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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.[1,2] 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,[3] sensors,[4] drug delivery,[5,6] templates[7] and scaffolds for cell culture.[8]

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.[9–11] 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[12,13] and drug delivery technologies[14] as they are fully biocompatible[15] and easily taken up by cells.[16] 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.[17] 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,[18] in single molecule spectroscopy and biosensing,[19,20] as nanocontainers in microfluidic systems[21] and in understanding the diffusion characteristics of phospholipids in cell membranes.[22]

Several methods to immobilize vesicles have been described in the literature, primarily using irreversible binding concepts including the use of DNA binding,[21–23] the biotin–streptavidin linkage,[21] biotin–avidin linkage[24] and various forms of covalent linkages.[18] The concept of patterning such attachment has been elegantly shown in the recent work by Hammer and coworkers[24] 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 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.[25–27] 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 Fig. 1c.[25] 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.[25,28] In earlier work,

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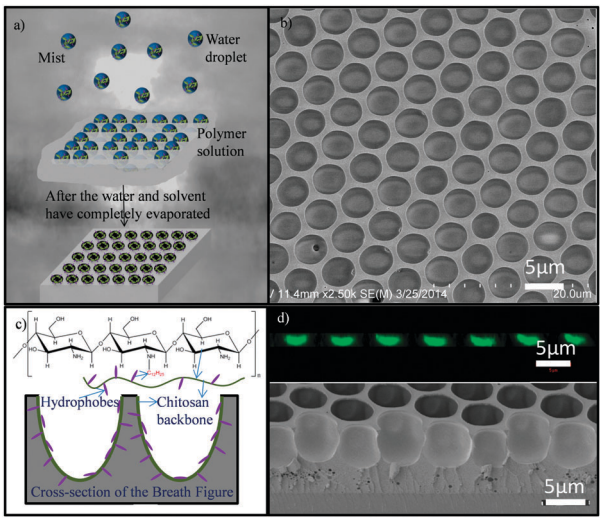

Fig. 1 (a) Schematic indicating incorporation of hm-chitosan into breath figures. (b) Top view of polystyrene breath figures containing hm-chitosan. (c) Structure of hm-chitosan (top) and a schematic of the cross section of the breath figure pores showing hm-chitosan anchored to the pore surface. (d) (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.

we have shown that hm-chitosan coated flat surfaces are capable of anchoring liposomes.[29,30] 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 Fig. 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^{-1} and with the tube diameter (leading from the nebulizer nozzle) of 27.2 mm, the superficial velocity of the mist was between 0.10045–0.1148 cm s^{-1}. All experiments were done at ambient temperature. The temperature of the evaporating film was monitored using an infrared gun (CEN-TECH 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[3] 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 Fig. 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 ESI,† but we note that the precursor solution of hm-chitosan was maintained at slightly acidic conditions below the pK_a 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. Fig. 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. Fig. 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[31,32] and are explained in detail in the ESI.† The green fluorescence from fluorescein-labeled hm-chitosan is observed in the confocal images of Fig. S2 (please refer to the ESI†).

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,† Fig. S2a–d). The cross-section of the breath figures was compiled from the z-stack images in Fig. 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 Fig. 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†).

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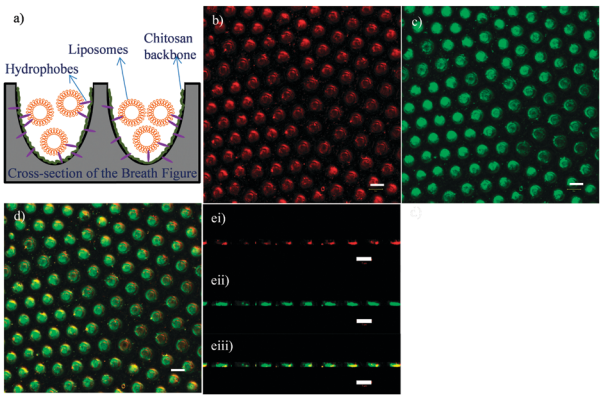

Fig. 2 (a) Schematic of the vesicle capture. Red fluorescent liposomes were incubated on breath figures made from green fluorescently labeled hm-chitosan. The corresponding confocal images are as follows: (b) red fluorescence from the labeled liposomes. (c) Green fluorescence from the labeled hm-chitosan.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

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 Fig. 2 illustrate that the pores of the breath figure now contain liposomes. Thus, Fig. 2b indicates the green fluorescence from the hm-chitosan, Fig. 2c displays the red fluorescence from the liposomes and Fig. 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.[29] The compiled cross-section from z-stack images is shown in Fig. 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 Fig. 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.[28,33] Recent work has shown that α-cylodextrins (α-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 α-CD.[28] The schematic in Fig. 3a is an illustration of the concept of vesicle release from breath figure films containing hm-chitosan. To prove the concept, breath figure films containing hm-chitosan were incubated

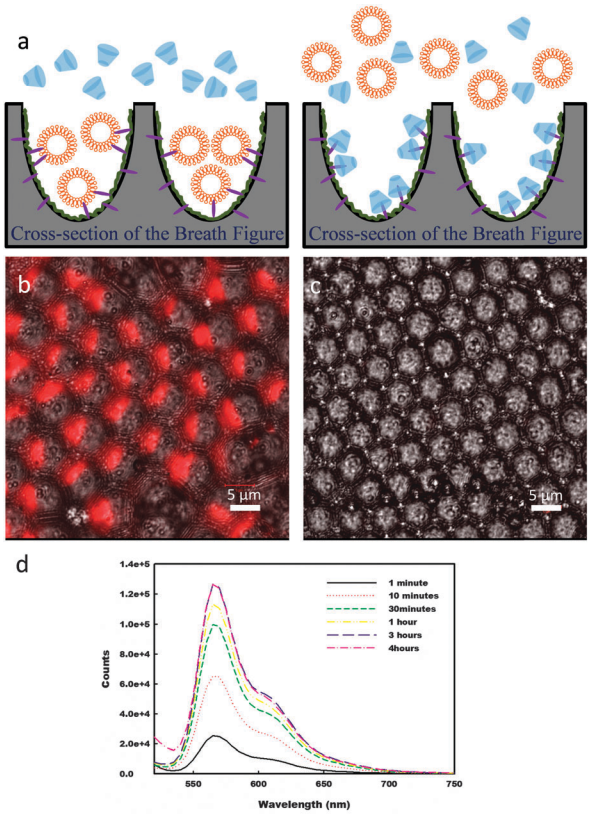

Fig. 3 (a) Illustration of the vesicle release from breath figure films containing hm-chitosan upon addition of alpha-CD. Confocal images of a breath figure film containing hm-chitosan tethered to red fluorescent vesicles were taken when (b) incubated in water and (c) when incubated in a solution of 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.

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) α-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 (Fig. 3d) and the films were imaged (Fig. 3b and 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 Fig. 3b, the films exposed to water retain the liposomes (retention of red fluorescence), while the films exposed to α-CD (Fig. 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

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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[5] and scaffolds for cell growth.[34] 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.[35] 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.[36] 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.

Acknowledgements

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Supplementary Information for

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

Jaspreet Arora, Thiruselvam Ponnusamy, Rubo Zheng, Pradeep Venkataraman, Srinivasa R. Raghavan, Diane Blake and Vijay John

Experimental Details:

Materials: Methylene chloride (organic solvent - ACS grade), Acetic acid (glacial) were obtained from Fisher Scientific, USA. Chitosan medium molecular weight, sodium cyanoborohydride, n-dodecyl aldehyde, carbon disulphide were obtained from Sigma Aldrich. Polystyrene (monocarboxy terminated, approx. M.W. 50,000) was obtained from Scientific Polymers INC., NY. "DiI" the fluorescent lipid 1, 1'-dioctadecyl-3,3,3,3'-tetramethylindocarbocyanine perchlorate was obtained from Invitrogen corporation, CA. L – α-phosphatidylcholine (soy PC 95%) was obtained from Avanti polar lipids. All chemicals were used as received, without further purification.

Synthesis of Hydrophobically Modified Chitosan (hm-chitosan): N-dodecyl tails were attached to low molecular weight chitosan by reacting its amine groups with n-dodecyl aldehyde. Briefly, Chitosan was dissolved in an acidic solution of acetic acid. A solution of the aldehyde in ethanol was then added followed by the addition of sodium cyanoborohydride such that the molar ratio of the aldehyde to the chitosan monomer was 2.5%. The pH was raised to 9 which led to the precipitation of the hm-chitosan. The obtained precipitate was purified by washing with ethanol followed by deionized water. Finally the hm-chitosan was dissolved in a solution of 1% acetic acid.

Fluorescent labelling of hm-chitosan and Chitosan: A solution of chitosan or hm-chitosan in dilute hydrochloric acid was cast in a petri dish and allowed to dry overnight. This film was then neutralized with sodium hydroxide. Then this film was labeled by reacting it with a solution of NHS-fluorescein in ethanol and dimethylformamide in the presence of phosphate buffer saline (PBS) for 30 minutes. After the reaction, the film was rinsed with PBS and lyophilized.

Fabricating the breath figures via a nebulization based process: Thin breath figures films were prepared using a spin coating procedure (spin coater model - WS-400-6NPP-LITE, Laurell Technologies Corporation, North Wales, PA). The polystyrene was dissolved in carbondisulphide to obtain a polymer concentration of 4% (w/v). A 22x22 mm glass coverslip, used as the substrate, was placed on the fragment adapter inside the spin coating chamber. The chamber's fragment adapter was kept under vacuum to hold the substrate while it spun. Through an orifice located on the top of the spin coater, an aerosol mist carrying hm-chitosan/chitosan was introduced at flow rates between 35-40 ml/min. The mist was administered at a room temperature of ~ 25°C

with the mist velocity between 0.087 – 0.099 cm/s normal to the cast polymer solution. The aerosol mist was produced using a nebulizer. The reservoir of the nebulizer was filled with hm-chitosan/chitosan solution and when air at a certain pressure was passed through it, a uniform aerosol mist of water droplets containing hm-chitosan/chitosan was generated. Polymer solution (0.4 ml) was dropped onto the substrate and spun to 2500 rpm for 30 seconds. During the spin coating process, the solvent was allowed to evaporate in the presence of the introduced aerosol mist in order to obtain a porous and opaque film. The films were rigorously rinsed with DI water and 1% acetic acid and further dried at room temperature prior to further analysis and use.

Liposome fabrication: The liposomes were prepared by thin-film evaporation. The phospholipids PC and DiI were mixed in the ratio of about 100:1 (w/w). Phospholipids (0.1g) was dissolved in chloroform and methanol (10 ml) mixture (2:1 v/v). The solution was evaporated inside a round bottom flask using a rotary evaporator (Buchi R-205) for 2.5 hours to obtain a dry lipid film. The lipid film was then hydrated for 1 hour with DI water (5 ml) at 50ºC and 125 rpm. The hydrated solution was extruded 11 times through a 400 nm polycarbonate membrane at 50ºC followed by the use of a 100 nm membrane (Whatman)

Vesicle capture: Once the polystyrene breath figure films with or without hm-chitosan were fabricated, they were rinsed with ethanol and incubated in a solution of liposomes for 45 minutes. Following incubation, they were rinsed thoroughly with DI water to remove untethered vesicles.

Vesicle release using α-cyclodextrin: α-CD was used to release the captured vesicles from the pores of the breath figures. Once the red fluorescently tagged liposomes were captured in the breath figure films, the films were placed in a small petri dish and a solution of 0.3% (w/v) α-CD (2ml) in DI water was added to the petri dish. At specified time intervals, an aliquot of the solution (150µl) was drawn from the petri dish, analyzed for fluorescence using a fluorescence spectrometer (Photon Technology International, New Jersey) and then placed back in the petri dish.

Characterization Methods: The surface morphology of the breath figure polystyrene films containing hm-chitosan/chitosan was characterized with a field emission scanning electron microscope (SEM) (Hitachi S-4800). Prior to the SEM imaging, all the samples were coated with a thin gold layer through sputtering (Polaron SEM coating system) set at 20 mA and 2.4 kV for a duration of 90 sec. The fluorescence of the red fluorescent labeled liposomes and the green fluorescently labeled chitosan were imaged using the 63x lens of a Zeiss LSM confocal microscope system (Carl Zeiss International, Germany).

Figure S1

The aerosol based process was tested to see the breath figure formation. Since hydrophobically modified chitosan (hm-chitosan) was dissolved in 1% acetic acid, an aerosol mist containing 1% acetic acid was created using a nebulizer and used to fabricate breath figures. These breath figures were compared with the ones made when a mist of hm-chitosan was used by scanning electron microscopy (SEM). As seen in FigureS1, the surface morphology of the breath figures created by employing a mist of acetic acid **and hm-chitosan is similar with identical pore size.**

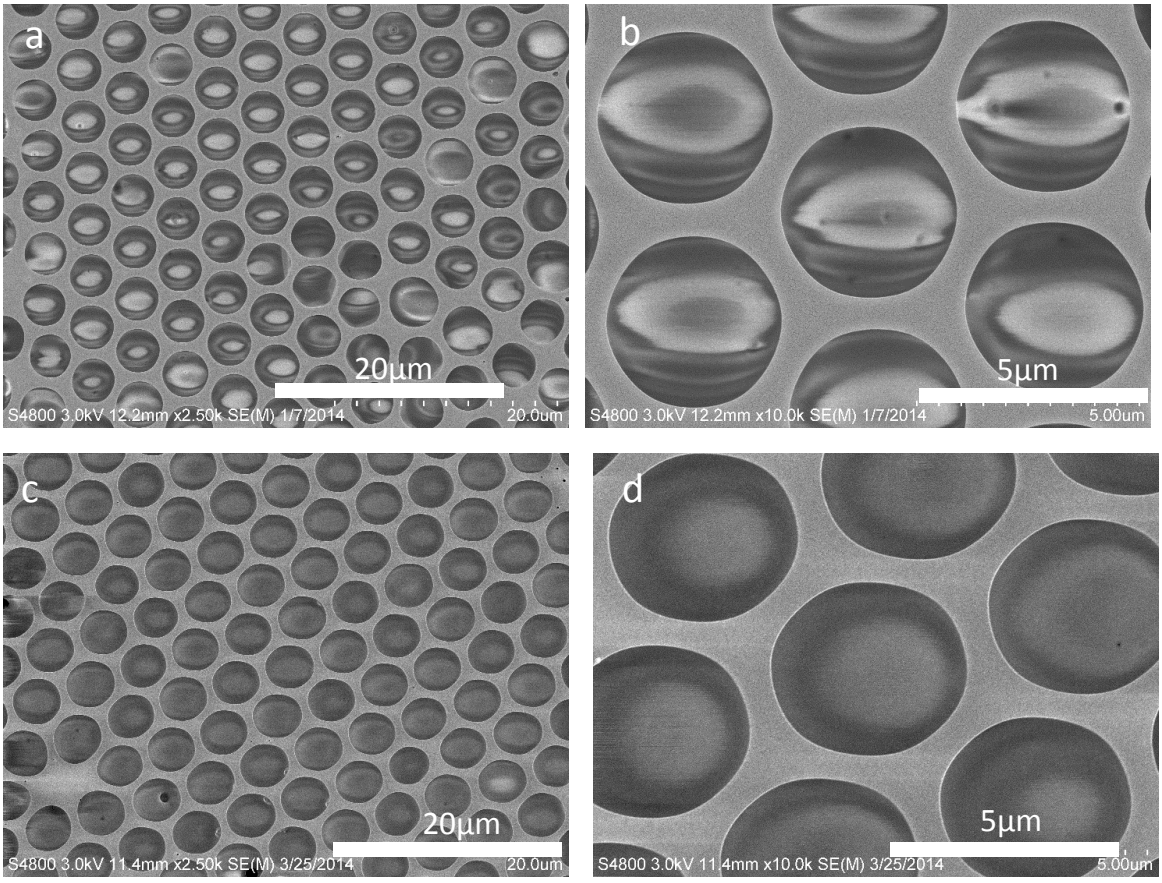

Figure S1: SEM images of the breath figures when a mist of 1%(v/v) acetic acid was employed a) and b) and 1%(w/v) of hm-chitosan was employed c) and d).

Figure S2:

To detect the presence of hm-chitosan in the pores of the breath figures, Fluorescein labeled hm-chitosan was used. Once it was deposited in the pores, confocal laser microscopy was used to detect it along the depth of the breath figures. In Figure S2, images were taken at an interval of 500nm. These images were used to compile Figure 1d (top).

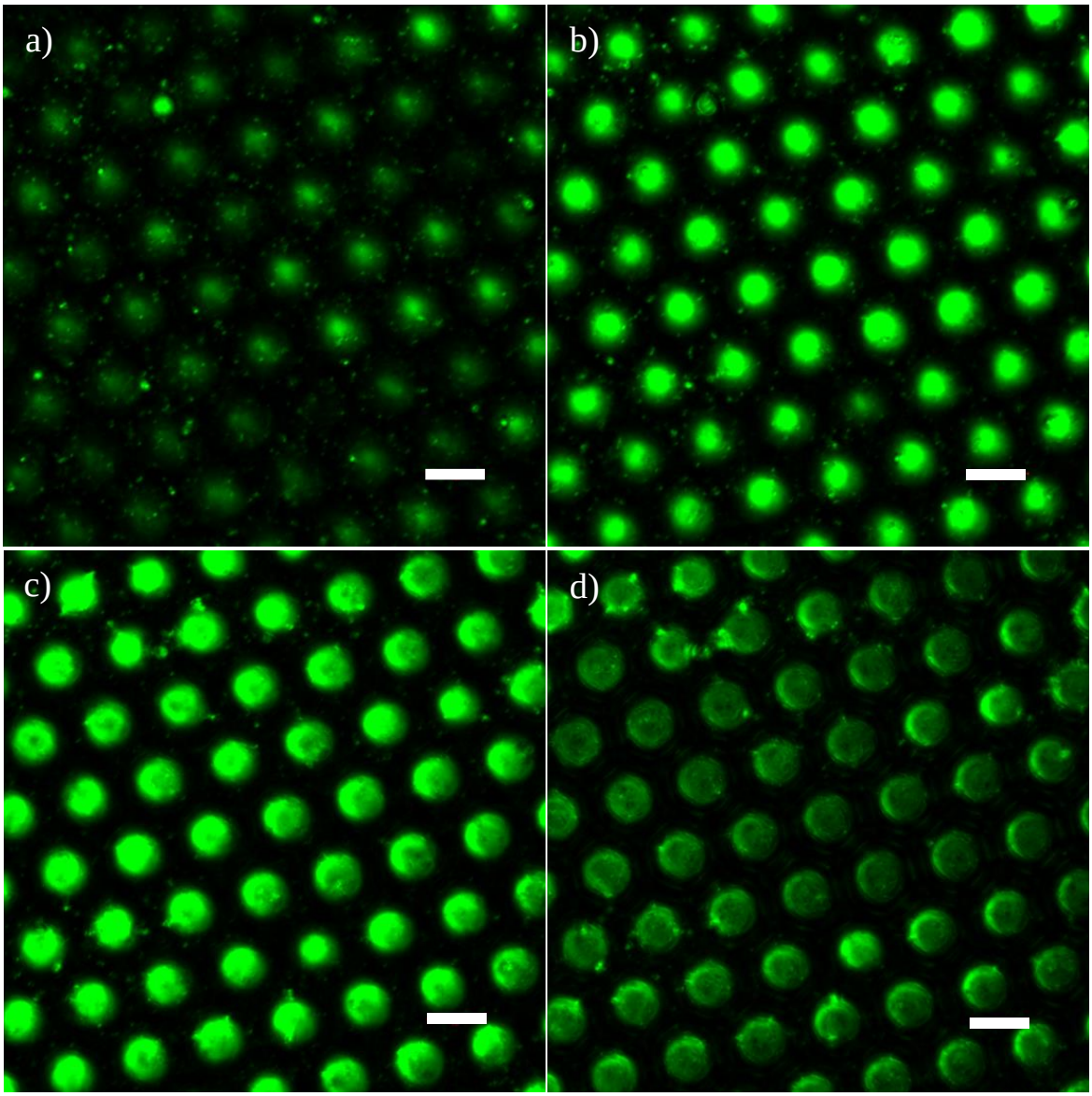

Figure S2: Fluorescein labeled hm-chitosan was employed to fabricate polystyrene breath figures. a) through d) are z-stack confocal microscopic images of the same xy frame of such a sample film. The z-distance between each of the frames is 0.5 µm. Scale bar in all images is 5 µm.

Figure S3:

To test the vesicle capture ability of hm-chitosan, fluorescein labeled hm-chitosan was used to fabricate breath figures. Then, red fluorescently tagged liposomes were incubated on top of these breath figures. The film was then rinsed thoroughly with distilled water to remove the unbound liposomes and analyzed using confocal laser microscopy. The green signal of the hm-chitosan and red signal from the liposomes is simultaneously detected in FigureS3 along the depth of the breath figure film at intervals of 500nm.

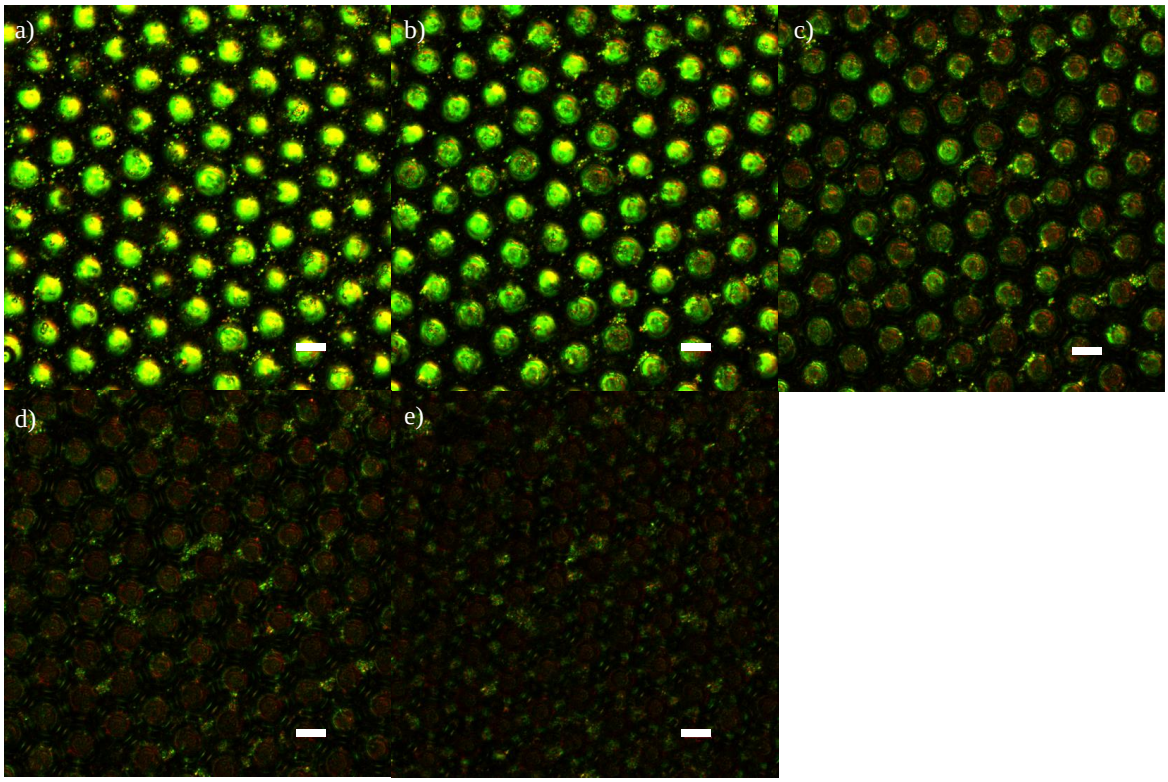

Figure S3: Green fluorescently labeled hm-chitosan was aerosolized to form polystyrene breath figures and red fluorescently labeled liposomes were incubated on these. a) through e) are multi-track z-stack confocal microscopic images of the same xy frame of such a sample film. The green and red fl. Signals are co-localized as in Figure2. The z-distance between each of the frames is 0.5 µm. The scale bar in all images is 5 µm. These images were used to compile Figure2e.

Figure S4:

PC liposomes were analyzed using Cryo-TEM and DLS. From the cryo-TEM image on the left it is clear that the liposomes are mostly unilamellar and spherical. The average diameter of the liposomes was estimated to be 94.27nm with a standard deviation of 22.13nm by Image J analysis**.** The DLS results of the same liposomes suggest an effective diameter of 118.94nm with a standard deviation of 1.55nm and polydispersity of 0.104. The intensity v/s diameter and the number v/s diameter plots above are in agreement with the value of polydispersity.. The vesicles were prepared via thin film hydration and then they were extruded through a polycarbonate membrane of 100nm to control their size. Extrusion decreases the polydispersity of liposomes to a large extent

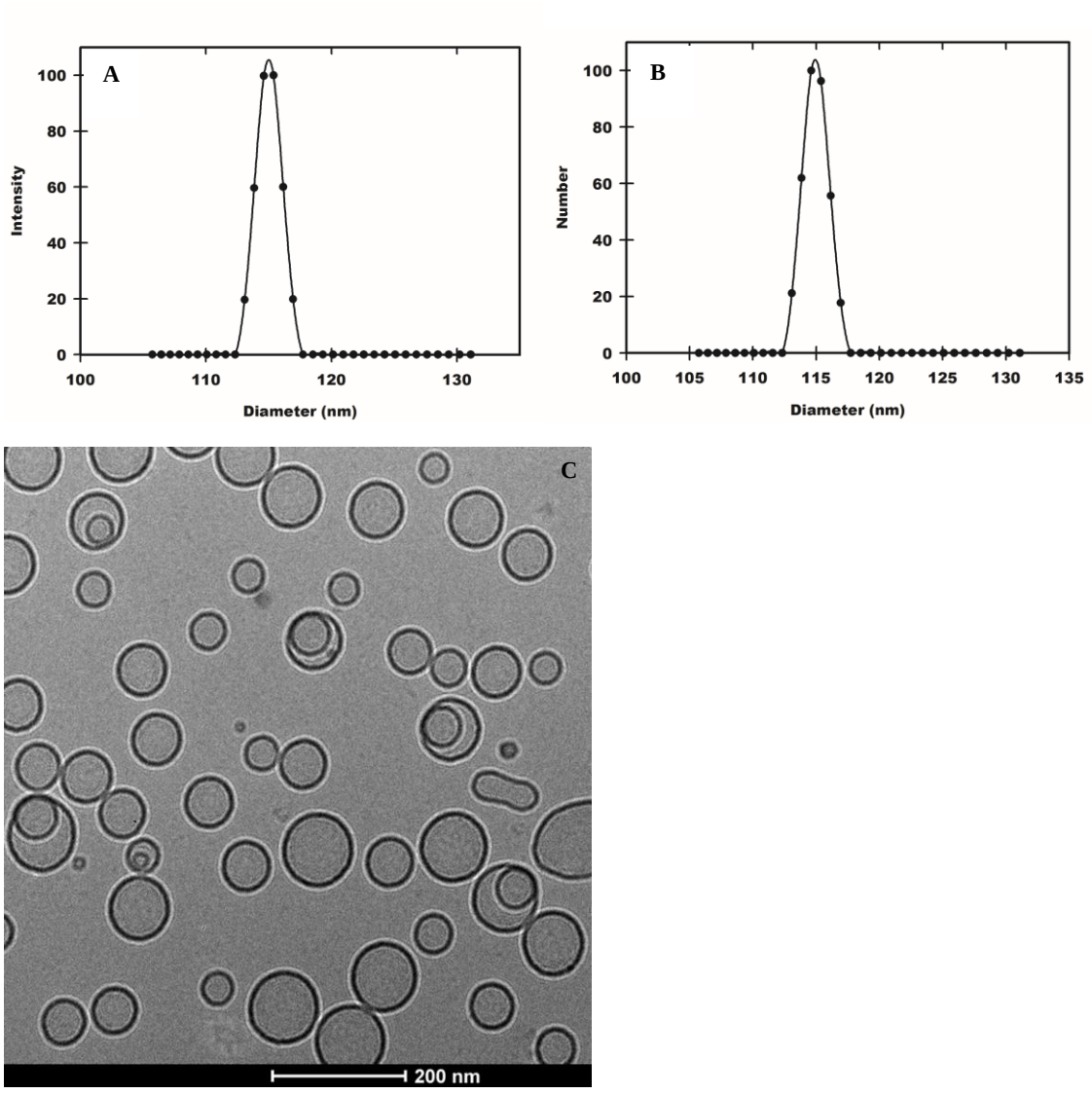

Figure S4: DLS results of the formulated PC liposomes a) Intensity v/s diameter plot, b) Number v/s diameter plot. c) Cryo-TEM image of the PC liposomes.