Structural Elucidation of Doxorubicin-Loaded Liposomes by Atomic Force Microscopy in air and water

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ABSTRACT

In this study, we are reporting the structural elucidation of doxorubicin-loaded liposomes (L-Dox) by atomic force microscopy (AFM) in water and air. Our results showed that liposome vesicles ruptured on the mica surface and resulted in Dox crystals exposed outside in water, enabling direct imaging by AFM. The height of such crystals was determined ranging from10 nm to 30 nm by the cross-section analysis. The first experimental proof of the hard nature of Dox crystals was confirmed in phase image by AFM. Consistent results were obtained in air scan when vesicles were dried. In conclusion, Dox crystals formed inside liposomes following encapsulation were visualized by AFM in water and air for the first time.

Keywords: Doxil, doxorubicin crystal, AFM, cancer, liposomes

Atomic force microscopy (AFM) is a powerful imaging technique to image and characterize nanoparticles, for example liposomes.[1,2] AFM has many advantages over electron microscopy since it does not require special sample processing and can be operated both in air and water, plus offering three-dimensional visualization.[1,2] AFM have been used to elucidate liposome-based structures in air, such as lipid-enveloped adenovirus[3] and functionalized QD-liposome hybrids.[4] AFM imaging of liposomes under fluid conditions is a very challenging task mainly due to their soft nature. So far, only several studies have attempted to image liposome vesicles in fluid, using tapping mode by AFM.[5-8] Very recently, two studies have pioneered to image more complicated liposome-based delivery systems in water, such as iron oxide-encapsulated liposomes and action-containing liposomes in fluid, using tapping-mode AFM.[9,10] Both studies have pointed out that the rupture of liposomes on mica in fluid resulted in the exposure of encapsulated entities outside.

Liposomes are one of the most-developed nanometer-scale drug delivery systems.[11] Liposomes encapsulating cytotoxic drugs have been widely used for the treatment of various tumors.[11-13] Liposomes (smaller than 200 nm in mean diameter) can preferentially accumulate in tumors *in vivo* due to enhanced permeation and retention effect (EPR).[11-14] Furthermore, the attachment of targeting ligands to the liposome surface facilitate their cellular uptake [15], resulting in a significant improvement in cancer therapy.

Doxil is a clinically used, FDA-approved liposomal product against cancer. Anticancer drug Dox is loaded into liposomses using the osmotic gradient technique, with high loading efficiency achieved over 95%.[16] Structural elucidation of L-Dox by cryo-EM showed that Dox formed crystal-like structures inside of liposomes.[17-19] Since 1992, cryo-EM has been the only technique used to visualize the Dox crystals inside of liposomes. The formation of such crystals has been reported due to the fact that Dox precipitates with sulphate inside of liposomes.[17] Li and co-workers pioneered to investigate physical state of Dox crystals inside of liposomes using cryo-EM and circular dichroism spectra, concluding that Dox crystals account for 99% of Dox molecules loaded inside of liposomes.[20] The formation of such crystals also lead to stable encapsulation of Dox inside of liposomes.[16] However, the physical properties of Dox crystals have to be fully studied yet and their residing inside of liposomes poses many difficulties.

In this study, we attempted to elucidate the structure of of liposomes encapsulating Dox by AFM. We first optimized the experimental conditions for both AFM in air and water to image PEGylated liposomes without leading to fusion between adjacent vesicles. Liposomes were deposited on mica surface and scanned using the tapping mode by AFM to avoid liposome damage. Our results showed that liposome vesicle deposition onto the mica surface resulted in Dox crystal exposure in fluid. The hard nature of Dox crystals was clearly shown in phase images where the height of such crystals was determined to range between 10nm to 30nm. The structure of liposomes scanned in air showed consistent results with that of in water scan. However, we found that the structure of liposomes in air was more dependent on the deposition time and the drying process applied. In conclusion, Dox crystals formed inside

liposomes following encapsulation were visualized by AFM in water and air for the first time. These optimized conditions in our study allow the structural elucidation of individualized liposome vesicles and indicate that AFM can be applied as a routine technique for the structural characterisation of soft nanoparticles such as hydrated lipid bilayer vesicles.

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