

Liposomes Embedded in Layer by Layer Constructs as Simplistic Exosome Transfer Model

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Abstract

Background. Exosomes are extracellular vesicles originating from the exfoliation of the cellular membrane. They are involved in cell-to-cell and cell-to-matrix signaling, exchange of bioactive molecules, tumorigenesis and metastasis, among others. To mitigate the limited understanding of exosome transfer phenomena, we developed a simplistic model that mimics exosomes and their interactions with cells and the extracellular matrix. The proposed model is a layer-by-layer (LbL) film built from the polycationic poly-L-lysine (PLL) and the glycosaminoglycan hyaluronic acid (HA) to provide ECM mimicry. Positively charged 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and N^1,N^1,N^{14},N^{14} -tetramethyl- N^1,N^{14} -ditetradecyltetradecane-1,14-diaminium dibromide (GS14) liposomes were embedded in this construct to act as exosome analogs.

Results. To simulate exosomes carrying substances, Nile Red was loaded as a model of lipophilic cargo molecules. The integration of each component was followed by quartz-crystal microbalance measurements, which confirmed the immobilization of intact liposomes on the underlying (PLL/HA)₃ soft film. The release of Nile Red from liposomes either embedded in the LbL construct or exposed at its surface revealed a fast first order release. This system was validated as a model for exosome/cell interactions by incubation with breast cancer cells MDA-MB-231. We observed higher internalization for embedded liposomes when compared with surface-exposed ones, showcasing that the ECM mimic layers do not constitute a barrier to liposome/cell interactions but favor them.

Conclusions. Our findings indicate that the developed model enhances the structural stability of liposomes and induces endocytosis from breast cancer cells. We envisage that the internalization can be tuned by exploring different levels of embedment to achieve a cellular uptake modulated in a spatiotemporal dependent manner. The versatility provided by the LbL technique will allow incorporating additional specialized biomaterials to better mimic the structure and composition of exosomes and their role in cell-to-cell communications.

Background

Exosomes are vesicular colloidal nanoparticles generated as byproducts of cellular activity[1–5]. During the invagination of late endosomes or multivesicular bodies, intraluminal vesicles are generated. These vesicles fuse with the plasma membrane, thus triggering the formation and secretion of exosomes[6] – closed lipid bilayers that carry bioactive molecules such as proteins and RNA[7]. The released exosomes and their cargo are transported by circulating body fluids, such as blood, and captured by host cells[8, 9]. The involvement of exosomes in different biological processes has been extensively researched and reviewed[10–13]. Once considered as “garbage bags” of cells[14], today exosomes are recognized as transport systems that carry and deliver biomolecules to modulate key signaling pathways in cell-to-cell communications[15, 16] and the propagation of numerous diseases[17, 18]. The exosomes capture is a largely variable event, due to the abundance of extracellular matrix (ECM) components from the donor cells presented at the surface of exosomes among other factors. As a result, the associated transfer and

cellular recognition processes remain unclear. Exosomes bioactivity could be further elucidated by developing new systems that mimic the interaction of vesicular structures with cells and the ECM.

Liposomes resemble the exosomes in different aspects: they are nanoparticles capable of interacting with cells as well as carrying bioactive molecules. Like exosomes, liposomes are formed from dispersion of lipids in aqueous media *via* self-assembly that results in closed lipid bilayers. The high thermodynamic stability[19] and biocompatibility[20] of liposomes, as well as their well-defined hydrophobic and hydrophilic domains, make them suitable systems to be loaded with and deliver bioactive molecules[21, 22]. Because of these similarities, liposomes have been used as exosome analogs[23].

Liposomes can be loaded with different biomolecules to simulate exosomes cargo and its transfer to ECM and/or cells. The ECM is one of the first contact points of newly generated exosomes and their environment: exosomes-to-matrix interactions can result in ECM remodeling[24], initiation of mineralization in cartilage or bone[25, 26] or conditioning of cancer metastasis[17]. Mimicking the ECM is a great challenge for tissue engineering because of its high complexity on structural and functional level[27]. Previously, we have shown that layer-by-layer (LbL) approach can be applied to generate reductionist ECM mimics[28, 29]. LbL allows assembling nanostructured films driven by – but not limited to – electrostatic interactions containing polyionic species, including building blocks with potential for biomedical applications[30–32]. Herein, we assembled ECM mimicking LbL from the polycation poly-L-lysine (PLL) and the polyanion hyaluronic acid (HA): PLL is a positively charged polypeptide known to promote cell adhesion, while HA is a negatively charged glycosaminoglycan that is one of the main components of the ECM[28, 29, 33]. The use of polyions allows us not only to build the LbL but also to immobilize a supported vesicle layer (SVL) of cationic liposomes (Scheme 1). Such system provides a simplistic model for exosome-like vesicles embedded in ECM and allows to study the interactions of these vesicles with other biologics and cells.

Methods

Materials. Zwitterionic lipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids, Inc. (USA). Poly-L-lysine hydrobromide (15–30 kDa), Nile Red, Dulbecco's Modified Eagle's Medium high glucose with phenol red were purchased from Sigma-Aldrich. Sodium hyaluronate (21–40 kDa) was purchased from Lifecore Biomedical (USA). Fetal bovine serum, antibiotic/antimycotic solution and TrypLE Express™ were bought from Gibco, and formalin from Thermo Scientific. The surfactant N^1,N^1,N^{14},N^{14} -tetramethyl- N^1,N^{14} -ditetradecyltetradecane-1,14-diaminium dibromide (GS14) was a generous gift from Prof. Luis García-Río's Group (USC) and synthesized as described elsewhere[34]. Gold-coated AT-cut quartz crystals were bought from AWSensors (Spain). Square cover glasses of $24 \times 24 \text{ mm}^2$ and $20 \times 20 \text{ mm}^2$ were purchased from Agar Scientific and employed to assemble films by dip coating. Ultrapure water was used for the synthesis of liposomes and for the preparation of the polyions solutions.

Formulation of DPPC-Based Nile Red-Loaded Liposomes (NR-Liposomes). Unless otherwise stated, liposomes were loaded with Nile Red. A thin-film hydration method[35] was used to prepare NR-Liposomes. Briefly, DPPC, GS14, and Nile Red (200:4:1, mol^{DPPC}/mol^{GS14}/mol^{NR}) were dissolved in chloroform in a round-bottom flask. Due to the great surface activity of GS14 and its low CMC[36], this surfactant was used to generate cationic liposomes, as proposed previously for DODAB surfactants[37]. A CMC of 27 μ M was determined (**Figure S1**). Chloroform was removed by a rotary evaporator (IKA RV10 AUTO model, Germany). Then, the dry deposited lipids, GS14 and Nile Red were hydrated with 0.15 M NaCl under sonication for 10 min, maintaining the temperature of the bath above the smectic-nematic transition[38] of the lipid bilayer ($T_m = 39.5$ °C), resulting in a multilamellar vesicle (MLV) dispersion. Finally, the Nile Red-loaded MLVs were extruded five times through polycarbonate membranes (Millipore, Bedford MA, USA) with a pore size of 100 nm. The extrusion process was carried out in a thermostatic bath at 60 °C, a temperature above the T_m to avoid the disruption of the liposomes.

Characterization of NR-Liposomes. Differential Scanning Calorimetry (DSC) and Dynamic Light Scattering (DLS) analyses were carried out to confirm the loading of Nile Red inside the lipid membrane. Data were compared with the characteristics of empty liposomes (diameter of 50–150 nm, **Figure S2**). A SETARAM Micro DSC-III heat flux microcalorimeter was used to determine the λ -transition that proves the existence of a lipid bilayer. The analysis was performed between 20 and 55 °C at a 0.2 °C•min⁻¹ of heating/cooling rate. Hastelloy batch vessels were used with 900 μ L of an NR-Liposome suspension in a sample cell and 900 μ L of 0.15 M NaCl in a reference cell. A Zetasizer Nano ZS (Malvern Instruments Ltd., UK) was used to determine the size, polydispersity index (PDI), and ζ -potential values. A sample of NR-liposomes of 1.5 mM of DPPC was diluted ten-fold in 0.15 M NaCl to reach optimal kilocounts per second (kcps > 2×10^4 kcps) in DLS measurements. Each analysis was done in triplicate at 25 °C. For both size and ζ -potential, the number of runs was set to automatic.

Molecular Dynamics Simulation of Nile Red Inside the Lipid Bilayer. To evaluate the stability of Nile Red inside the lipid bilayer of DPPC, computational molecular dynamics simulations were performed. The GROMOS force-field 54a7[39] was used for the topology building of Nile Red and GS14 molecules using Automated Topology Builder (ATB)[40]. A solvated bilayer system was constructed with 8 Nile Red molecules, 128 DPPC lipids, and 1 GS14 surfactant. All models were simulated with a 2 fs time-step, and 300 ns production runs were conducted at different temperatures, below and under transition melting temperature of DPPC. Particle-mesh Ewald method was used to obtain long-range electrostatics[41] and periodic boundary conditions were applied for all simulations in the isobaric-isothermal ensemble[42]. The cutoff for the Lennard-Jones potential was set at 12 Å[43]. The pressure was kept at 1 atm, and the temperature was set to either 293 K or 323 K. The density profiles of water, Nile Red and phosphate were obtained by average of simulated trajectories, computing partial densities across the Z-axis in the simulation box.

Assembly of LbL Constructs with Surface-Exposed and Embedded NR-Liposomes. A quartz crystal microbalance with dissipation monitoring (QCM-D) (Biolin Q-Sense E4 model, Sweden) was used to

follow in real-time the deposition of layers of PLL, HA, and NR-Liposomes (software QSoft401, v 2.7.2.867, Biolin Q-Sense). Aqueous solutions of PLL ($1 \text{ mg}\cdot\text{mL}^{-1}$) and HA ($1 \text{ mg}\cdot\text{mL}^{-1}$), and a suspension of NR-liposomes ($1 \text{ mg}\cdot\text{mL}^{-1}$ of DPPC) were prepared in 0.15 M NaCl at pH 7.4. The solutions of PLL and HA were injected alternately into flow chambers containing gold-coated quartz crystals oscillating at a fundamental frequency of 5 MHz and its overtones (15, 25, 35, 45, 55 and 65 MHz) at a constant temperature of 25 °C to assemble 3 bilayers (PLL/HA)₃ (Scheme 1A). The flow was assisted by a peristaltic pump operating at $50 \text{ }\mu\text{L}\cdot\text{min}^{-1}$ until a plateau was reached. After each deposition, a rinsing step with 0.15 M NaCl was made for 10 min. In the last step an overlayer of cationic NR-liposomes was deposited at the same conditions to generate (PLL/HA)₃/NR-Lip system (Scheme 1B). The interaction of the building blocks with the surface of the crystals was detected by observing the acoustic variations of frequency (ΔF) and dissipation (ΔD). The sensors were previously cleaned in acetone, ethanol, and isopropanol in an ultrasound bath (40 °C, 5 min per solvent). For simplicity's sake, this construct is henceforth referred to as surface-exposed liposomes. Additional HA/PLL/HA layers were assembled further to embed the NR-liposomes into the LbL construct (Scheme 1C). These are henceforth designated as embedded liposomes.

Estimation of the Viscoelastic Properties of LbL Constructs with Immobilized NR-Liposomes. The viscosity (μ), elastic modulus (η), areal mass and thickness of the assembled constructs were estimated from the raw ΔF and ΔD data using the Voigt-based viscoelastic model integrated with the DFind analysis software (version 1.2.1, Biolin Q-Sense). The estimations were made with the BroadFit mode using the prebuilt density values for PLL (hydrated protein, $1100 \text{ g}\cdot\text{L}^{-1}$), HA (hydrated sugar, $1050 \text{ g}\cdot\text{L}^{-1}$), and NR-Liposomes (fat, $910 \text{ g}\cdot\text{L}^{-1}$). The solvent density was set to $1005 \text{ g}\cdot\text{L}^{-1}$.

In Vitro Release of Nile Red. The build-up of LbL constructs with surface-exposed and embedded liposomes was replicated using negatively charged cover glasses as substrates. Cover glasses were placed on a vertical stand and submerged in containers with PLL, HA, or NR-Liposomes in the same sequence as in the QCM-D studies. The times for deposition of each layer was set based on the QCM-D measurements (6 min for PLL and HA, 20 min for NR-Liposomes, intercalated with 0.15 M NaCl for 10 min). The assembled constructs were placed in a beaker at 37 °C with 500 mL of 0.15 M NaCl to satisfy sink conditions. For the release studies (up to 300 min), cover glasses were taken from the beaker at different time-points and dried. Then they were placed in separate containers and DMSO (3 mL) was added to dissolve the dry films (sonication for 10 min at room temperature). The amount of Nile Red in each film was determined from the intensity of the peaks of the emission spectra ($\lambda_{\text{ex}} = 550.0 \text{ nm}$; $\lambda_{\text{em}} = 617.8 \text{ nm}$) of the dissolved film and comparing the data with a calibration line of Nile Red in DMSO (**Figure S3**). The total mass of Nile Red entrapped in the LbL constructs (i.e., encapsulation efficiency) was determined at time-point “0 min” using the same film dissolution method, resulting in approximately 1.9 ng per coverglass.

In vitro NR-liposome internalization by MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cell lines were kept in culture on tissue polystyrene (TCPS) in Dulbecco's Modified Eagle's Medium high

glucose ($4.5 \text{ g} \cdot \text{L}^{-1}$) with phenol red, supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) of antibiotic/antimycotic solution (10 000 units/mL penicillin G sodium, 10 000 units/mL streptomycin sulfate and $25 \text{ mg} \cdot \text{mL}^{-1}$ amphotericin B) at 37°C in 5% humidified atmosphere. Upon confluence, cells were detached with TrypLE Express™ (5 min, 37°C in a 5% humidified atmosphere) and subcultured.

For internalization assays, cells were detached using 4 mM of EDTA (10 min, at 37°C in a 5% humidified atmosphere) and seeded at 5×10^4 cells/well in 6-well plates. The internalization of NR-Liposomes was evaluated under three conditions: NR-Liposomes in suspension, surface-exposed liposomes, and embedded liposomes. To evaluate the internalization of NR-liposomes in suspension, MDA-MB-231 cells were seeded on TCPS for 24 h and then the NR-Liposomes at concentration of $150 \mu\text{M}$ of DPPC were added to the culture. To evaluate the internalization of surface-exposed and embedded liposomes and cell adhesion, MDA-MB-231 cells were seeded on these substrates. After incubation periods of 1 h, 3 h and 7 h, the cell culture medium was removed and cells were fixed with 10% formalin at 4°C for 1 h. Nucleus and cell glycocalyx were counterstained with DAPI ($1 \mu\text{g} \cdot \text{mL}^{-1}$ in PBS) and wheat germ agglutinin Alexa Fluor® 488 conjugate ($1.25 \mu\text{g} \cdot \text{mL}^{-1}$ in PBS), respectively. Coverslips were mounted with Vectashield® mounting medium (Vector Laboratories), and images were acquired in confocal laser scanning microscope (CLSM) TCS SP8 (Leica Microsystems).

Results And Discussion

NR-Liposome formation and characterization

Liposomes can be assembled from various lipids. Among different options, the DPPC used by us is a common choice due to its biocompatibility and smectic-nematic transition temperature (temperature at which transition from a rigid to a fluid lipid bilayer occurs) near to the human physiological temperature[44]. The incorporation of the liposomes into LbL constructs was performed via electrostatic interactions: we used the gemini surfactant GS14 to render positive (ζ -potential of $+34.0 \pm 2.8 \text{ mV}$) the surface of these vesicular structures[19, 37]. Loading with Nile Red – a conventional lipophilic, low molecular weight substance that allows fluorescence quantification – was used to simulate exosomes carrying substances. Nile Red incorporation did not affect the good stability of the colloidal dispersion and the positive charge of the liposomes (ζ -potential of $+38.2 \pm 3.8 \text{ mV}$). As we demonstrate, the ζ -potential differences were not significant. This implies that Nile Red did not change the orientation of the zwitterionic heads of the lipids. The size of NR-Liposomes also was not affected expressively by the Nile Red loading (Fig. 1A): a monodisperse population with an average hydrodynamic diameter (D_H) of 91.3 nm and polydispersity index (PDI) of 0.161 was determined for NR-Liposomes. These data were similar for empty liposomes (111.3 nm, PDI of 0.167). The expected endothermic λ -transition of empty liposomes was determined at a T_m of 41.2°C and is in agreement with the supplier's data ($T_m = 41^\circ\text{C}$) (Fig. 1B). The T_m of NR-Liposomes was 39.5°C , slightly less than a pure DPPC bilayer, indicating the presence of the Nile Red inside the membrane. These calorimetric results provide further evidence of the successful formation of liposomes in the presence of Nile Red.

We also ran computational simulations to predict the location of Nile Red inside the liposomes. The poor water solubility of Nile Red and the high partition coefficient in organic solvents[45] indicate that Nile Red should be positioned in the inner part of the lipid bilayer. The simulations at different temperatures (below and above the melting transition $T_m = 39.5\text{ }^{\circ}\text{C}$ or 312.65 K) showed the smectic (Fig. 1C) and nematic phases (**Figure S4**) of the alkyl chains in the lipids. The results demonstrated that the Nile Red molecules can be successfully loaded in between the phosphate head groups (*i.e.* the hydrophobic region of the liposome membrane), thus showcasing the expected capacity for liposomes to entrap hydrophobic substances[38].

LbL assembly of ultrathin biomimetic films with immobilized NR-Liposomes

The manipulation of the liposomes may compromise their stability. As an example, we observed that NR-Liposomes lose their integrity when they become in contact with a rigid surface such as gold (**Figure S5**): QCM data showed adsorption of mass (ΔF decrease) and an initial ΔD increase associated with liposome deposition on the substrate. The sudden reversal of this trend indicates the formation of supported phospholipid bilayers and is associated with the release of the water entrapped within the core of the liposomes (ΔF increased) and damping decrease[46].

The observed results were different when NR-Liposomes were deposited on an LbL substrate (Fig. 2A); liposomes retain their integrity on these soft substrates[47]. Indeed, ΔF decreased and ΔD increased in all adsorption stages (excluding rinses), contrary to the sudden trend reversal with NR-Liposomes on gold. First, the assembly of the precursor (PLL/HA)₃ film (region I) was confirmed by the successive decreases of ΔF with each layer deposition that were accompanied by increases of ΔD , confirming a viscoelastic behavior of the assembled construct. The adsorption of NR-Liposomes onto the (PLL/HA)₃ film (region II) was then evidenced by the monotonic decrease of ΔF accompanied by a similarly monotonic increase of ΔD until saturation was reached. The subsequent rinsing did not cause substantial changes in ΔF and ΔD , showing the stability of the deposited layer, *i.e.* intact NR-Liposomes were adsorbed in the form of an SVL.

To provide ECM mimicry, NR-Liposomes were further covered by an LbL film *via* assembling of additional HA/PLL/HA layers (region III) on top of them. Observed changes of ΔF and ΔD corresponding to this deposition were similar to region I, confirming the existence of electrostatic interactions of these layers with NR-Liposomes. This behavior may also allude to a potential strong binding of our vesicular structures to glycosaminoglycans of the ECM. In fact, about one third ($2.7\text{ }\mu\text{g}\cdot\text{cm}^{-2}$) of the total estimated mass of the LbL construct ($7.9\text{ }\mu\text{g}\cdot\text{cm}^{-2}$) corresponded solely to the ECM mimic film (Fig. 2B).

Figure 2C depicts the estimated viscosity (μ) and elastic modulus (η) upon the adsorption of successive layers. The overall increasing values reveal a viscoelastic behavior, as expected. However, additional information can be gained using the μ/η ratio, where the viscous behavior is weighted against the elastic modulus. Indirectly, it establishes a relationship between the water content and the rigidity of the film. As

observed, this ratio showed a tendency to decrease until the adsorption of NR-Liposomes was completed. At this point, the μ/η ratio reached a minimum, with a viscosity at 2.9 mPa•s and an elastic modulus at 116.4 kPa. Teixeira et al.[33] previously demonstrated that the LbL assembly of PLL/HA films follows a biphasic growth/densification behavior, showing a μ/η decreasing tendency like the reported herein. These data suggest a gradual compaction of the films with each layer deposition and subsequent release of water. Furthermore, the minimum μ/η ratio calculated upon the formation of the SVL is aligned with the rigid behavior of the smectic phase of liposomes below T_m . The data obtained from the QCM-D allows us to conclude the successful electrostatically driven interactions between the various building blocks of the proposed model, as presented in Scheme 1.

Retention of Nile Red by surface-exposed and embedded liposomes

The capacity of the surface-exposed and embedded NR-Liposomes to retain and release the cargo molecules was studied at constant temperature (37 °C) and saline conditions (0.15 M NaCl) - conditions close to those of physiological environments (Fig. 3).

The release from surface-exposed liposomes was faster than from the embedded ones demonstrating that the polymeric layers assembled on top of the SVL play an essential role as a barrier for the diffusion of Nile Red to the bulk medium. The surface-exposed liposomes showed a pronounced burst release: after 23 min about 65% of Nile Red had been released, whereas only 41% release was estimated for the embedded liposomes. The inset shows the release from both surface-exposed and embedded liposomes following a first order kinetics. A release of this type indicates that the diffusion across the lipid bilayer and the additional ECM mimic layers is largely dependent on the cargo concentration. Indeed, the osmotic pressure and ion movements should create a partition coefficient that drives the diffusion of Nile Red molecules from the inner part of lipid bilayer to the bulk medium. We further analyzed the mode of release by determining the correlation coefficients with this and other models – zero order, Hixson-Crowell and Higuchi (**Figure S6, Table S1**). We observed that the release from embedded liposomes provided an approximation to the Higuchi model similar to the first order kinetics. This model is also used to describe the release of a drug dispersed in a matrix. In our system, the HA/PLL/HA top layers can be considered a noncovalent bonded polymeric matrix assembled by LbL. Since Nile Red in embedded liposomes must diffuse across this additional barrier, the observed release agrees well with the interpretation of the Higuchi model and with the successful assembly of the ECM mimic layers.

Degree of internalization by MDA-MB-231 breast cancer cells

Since exosomes have been implicated in tumorigenesis and metastasis, we used MDA-MB-231 breast cancer cells to track the internalization of our analogs via the fluorescence emitted by Nile Red (Fig. 4). The internalization of exosome-like systems is predominantly a non-specific endocytosis-mediated event[48, 49] during which cells uptake vesicles by engulfing them with the cellular membrane, generating endosomes. When a suspension of NR-Liposomes was supplemented to the culture medium, we

observed a colocalization of the fluorescences of Nile Red and the glycocalyx (stained in green) inside the cells. This is consistent with cells taking in liposomes loaded with Nile Red, and it was visible from the first hour of incubation (arrows in Fig. 4D, **Figure S8**).

We evaluated the capacity of spreading and internalization of cells adhered to both tested LbL substrates, *i.e.* the ones with surface-exposed and with embedded NR-Liposomes. At short culture times (1 h and 3 h) few cells were observed on the substrate and they presented a round shape, which agrees with the first steps of cell adhesion to soft substrates (Fig. 4G, H, J, K). At longer periods of incubation (7 h, Fig. 4I, L), their morphology was similar to the controls at early time points (Fig. 4A, D): cells became more elongated and formed protrusions to increase adhesion to the surface. These morphological differences are due to the higher stiffness (which promotes spreading)[50] of TCPS as compared with LbL substrates. The internalization of free and immobilized NR-Liposomes by MDA-MB-231 cells was also different: faster and higher internalization was observed for NR-Liposomes in suspension than for surface-exposed or embedded liposomes. This is an important result, which demonstrates that liposome immobilization is a means to modulate cellular uptake in a spatiotemporal dependent manner.

The interaction between exosomes and cells can be specific (by ligand-receptor interaction) or non-specific (by endocytosis or membrane fusion)[48]. For embedded liposomes, we observed vesicles stained by Nile Red surrounded by glycocalyx stained with WGA inside the cells, which is consistent with endocytosis (Fig. 4J-L). We also found that internalized NR-Liposomes from the embedded structure migrated from regions near the membrane of the cells to the nucleus surroundings as the time of incubation increased, showing the integration of these vesicles and their cargo in endocytic pathways. On the other hand, cells seeded on surface-exposed liposomes presented a Nile Red staining throughout the cytoplasm but it was not engulfed in glycocalyx (Fig. 4G-I). The intensity profile also agrees with a more homogeneously distributed Nile Red (**Figure S9**). These observations contrast with the punctuate fluorescent pattern of embedded NR-Liposomes, suggesting that Nile Red might enter the cells in free-form, *i.e.* unbounded from liposomes. In fact, the fast release of Nile Red from surface-exposed liposomes can result in direct cell internalization of the compound by diffusion through the cellular membrane. Nile Red may also enter by membrane fusion due to the close contact of cells with the exposed liposomes. Gemini amphiphiles, such as GS14, are used as adjuvants in membrane fusion[51], during which the lipid bilayer of liposomes is destabilized and the cargo is released to the interior of the cell.

Of note, embedded liposomes are not directly exposed to be internalized but are covered by layers containing HA. Most tumor cells degrade HA into smaller fragments by enzymatic action. LbL films are also degraded by hyaluronidases and the degradation rate can be controlled by the order of layers: faster degradation is achieved when the construct has a surface exposed layer of HA[52]. These previous results indicate that MDA-MB-231 cells will act upon gradual disassembly of the layers and liberation of NR-Liposomes from the substrate. The observed endosomes indicate that the ECM mimic layers enhanced the stability of NR-Liposomes but, similar to LbL films containing lipoplexes[53], did not obstruct their internalization. On the contrary, they endorse an internalization *via* endocytosis. Such a

result validates our hypothesis that new exosome models can benefit from adding ECM mimicking functions to vesicular structures.

Conclusions

We developed and characterized a simplistic model to mimic the interactions between exosomes, cells, and the ECM. The LbL technique allowed assembly of a precursor soft film to support an intact SVL that mimics ECM. We demonstrated that the structure and the interaction with cells is reminiscent of native exosomes: the presentation of ECM components enhances structural stability of liposomes and drives cellular uptake by endocytosis. We forecast that better control of the internalization rate can be reached over more extended periods by exploring different levels of embedment and liposome surface functionalization. The proof-of-concept was shown for cancer cells due to their affinity towards HA. However, the versatility of LbL allows modulation of macromolecular building blocks to increase the affinity to cells from different lineages. Therefore, our model opens the door to reach a better understanding of exosomes transport phenomena in cell-to-cell communications, not only in the deleterious effects of disease propagation but also in the exploitation of such phenomena for treatment and diagnosis.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Figures

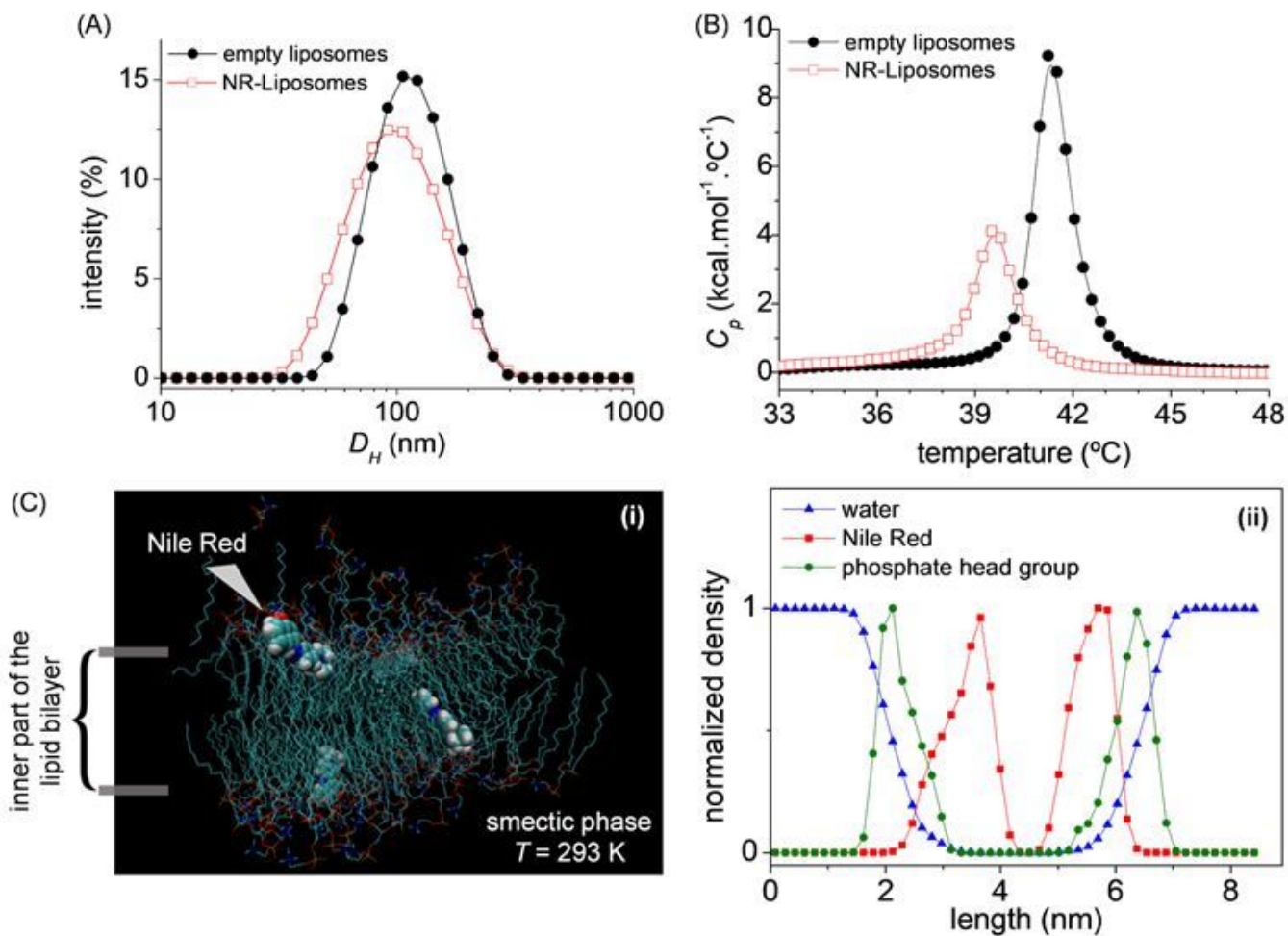


Figure 1

(A) DLS analysis of NR- and empty liposomes in 0.15 M NaCl at 25 °C. The average hydrodynamic diameters (D_H) by intensity distribution of three measurements are represented. (B) DSC of empty and Nile Red-loaded liposomes. (C) Computational molecular dynamics simulations of the lipid bilayer loaded with Nile Red at 293 K: (i) 3D visualization and (ii) normalized density of water, Nile Red and the phosphate head groups are represented.

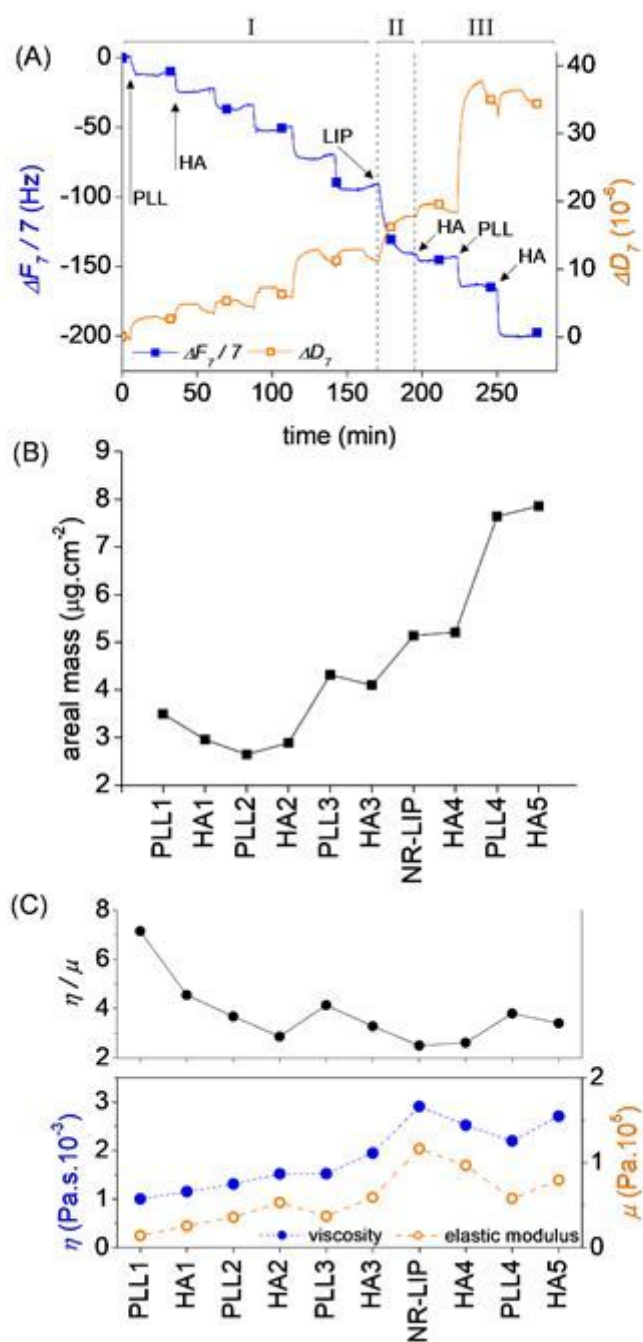


Figure 2

(A) QCM-D variations of normalized frequency ($\Delta F_7/7$) and dissipation (ΔD_7) during the assembly of the precursor (PLL/HA)3 (region I), NR-Liposomes (region II) and HA/PLL/HA (region III). The values are normalized to the 7th overtone (oscillation of 35 MHz). (B) Cumulative areal mass of the model for each stage of construction. (C) Cumulative viscosity (η) and elastic modulus (μ) of the model and representation of η/μ ratio upon the adsorption of each layer. Data in B and C were fit with the Voigt-based viscoelastic model (total $\chi^2 \approx 7.3 \times 10^4$).

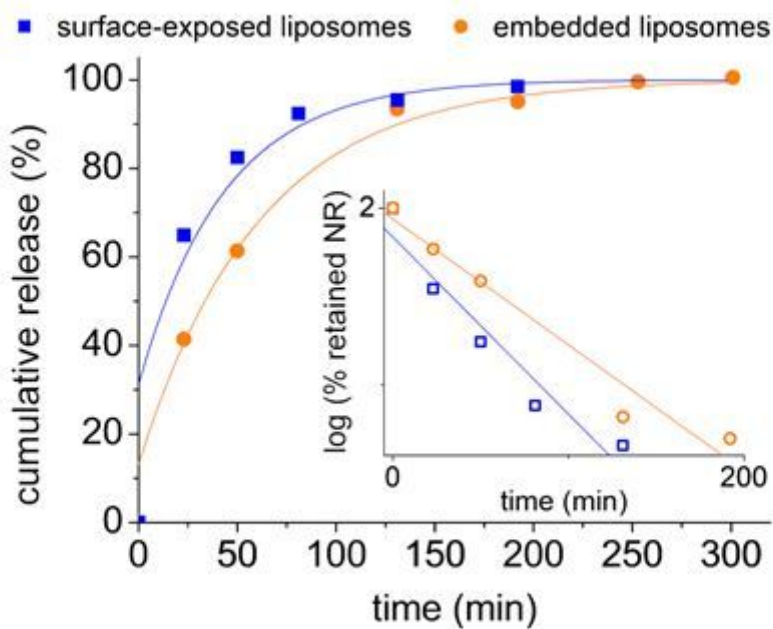


Figure 3

Cumulative release profiles of Nile Red from surface-exposed and embedded NR-Liposomes. The blue and orange curves represent the regression analysis fitting to a first order release. The inset shows the logarithm of the remaining unreleased fraction as a function of time. Fitting was made for 95% of cumulative release. Data points were determined with a sensitivity of $\pm 5\%$.

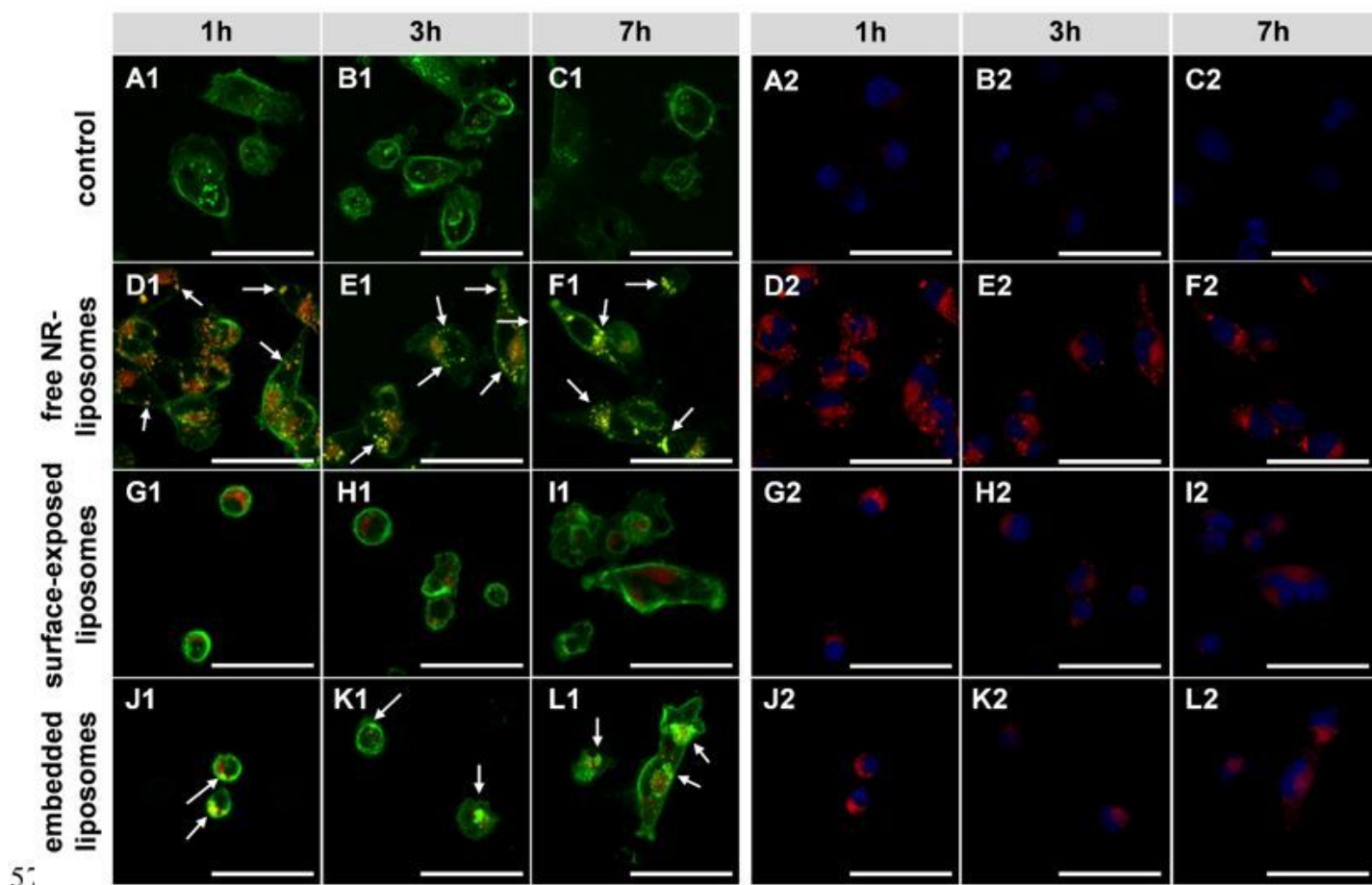


Figure 4

CLSM images of MDA-MB-231 breast cancer cells cultured at different conditions: (A-C) cells on TCPS without NR-Liposomes, (D-F) cells on TCPS treated with free NR-Liposomes, (G-H) cells seeded on surface-exposed liposomes, and (J-L) cells seeded on embedded liposomes. The fluorescence of Nile Red (red), wheat germ agglutinin (WGA, green), and DAPI (blue) are shown after 1 h, 3 h and 7 h of incubation. Arrows point to representative regions of colocalized Nile Red and WGA. Scale bars: 50 μ m. The isolated Nile Red channel is found in Figure S7.

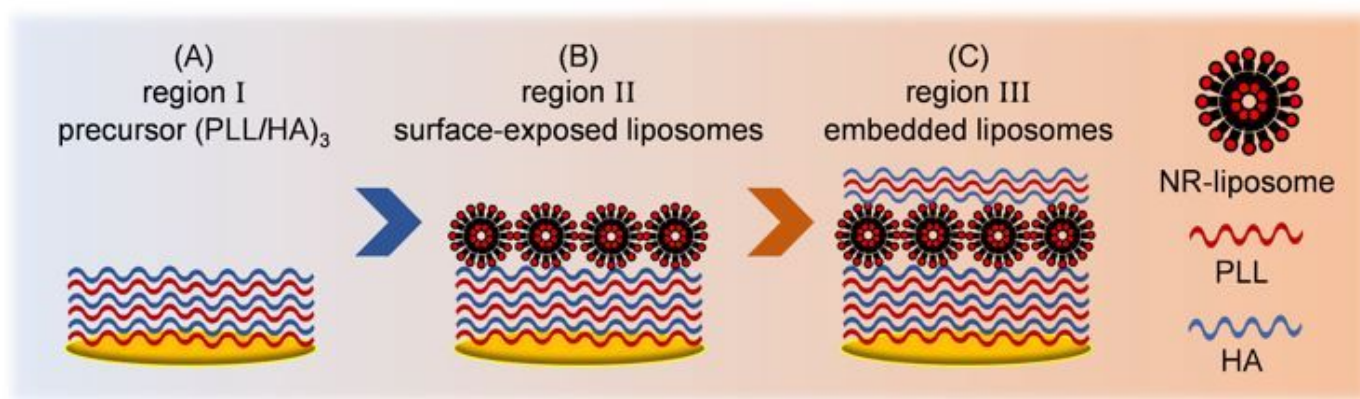


Figure 5

Scheme 1. Schematic presentation of the developed simplistic exosomes transfer model: (A) assembly of a precursor LbL substrate; (B) deposition of liposomes; (C) assembly of the extracellular matrix mimics. The picture is simplified to facilitate observation (i.e. building blocks are not at scale, and interpenetrations between adjacent layers are omitted).

Supplementary Files

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