Research Article

Novel method for visualizing of the coated microbubbles using the electron microscopy

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Abstract

The microbubbles have many important acoustics applications, some of these applications are in medical uses such as ultrasound contrast agents and drug delivery. The efficiency and stability of the microbubbles are depending on many factors one of them is the structure of their shells. There is a significant need to visualize this structure to understand and optimize the bubbles behavior. Direct observation such scanning electron microscopy (SEM) and transmission electron microscopy (TEM) pose a big technical challenge because of the vacuum environment required by these devices. In addition, the freeze-fracture technique with TEM is long and expensive. In the present study, a novel modified method was used to overcome these challenges and consider a simple, low cost, and direct one to visualize the microbubbles with TEM.

Key words: Coated microbubbles , electron microscopy

INTRODUCTION

The microbubbles are consisting of gas core which is normally stabilized by a thin shell of protein, lipid or polymer layer. Compared to other particles, gas bubbles in liquids are unique as they are highly efficient scatterers of sound [1]. Because of this strong acoustic scatter, bubbles have many important acoustics applications; some of these applications are the medical ultrasound contrast agents [2] and drug delivery potential under ultrasound exposure. The advantage of microbubbles for a specific application is intrinsically related to their structure and stability, which in turn are highly sensitive factors in fabrication techniques of microbubbles. There are several models describe the dynamic behavior of these microbubbles under ultrasound filed. Most of these models need to determine the physical and functional qualities of the bubble and its shell such as the bubble diameter and its shell thickness (the shell thickness is usually in nanometer scale). So, there is a significant need for visualization of the microbubbles structure. Several methods have been applied to characterize these microbubbles, such as, optical and atomic force microscopy, but these methods are depends on indirect observation of the nanostructure of the coated microbubble shell [3]. The major challenge to imaging the microbubbles with SEM and TEM is the tendency of the microbubbles to collapse under vacuum. This challenge was overcome by using the freeze-fracture technique with SEM and TEM [4,5]. Another technique

was developed that allows observed the nanostructure shell of the microbubbles via SEM after drying and coating with a mixture of gold and palladium nanoparticles, but this technique is specific for bound microbubbles, the details of nanoscale surface could not be visualized by this technique [6].

Others were tried to use the TEM to confirm attachment of a gold labeled antibody to the surface of microbubble [7]. However, the microbubbles were not stabilized in the vacuum environment in this technique. On the other hand, another team of researchers were tried to used the freeze-fracture technique with TEM, they actually succeeded to visualized the nano structures of the shell and its surface [4,5,8]. However, it is an expensive technique and has not been commonly used to characterize the microbubble.

The aim of the current study was to use a conventional TEM to visualize microbubbles by adapting a modified method of sample preparation to concentrate the microbubbles on the electron microscope mesh by taking the advantage of their buoyancy.

PATIENTS AND METHODS

Using medical syringe, aliquot of a solution (Lipofundin® MCT/LCT 10%) has been selected to be a shell of the microbubbles and was transferred by injection into single use sealed glass vial which has been previously cooled and filled with "HFC-134a" gas. Coated microbubbles with gas-filled were formed by shaking the vial by high speed mixer unit (CapMix™) for one minute at 4,300 rpm at room temperature. The solution intended for microbubbles preparation was placed in the refrigerator at 5 °C to be ready for testing. The refrigerated microbubbles solution then is activated again at room temperature with a high speed mixer unit for approximately 30 sec and was left at rest for 5 minutes, this enable to removes the large bubbles by a flotation technique. A drop from the prepared samples was collected from the bottom of the vial. This drop was examined by optical microscopy under cover slip (Fig.1). The size distribution of the microbubbles seen by the microscopic image was calculated by using Image J software 1.50i (National Institutes of Health USA) which run under Windows operating system. This sample was served as control for the sizes distribution of microbubbles.

Another drop from the prepared samples was collected from the bottom of the vial and mounted in a similar manner to liposomes over two 3.05 mm copper grids of 200 mesh (Emscope laboratories Ltd.) which had been coated by "formvar film 15/95 E free flowing powder" (polyving formal. Gurr microscopy materials. BDH Ltd. Poole England) and gently cleaned by UV light for Approximately 5 minutes. The first mounted copper grid was examined under TEM at 80 kV (Phillips CM10) by conventional technique of liposomes [9].

The second mounted copper grid was turned upside down through the holder for approximately 2 minutes to allow a high concentration of microbubbles to float upside, coalesce and accumulate on the grid surface. Then the grids was negatively stained using incubation with drop of 4% weight per volume of uranyl acetate for 30 seconds. Re-inverted back and left over a filter paper for about one minute to dry. The grid was examined under TEM at 80 kV (Phillips CM10). Acquired images by TEM were at a magnification of 25000 and were filmed by Sony CCD camera.

RESULT

The distribution size of microbubbles obtained by Image J software image analysis was shown in table 1. This result shows that the mean of the diameter was 3.87 ±1.96 (SD).

Table (1): microbubble size distribution data.

Concentration[MB/ml]	Min diameter [um]	Max diameter [um]	Mean Dist. diameter [um]	Standard deviation [um]
$4 * 10^8 \pm 95000$	1	13	3.87	1.96

This result cannot directly compare with the others because of the specificity and the criteria of the solution used in the present study that was not used by other researches [10]. TEM images of the first non-inverted copper grid did not show any microbubbles that could be clearly recognizable (Fig. 2). Instead, various shapes and irregular structures were seen. This could be due to the collapsing of microbubbles under the vacuum of the TEM, which is in consistent with previous experiments and studies [9]. In contrast, the TEM images of the second inverted copper grid showed clear and sharp edge microbubbles (Fig. 3) that are consistent with the microbubble size distribution obtained from optical microscopy (Fig. 1). In addition, their nanostructures are clearly seen as indicated by the arrow in (Fig. 3).

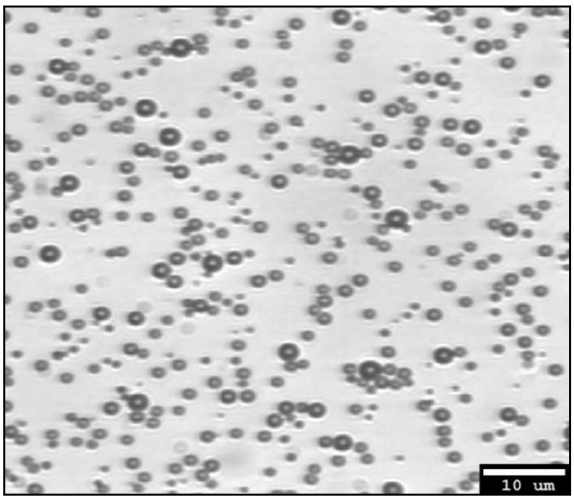

Figure 1: The image of the microbubbles obtained from optical microscopy

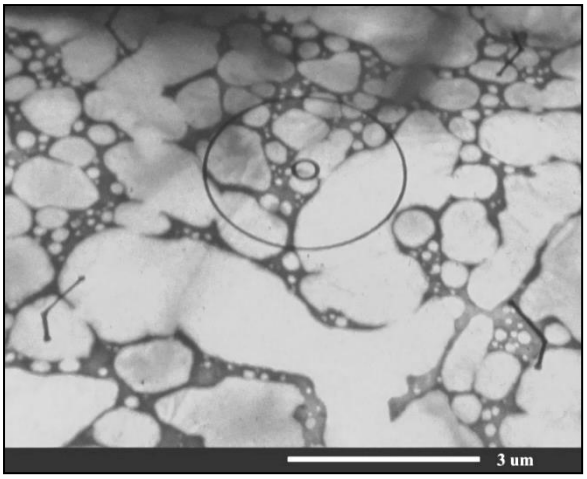

Figure 2: The TEM image of the non-inverted copper grids sample, the microbubbles could not be recognized. Instead, they appear as an irregular structures.

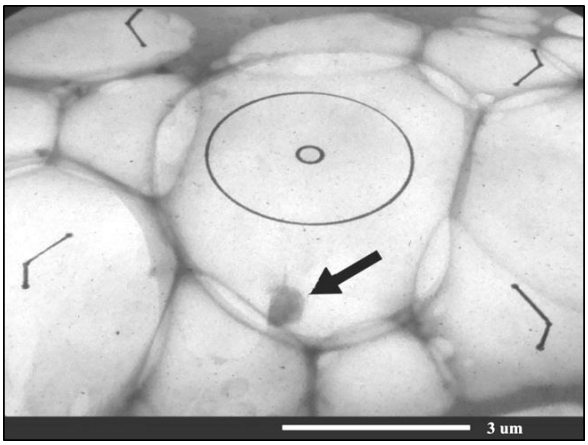

Figure 3: The TEM image of the microbubbles for the inverted copper grids sample. It's clearly showed the microbubbles that were resist the vacuum of TEM and accumulated on each other. Black arrow shows an area that could be residue of the staining with uranyl acetate

DISCUSSION

With this alternative modified technique, the microbubbles became stable and resist the vacuum environment of TEM and the nanostructure features of their shells became clearly visualized. Therefore, this technique has the potential to give a good perception of the microbubble structures and their validity. By inverting the sample and exploit the microbubbles buoyancy, high concentration of microbubbles are accumulated on the copper grid surface. Staining with uranyl acetate allows the microbubbles structure to resist the vacuum environment during TEM imaging.

In conclusion, the technique adapted by this study allows the nanostructure surface of the shell to be observed. This allows the nanoparticles which are embedded within the bubble shell to be directly visualized. This is a simple, low cost, and direct technique to characterize the nanostructure of the microbubble and its shell.

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