

# Soft Matter

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## COMMUNICATION

## Spatially Directed Vesicle Capture in the Ordered Pores of Breath-Figure Polymer Films

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**This work describes a new method to selectively capture liposomes and other vesicle entities in the patterned pores of breath-figure polymer films. The process involves the deposition of a hydrophobe containing biopolymer in the pores of the breath figure, and the tethering of vesicles to the biopolymer through hydrophobic interactions. The process is versatile, can be scaled up and extended to the deposition of other functional materials in the pores of breath figures.**

Breath figures, also known as honeycomb structures, are obtained when polymer films are fabricated through solvent evaporation under humid conditions.<sup>1,2</sup> The surface cooling created by the evaporating solvent leads to water droplet condensation onto the surface of the immiscible solvent. The droplets are stabilized by thermo-capillary convection and do not coalesce. Eventually after complete evaporation of solvent and water, the polymer film has an ordered honeycomb type structure with a dense polymer base. The ease of forming such microstructures without the need for microfabrication has led to several applications of breath figure morphologies in the development of photonic band gap materials,<sup>3</sup> sensors,<sup>4</sup> drug delivery,<sup>5,6</sup> templates<sup>7</sup> and scaffolds for cell culture.<sup>8</sup>

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To generate useful properties to the breath figures, the pore structure is designed to contain materials that generate functionality. This is usually done by mixing in an interfacially active functional material (polymers or nanoparticles) that segregates to the surface of the breath figure structure in the final phase of solvent evaporation.<sup>9</sup> We show in this paper, an alternate way to introduce relatively fragile self-assemblies such as liposomes into the pores of breath figure and keep them anchored to the pores. Such lipid based vesicles are of significant interest in a variety of drug encapsulation<sup>12,13</sup> and drug delivery technologies<sup>14</sup> as they are fully biocompatible<sup>15</sup> and easily taken up by cells.<sup>16</sup> Due to structural similarity with cell membranes, and their ability to encapsulate drugs and biomolecules and deliver such cargo to cells through fusion with cell membranes, liposomes are of vast application potential in drug delivery and as a cell model in biophysical studies of phospholipid bilayers.<sup>17</sup> While typically used in solution for their drug delivery potential in circulation, recent work has shown several applications of using immobilized liposomes. These include applications of liposomal coatings on biomedical devices for focussed drug delivery,<sup>18</sup> in single molecule spectroscopy and biosensing,<sup>19,20</sup> as nanocontainers in microfluidic systems<sup>21</sup> and in understanding the diffusion characteristics of phospholipids in cell membranes.<sup>22</sup>

Several methods to immobilize vesicles have been described in the literature, primarily using irreversible binding concepts including the use of DNA binding,<sup>21-23</sup> the biotin-streptavidin linkage,<sup>21</sup> biotin-avidin linkage<sup>24</sup> and various forms of covalent linkages.<sup>18</sup> The concept of patterning such attachment has been elegantly shown in the recent work by Hammer and coworkers<sup>24</sup> through the attachment of polymersomes onto microfabricated arrays using the biotin-avidin binding. In all these cases, the process involves functionalization to couple chemical moieties in the liposome hydrophilic head groups to

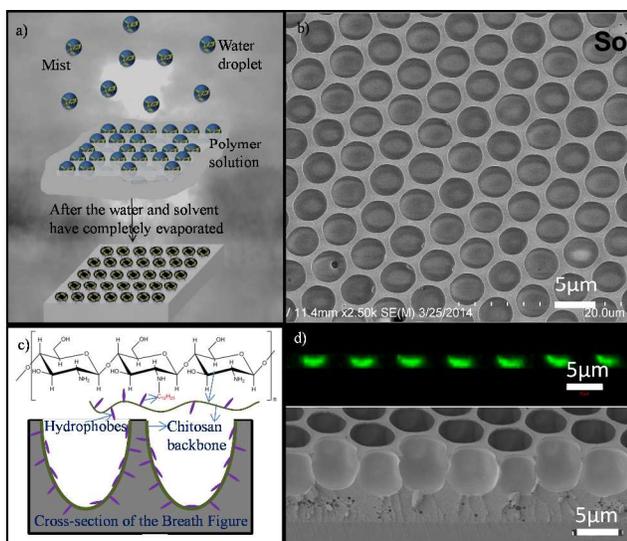


Figure 1: (a) Schematic indicating incorporation of hm-chitosan into breath figures. (b) Top view of polystyrene breath figures containing hm-chitosan. (c) Structure of hm-chitosan (top) and a schematic of the cross section of the breath figure pores showing hm-chitosan anchored to the pore surface. (d) (top) Compiled cross-section from the z-stack images of hm-chitosan in the pores and SEM image (bottom) of the pores containing hm-chitosan.

chemical moieties on the surface. In addition to being laborious from a scale-up perspective, the introduction of chemical moieties into the bilayer may lead to disturbance of the native bilayer structure.

In this paper we show a fundamentally different and extremely simple concept to tether liposomes to the surface of breath figure pores by exploiting the hydrophobic effect wherein long-chain alkyl moieties of polymer side chains insert themselves into the bilayers of liposomes.<sup>25-27</sup> The concept is most elegantly expressed in the application where a biopolymer chitosan, is functionalized with alkyl groups on a small fraction of its amine moieties (by covalent bonds), as shown in Figure 1c.<sup>25</sup> Such hydrophobically modified chitosans (hm-chitosan) have been shown to crosslink liposomes in solution leading to a dramatic gelation of liposomal solutions without affecting liposome functionality.<sup>25, 28</sup> In earlier work, we have shown that hm-chitosan coated flat surfaces are capable of anchoring liposomes.<sup>29, 30</sup> The technology described in this paper is based on the concept of placing a layer of hm-chitosan in the pores of breath figures to capture and tether intact liposomes. Combining the breath figure pore morphology with the vesicle tethering capability offers a simple way to pattern liposomes on a surface. While it is also possible to do this with lithography, the breath figure technique is very simple to implement. Additionally, the dual concept of fabricating the pores together with integration of hydrophobically modified chitosan (hm-chitosan) is novel and represents a one-step procedure of including functionality to the film. But perhaps the most significant advantage of the breath figure over a flat surface is the fact that the liposomes are exclusively within the pores and therefore inaccessible to cells that are too large to enter the pores. This may present a number of potential applications (i) to deliver nutrients to cells growing on the surface of the breath figure (ii) to exclusively capture extracellular smaller vesicular bodies such as exosomes.

The method we used therefore, was one where the water soluble hm-chitosan was introduced through the water droplets. This was done through an aerosol generated mist of water droplets containing hm-chitosan as shown schematically in Figure 1a. Briefly, the procedure involved the fabrication of breath figures films of polystyrene through spin coating over a glass coverslip substrate, using carbon disulfide as the solvent. Through an orifice located on the top of the spin coater, a nebulizer generated aerosol mist was introduced. The flow rate was measured using a compact shielded flow meter (Gilmont Instruments) to which the nozzle of the nebulizer was connected. The typical measured flow rates were between 35-40 ml/min and with the tube diameter (leading from the nebulizer nozzle) of 27.2mm, the superficial velocity of the mist was between 0.10045 – 0.1148 cm/s. All experiments were done at ambient temperature. The temperature of the evaporating film was monitored using an infrared gun (CENTECH Infrared thermometer). The entire process of spin coating takes 45 seconds during which the film surface temperature ranges from close to 0°C (initial solvent evaporation) to about 10°C when the film starts drying out. Similar temperature drops have been observed by Battenbo and coworkers<sup>3</sup> in fabricating breath figures of polystyrene initially dissolved in carbon disulfide. The polymer solution was exposed to the mist for 40 seconds before spinning. Following a 15 second programmed ramp to 2500 rpm, spinning was continued for another 30 seconds leading to reproducibility and consistency in the formation of a dry breath figure with the pore morphology shown in Figure 1. The films were extensively rinsed with DI water and 1% acetic acid to remove any free hm-chitosan, and further dried at room temperature prior to analysis and use. Further details of the aerosol process are described in the electronic supplementary information section (ESI), but we note that the precursor solution of hm-chitosan was maintained at slightly acidic conditions below the pKa of chitosan (6.5) to maintain polymer solubility during aerosolization. Since the hm-chitosan is in the water droplet, our hypothesis was that the polymer would deposit exclusively in the pores of the breath figure structure upon evaporation of all solvents. Figure 1b shows the breath figure formed with an aerosolized mist of hm-chitosan. To the best of our knowledge, this is the first instance of introducing water droplets through an aerosol process to form breath figures, rather than just exposing the evaporating polymer solution to humid air. The use of the aerosol process allows delivery of a variety of water soluble or water dispersible materials to the pores of the breath figure structure. However, to ensure adhesion of the deposited polymer in the pores, our hypothesis was that some of the hydrophobes of hm-chitosan would anchor to the surface of the hydrophobic polystyrene film as it was being formed. Figure 1c (bottom) is a schematic of the polymer layer in the pores showing the hydrophobes inserted into the polystyrene film.

To verify polymer retention in the pores, we used fluorescently labeled hm-chitosan and were able to clearly visualize retention of fluorescence even after washing the system repeatedly with 1% acetic acid to remove all free polymer. The protocols for

hydrophobically modifying chitosan and fluorescently labelling chitosan were adapted from the literature<sup>31, 32</sup> and are explained in detail in the ESI. The green fluorescence from fluorescein-labeled hm-chitosan is observed in the confocal images of Figure S2 (please refer to the ESI).

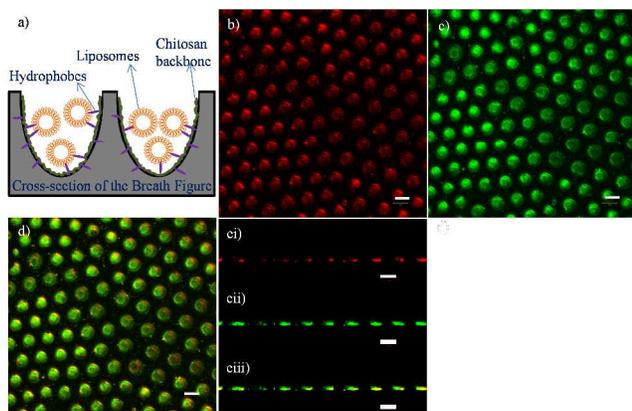


Figure 2a) Schematic of the vesicle capture Red fluorescent liposomes were incubated on breath figures made from green fluorescently labeled hm-chitosan. The corresponding confocal images are as follows:  
 b) Red fluorescence from the labeled liposomes  
 c) Green fluorescence from the labeled hm-chitosan  
 d) Both the green and the red fluorescence  
 e) The compiled cross section of the breath figure (from the z-stacks) showing the red fluorescent signal along the depth of the film  
 eii) Cross section image showing the green fluorescence  
 eiii) Cross section image showing both the green and red fluorescence signals. Scale bar in all images is 5 microns

Z-stack images along a row of pores in the (1,0) direction clearly show the presence of hm-chitosan exclusively in the pores (details of the z-stack images are in the ESI, Figure S2a-2d). The cross-section of the breath figures was compiled from the z-stack images in Figure 1d (top) and the width of the observed fluorescence is comparable to the dimensions of the pore width.

Subsequent to hm-chitosan deposition in the pores, the next step was the capture of liposomes, as schematically illustrated in Figure 2a. The liposomes were prepared by the thin film hydration method followed by extrusion through a 100 nm membrane to reduce polydispersity. DLS and cryo-TEM imaging shows liposomes in the 100 nm size range (details of the liposome preparation and the size distribution are in the ESI). To confirm the capture of liposomes, fluorescein (green) labeled hm-chitosan in the breath figure pores was incubated with a solution of neutral liposomes, tagged with a red fluorescent indocarbocyanine dye (DiI). The films were then extensively rinsed using DI water, followed by confocal microscopy analysis. The images in Figure 2 illustrate that the pores of the breath figure now contain liposomes. Thus, Figure 2b indicates the green fluorescence from the hm-chitosan, Figure 2c displays the red fluorescence from the liposomes and Figure 2d displays both the red and green fluorescent signals by the merging of both signals. Both sets of fluorescence from the liposomes and from the hm-chitosan

indicate localization within the pores of the breath figure suggesting capture of the liposomes by hm-chitosan. Repeatability was ensured by using multiple samples (>10) of breath figures with the fluorescence characteristics being consistent over all samples. Control experiments with native chitosan in the breath figures show no capture of liposomes.<sup>29</sup> The compiled cross-section from z-stack images is shown in Figure 2e, again illustrating the co-localization of liposomes and hm-chitosan in the cross-sectional frame of the breath figure. xy images at various z-values are shown in the Figure S3 (please refer to the ESI).

The aerosolized mist therefore represents a unique way to deposit water soluble functional polymers such as hm-chitosan into the pores of breath figures. An interesting aspect of vesicle capture through the hydrophobic interaction is the possibility of reversing the capture and releasing vesicles. Cyclodextrins interact strongly with the hydrophobes of hm-chitosan, sequestering them within their hydrophobic cavity.<sup>28, 33</sup> Recent work has shown that  $\alpha$ -cyclodextrins ( $\alpha$ -CD) compete effectively with vesicles for the hydrophobes of hm-chitosan, and this results in the breaking of vesicle gels formed by hm-chitosan upon adding  $\alpha$ -CD.<sup>28</sup> The schematic in Figure 3a is an illustration of the concept of vesicle release from breath figure

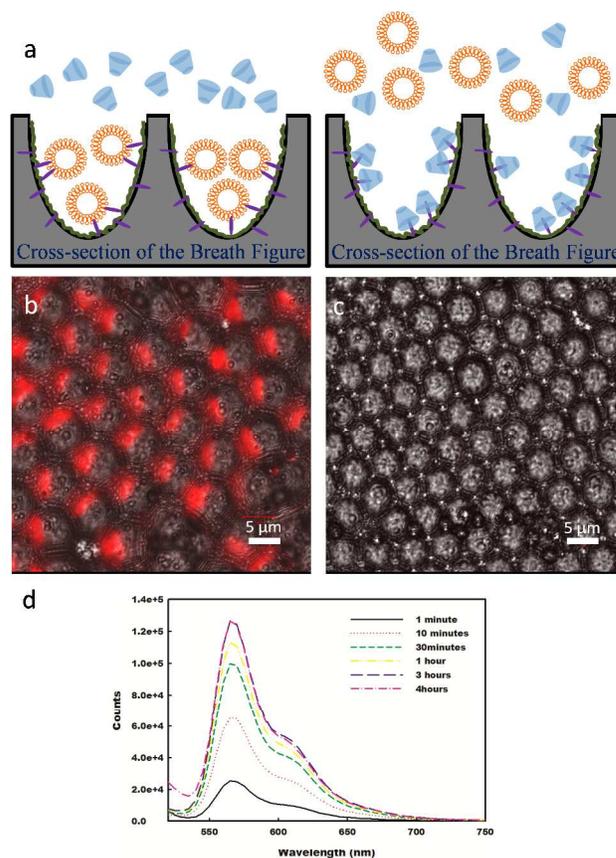


Figure 3: a) Illustration of the vesicle release from breath figure films containing hm-chitosan upon addition of alpha-CD. Confocal images of a breath figure film containing hm-chitosan tethered to red fluorescent vesicles were taken when b) incubated in water and c) when incubated in a solution of alpha-CD. d) The fluorescence spectra of the solution of alpha-CD incubated on the liposome containing breath figure was measured as a function of time.

films containing hm-chitosan. To prove the concept, breath figure films containing hm-chitosan were incubated with red-fluorescent (DiI-tagged) liposomes and then rinsed thoroughly to remove untethered liposomes. The films were exposed to a solution of 0.3% (w/v)  $\alpha$ -CD and the supernatant was examined at periodic time intervals to monitor the release of liposomes. After about 3 hours, the solution fluorescence saturates to an asymptotic value (Figure 3d) and the films were imaged (Figure 3b-c). Reproducibility was again ensured using multiple breath figure samples (>5). While the release kinetics vary between samples, all liposomes are released within 3 hours and the maximum peak intensities of the final fluorescence values are consistent between samples. The control experiment was one where the films were exposed to water over the same time period; in this case, no fluorescence was detected in solution, indicating that the liposomes remained stable and bound to the surface. As seen in Figure 3b, the films exposed to water retain the liposomes (retention of red fluorescence), while the films exposed to  $\alpha$ -CD (Figure 3c) show no fluorescence indicating the complete release of liposomes.

## Conclusions

The hm-chitosan coated breath figures represent a platform for isolating vesicles on an ordered porous surface, and releasing them quickly when required. The method involves the synergism of three concepts (a) the creation of ordered pores in a polymer film using the breath figure method of condensing water droplets onto an evaporating polymer solution, (b) the introduction of materials into the water droplets which then result in specific placement in the pores of the final structure and (c) the exploitation of the hydrophobic effect where the deposited material is a polymer that exposes hydrophobes capable of tethering liposomes. The patterned pores of the breath figures and the selective deposition of hm-chitosan in the pores lead to spatially directed liposome capture.

Such concepts have significant application potential. The masking of specific regions of the hm-chitosan containing breath figures allows capture of distinct and separate vesicle systems in various regions of the polymer film. Furthermore, incorporating vesicle capture functionality in the pores of breath figures may lead to developing sophisticated drug delivery systems. Previously our group has used breath figures made from biodegradable polymers such as poly(lactic-co-glycolic acid) as implant coatings<sup>5</sup> and scaffolds for cell growth.<sup>34</sup> Tethering antibiotics carrying liposomes to the surface of breath figure implant coatings may prevent the development of post-surgical infections. The sequestration of liposomes in the pores may have implications to shielding liposomes from macrophage ingestion.<sup>35</sup> Liposomes tethered to breath figure scaffolds may be used to deliver biomolecules for cell growth and differentiation, for example the delivery of cytokines for stem cell differentiation.<sup>36</sup> Thus, a platform for tethering, isolating, sensing and analysing an array of vesicles on a small film may be realized through the simple fabrication methods described.

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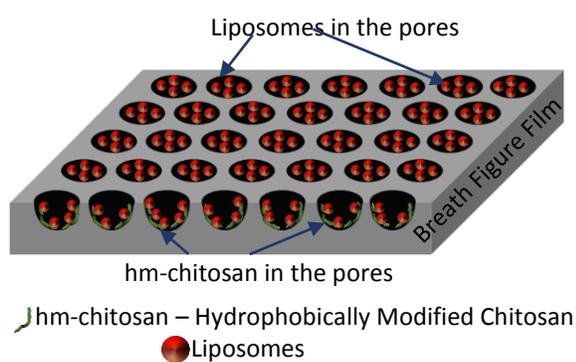
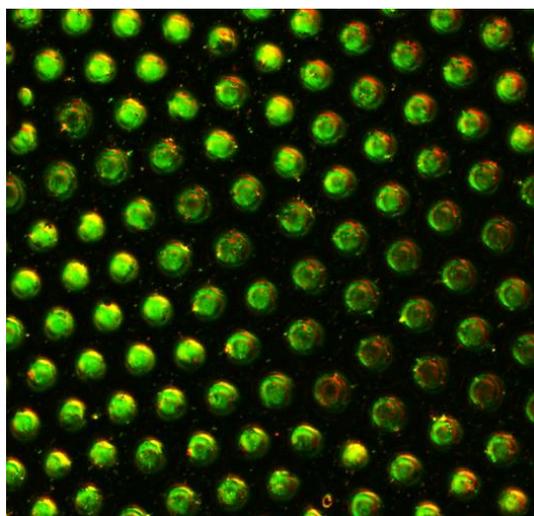
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## Spatially Directed Vesicle Capture in the Ordered Pores of Breath-Figure Polymer Films

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A hydrophobically modified biopolymer (chitosan) when deposited in the pores of patterned polymeric breath figures inserts its hydrophobes into liposomal bilayers. This is a facile method to capture and tether liposomes in a patterned porous polymer film.