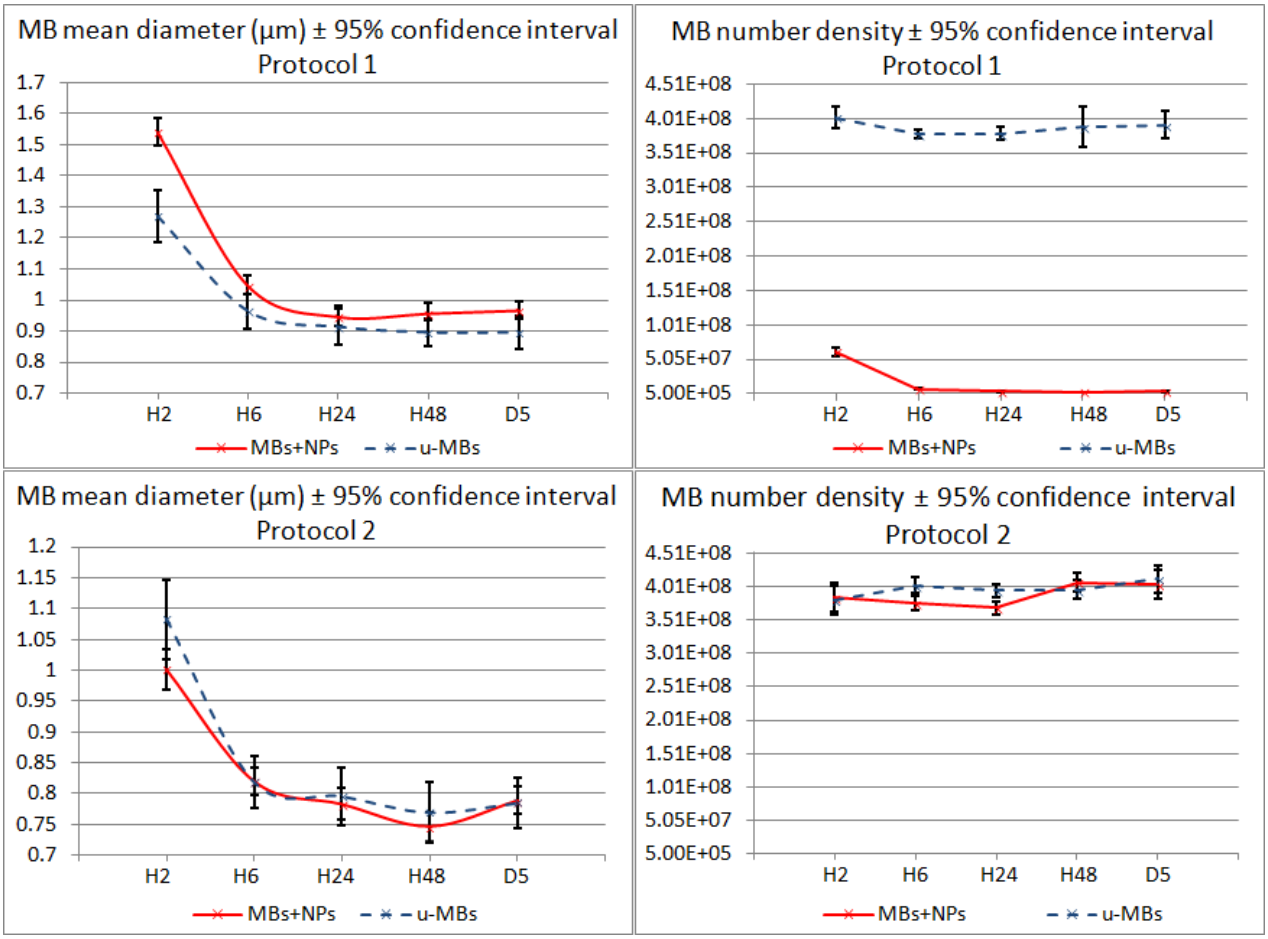

Fig. 2: Loaded and u-MB mean diameter (A & C) and number density (B & D) as a function of time for Protocols 1 (A & B) and 2 (C & D). Error bars represent 95% confidence intervals.

IV. Discussion and Conclusion

A. *Discussion*

MB size evaluation demonstrated that loaded and u-MBs achieved temporal stability several hours after their production. This delay appears to be related to the presence of larger sized MBs immediately following their production. Indeed, even if the approach to make MBs aims to produce mainly 1 µm MBs, it also produces larger and unstable MBs that tend to disappear a few hours after the production process is complete [1]. Also, independent of protocol, loading NPs did not affect the MBs size.

MB number density evaluation demonstrated that for Protocol 2, loaded MBs did not achieve a comparable number density as did Protocol 1. On the other hand, for both protocols, u-MBs were assumed to exhibit similar number: sonicating NPs lead to poor loading capacity. A possible explanation lies in the extreme conditions of the sonication step that can adversely affect the payload. In addition, Protocol 1 number density was markedly lowered by embedding NP into MB shell: loaded MB number density was 10 times less than u-MBs. Conversely, for Protocol 2, loaded and u-MBs achieved similar numbers density: attaching f-NPs at the MB surface did not lowered loaded MBs number density.

B. *Conclusion*

A new production approach to make MBs loaded with NPs (Protocol 1) was evaluated and compared with the more common procedure that was based on covalent linking of f-NPs to MBs (Protocol 2). Protocol 1 succeeded in loading NPs in MBs. However, NP-loaded MBs disappeared after several hours, making them unsuitable for future drug delivery studies: the more common approach of linking functionalized NPs to the MB surface (Protocol 2) was much more efficient than directly embedding NPs in the MB shell.

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