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Journal:	*Nano Letters*
Manuscript ID:	Draft
Manuscript Type:	Communication
Date Submitted by the Author:	n/a
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Multifunctional nanovesicle-bioactive conjugates prepared by a one-step scalable method using CO2-expanded solvents

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ABSTRACT. Integration of therapeutic biomolecules, such as proteins and peptides, in nanovesicles is a widely used strategy to improve their stability and efficacy. However, translation of these promising nanotherapeutics to clinical tests is still challenged by the complexity involved in the preparation of functional nanovesicles, their reproducibility, scalability and cost production. Here we introduce a simple one-step methodology based on the use of CO_2-expanded solvents to prepare multifunctional nanovesicle-bioactive conjugates. We demonstrate high vesicle-to-vesicle homogeneity in terms of size and lamelarity, batch-to-batch consistency and reproducibility upon scaling-up. Importantly, the procedure is readily amenable to the integration/encapsulation of multiple components into the nanovesicles in a single step and yields sufficient quantities for clinical research. The simplicity, reproducibility and scalability render this one-step fabrication process ideal for the rapid and low cost translation of nanomedicine candidates from the bench to the clinic.

KEYWORDS: Compressed fluids, bioconjugates, nanovesicles, liposomes, nanomedicine, scale-up

Liposomes, and in general vesicles, are undoubtedly one of the most promising supramolecular assemblies for nanomedicine due to their great versatility respect to size, composition, surface characteristics and capacity for integrating and encapsulating bioactive molecules. Their membranes can be efficiently functionalized with different targeting units like peptides, antibodies, etc., that promote specific and increased accumulation of the drug or the bioactive molecule in the target cells. Besides, they are well recognized as pharmaceutical carriers because of their biocompatibility, biodegradability and low toxicity.[1,2] This has prompted their use in the treatment of some major health threats including cancer, infections, metabolic and autoimmune diseases, and has even led to first marketed products.[3,4] Important pharmacological specifications like stability, loading capability, leakage kinetics of entrapped substances, etc, are determined by the structural characteristics (e.g. size, morphology, supramolecular organization, structural homogeneity) of these nanocarriers.[5] For instance small unilamellar vesicles (SUVs) with sizes around 100 nm, have attracted great attention in the drug delivery field. These vesicles are large enough to avoid the first-pass elimination through the kidneys but sufficiently small to present a minimal uptake by the mononuclear phagocytic system, facilitating their longer circulation lifetimein the body and hence higher possibility to reach the target cells. Moreover, due to their nano-scale size SUVs can accumulate within tumours through the enhanced permeability and retention (EPR) effect and thereby be applied in cancer therapy.[6] Methods to produce SUVs commonly use conventional techniques for vesicle formation such as lipid thin-film hydration[7,8] or reverse-phase evaporation,[9,10] and further post-formation steps (sonication,[11,12] extrusion,[13,14] etc) for size reduction and homogenization. These conventional processes are generally complex, multi-step, time consuming, not easily scalable and might damage the functionality of the bioactive molecules. All these drawbacks are particularly relevant in the preparation of colloidal

bioconjugates with expensive and/or fragile active biomolecules, such as proteins, peptides, enzymes, hormones, etc. Thus, it is crucial the development of simple and mild processes that allow the control of the structure at the micro-, nano- and supramolecular levels and are amenable to be scalable.[15]

Compressed fluid-based methodologies, also named as dense gas technologies, are attracting increasing interest for the direct preparation of micro/nanoparticulate materials with structural characteristics not reachable by already existing particle production procedures using liquid solvents.[16,17] Compressed fluids are defined as substances that at normal conditions of pressure and temperature exist as gases but increasing the pressure can be converted into liquids or supercritical fluids, and be used as solventmedia for chemical and material processing.[18,19]

Recently, we have developed a procedure called DELOS-SUSP (Depressurization of an Expanded Liquid Organic Solution-Suspension) for the preparation of dispersed systems. Using this methodology the straightforward synthesis of cholesterol-rich SUVs with controlled size distributions, uniform shapes and good stability in time, has been achieved.[20] In addition, recent studies have shown that vesicular systems prepared by this method have a vesicle-to-vesicle homogeneity degree, regarding membrane supramolecular organization, more than double than those prepared by thin-film hydration.[21]

Here we demonstrate the potentiality of the DELOS-SUSP method as a simple, robust, scalable and one-step process to prepare a variety of multifunctional SUV-biomolecule conjugates with high structural homogeneity. In particular, we report the straightforward functionalization of vesicle membranes with two different molecular units: 1) the hydrophilic poly(ethylene glycol) (PEG) polymer, a stealth agent widely used to prolong circulation time by stabilizing and protecting the vesicles against phagocytosis, and 2) a targeting RGD cyclic

peptide known to increase the cell penetration via RGD/integrin recognition. We also present the one-step integration of two hydrophilic model proteins: 1) the green fluorescence protein (GFP) and 2) the bovine serum albumin (BSA) as well as the preparation of multifunctional vesicles integrating simultaneously two active molecules. As depicted in Figure1, nanovesicle-bioactive conjugates were prepared using two different membrane chemical compositions. In one formulation the membrane was formed by cholesterol and the phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) yielding SUVs, from now on called Liposomes. The second formulation, much less common and presenting a positively charged membrane, is composed by cholesterol and hexadecyltrimethylammonium bromide (CTAB) surfactant, yielding SUVs named as Quatsomes.[22]

All bioconjugates were prepared several times following the procedure schematically represented in Figure2, always under mild conditions to preserve the activity of labile biomolecules (See Table 1 and Supporting Information Figure 1). Briefly, the method consists in loading a solution of the membrane lipid components and the desired hydrophobic bioactives in an organic solvent (e.g. ethanol) into a high-pressure autoclave and pressurizing it with a large amount of compressed CO_2. Vesicular nanoconjugates are formed, by depressurizing the resulting CO_2-expanded solution over an aqueous phase, which might contain water soluble surfactants and hydrophilic bioactives (Supporting Information). The experimental conditions used for the preparation of the organic and aqueous phases of each formulation are given in Table 2. No further energy input is required for achieving the desired SUVs structural characteristics, neither for increasing the loading or functionalization efficiencies. The CO_2 here acts as a co-solvent and its evaporation from the organic expanded solution during the depressurization stage produces a fast, large and homogeneous cooling responsible of the high vesicle-to-vesicle structural

homogeneity in comparison to that reached by conventional thin-film hydration. It should be pointed out, that lipids, such as cholesterol, have a great sensitivity to solvent media variations.[23] Therefore, homogeneous vesicle formation paths are required to guarantee a high degree of structural homogeneity.

Nanovesicle-PEG conjugates

One of the most spread strategies to avoid the fast clearance of vesicles by opsonisation and to increase the blood circulation periods in the body is the coating of the carrier membranes with hydrophilic biocompatible polymers such as polyethylenegycol polymers (PEGs).[24] These so-called long-circulating or stealth liposomes experience a "passive" accumulation in tumours and inflammations, enhancing the drug delivery in these affected parts.[25] Conventional methodologies like lipid thin-film hydration or reverse-phase evaporation are generally employed to prepare PEGylated liposomes using small concentrations ($\leq$ 7 mol %) of short-chain PEGs (1000-4000 Daltons) covalently linked to selected lipid membrane constituents. Nanosized PEGylated Liposomes and PEGylated Quatsomes were straightforward produced by DELOS-susp, adding cholesterol_PEG$_{1000}$ and cholesterol_PEG$_{2000}$ as part of the vesicle membrane components (Figure 1). To prepare the Quatsome-PEG conjugates, a CO_2-expanded solution of cholesterol and cholesterol_PEG (6:1 molar ratio) in ethanol was depressurized over an aqueous solution containing CTAB. In the case of Liposome-PEG conjugates, the CO_2-expanded alcoholic phase, composed by cholesterol, DPPC and cholesterol_PEG, was depressurized over water. In all experiments the molar ratio between cholesterol and cholesterol_PEG was 6:1. Cryo-transmission electron microscopy (Cryo-TEM) images of the resulting PEGylated SUVs, disclosed homogeneous, spherically shaped and unilamellarnanovesicles in all cases (Figure 3a-3d). Their size distribution, polydispersity index and Z potential were determined using dynamic

light scattering (DLS) and are reported in Table 1. The Quatsome-PEG conjugates presented smaller particle sizes and polydispersity indices. Besides their absolute Z potential values were larger than 30 mV consistent with a higher stability under storage conditions (Supporting Information). Indeed, no changes in size and morphology were observed for more than one year, indicating that these vesicular systems are very stable and do not suffer aggregation upon long periods of time. This stability was somewhat smaller in the case of Liposome-PEG formulations which presented Z potentials between 7 and 13 mV. Nevertheless, the suspensions were macroscopically stable during at least 30 days, with no evidence of solid deposition. The high stability of Quatsome-PEG conjugates is explained by the particular self-assembling of cholesterol and CTAB molecules to form vesicular structures with outstanding stability.[22]

Nanovesicle-RGD conjugates

"Active targeting" through the incorporation of specific molecules on the outer surface of vesicles can provide more effective therapeutic action to a nanomedicine.[1] In the last years the RGD-peptide has become the ligand of choice for the labelling of liposomes due to its capacity of binding integrin receptors, over expressed in tumor cells.[26] Among the different types of RGD peptides available we chose the cRGDfK to functionalize our Liposomes due to several advantages related with its cyclic structure.[27] We synthesized a cholesterol_PEG_{200}_RGD molecule, in which the cholesterol was first attached to a PEG_{200} unit through an ether bond and the cRGDfK peptide was coupled to this unit through a carbamate bond (Supporting Information). For the one-step preparation of Liposome-RGD conjugates, a mixture of cholesterol, DPPC and cholesterol_PEG_{200}_RGD in a molar ratio of 6:10:1 was dissolved in ethanol and then expanded with CO_2. Once the depressurization over water took place, a suspension of functionalized nanovesicles was obtained with a narrow particle size distribution

centered in 144 nm and macroscopically stable for at least 30 days (Table 1, Supporting Information). Cryo-TEM images (Figure 3e) revealed a much more homogeneous unilamellar system when cholesterol_PEG$_{200}$_RGD was inserted in the membrane compared to plain liposomes, which was also confirmed with small-angle X-ray scattering (SAXS) measurements (Supporting Information and Supporting Information Figures S3 and S5). Furthermore, an increase in the Z potential from less than +10 mV up to +30 mV was observed when cholesterol_PEG$_{200}$_RGD was present in the formulation (Table 1), leading to a higher stability of the vesicular system along time. To enquire whether this higher structural homogeneity was related to the use of the DELOS-SUSP method to produce the liposomes or exclusively due to the presence of the peptide, we prepared the Liposomes containing cholesterol_PEG$_{200}$-RGD using the conventional lipid thin-film hydration methodology (Supporting Infromation). The resulting formulation was less homogeneous, highly unstable and multilamellar showing a size distribution centered at 1926 nm (Supporting Information Figure S4). This demonstrates that both, the presence of the cholesterol_PEG$_{200}$_RGD molecule as well as the preparation methodology are key ingredients for the synthesis of these highly homogeneous conjugates. It is worthy to say that while it took 2 hours to prepare 25 mL of this nanoconjugate suspension by the CO$_2$-based procedure, 2 days were required for the preparation of 2mL of nanovesicles-RGD by thin-film hydration plus post-formation steps.

To determine the amount of cholesterol_PEG$_{200}$_RGD incorporated into the membrane, the fraction of non-integrated molecules was separated from the total sample using centrifugal filter devices (Centricons) of 30 kDa and then analyzed by HPLC (Supporting Information). The analysis showed the absence of free peptide in the mother liquors, resulting in almost a 100% incorporation of the cholesterol functionalized with the peptide within the lipid bilayer. A similar

high degree of RGD incorporation was achieved by Schiffelers *et al*. using the conventional lipid thin-film hydration methodology.[28] Thus the DELOS-SUSP methodology allows a one-step production of unilamellar conjugates in smaller processing times, with minimum material loss and high yields of the ligand in the final formulation.

To investigate whether the activity of the integrated biomolecule is maintained during the processing with CO_2-expanded solvents, we examined the internalization capabilities of the new Liposome-RGD conjugates on endothelial (HMEC-1) cells which express high levels of $\alpha_v\beta_3$ integrins on both their apical and basal membranes[29] (Supporting Information Figure S4). For this study the 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) fluorescent dye was integrated into the membranes of Liposomes and Liposome-RGD conjugates at a concentration of 0.6 nM (Supporting Information Figures S7 and S8), taking advantage of its lipophilicity. HMEC-1 cells were then incubated with DiD-labelled liposomes with and without RGD, for 3 hours at 37ºC, to induce internalization and were subsequently inspected by laser scanning confocal microscopy (LSCM). Liposome-RGD conjugates were rapidly uptaken by the cells (Figure 4a) whereas the control plain Liposomes were barely internalized (Figure 4b). Importantly, a fraction of Liposome-RGD conjugates trafficked to endosomal/lysosomal compartments as judged by colocalization studies with the DiD (red) fluorophore and the Lysotracker (green), a fluorescent probe which labels and tracks acidic organelles in live cells. Indeed, three-dimensional reconstructions of z-stacked fluorescence images of live HMEC-1 showed colocalization between DiD-labelled Liposome-RGD conjugates and Lysotracker after 3 h of incubation at 37ºC (Figure 4c). These data strongly indicate that the presence of RGD peptides on the liposomes membrane enhance their binding and uptake by HMEC-1 via $\alpha_v\beta_3$ integrin-mediated endocytosis. The results were further confirmed on a large population of cells

using flow cytometry analysis. Indeed, about 85% of the cells showed enhanced fluorescence when incubated with DiD-labelled Liposome-RGD conjugates at 37ºC, whereas this percentage was reduced to 4% for cells incubated with DiD-labelled plain Liposomes (Figure 4d). Furthermore, a decrease in the fraction of positive cells was observed when cells were pulsed with DiD-labelled Liposome-RGD conjugates at 16ºC, a temperature that reduces endocytosis (Figure 4d). The mean fluorescence intensity (MFI) values measured for HMEC-1 incubated with DiD-labelled Liposome-RGD conjugates were 30-fold higher than those of the cells incubated with DiD-labelled Liposomes (Figure 4e), demonstrating that the presence of the peptide is responsible for the enhanced liposome uptake levels. These studies also confirm that the DELOS-SUSP methodology did not affect the bioactivity of the RGD peptide after its processing with compressed CO_2. Further *in vitro* assays showed that these nanocarriers were non-toxic, non-hemolytic ($\leq$ 2 %) and sterile, rendering them as excellent candidates for the specific delivery of active molecules to targeted cells (Supporting Information Figures S9 and S10).[30]

Nanovesicle-protein conjugates

Nowadays, therapeutic proteins are attracting the attention of clinicians for the treatment of many diseases as they are well tolerated by the body and have the ability to perform specific functions without interfering with normal biological processes.[31] However, the inherent lability associated with proteins, including thermal instability, degradation by proteolysis, rapid body excretion and low solubility, hinder the rapid progression of this field. Some drug delivery systems such as polymeric nanoparticles or liposomes have been used to overcome these limitations.[32] Indeed, there are several examples of enzymes and hormones, among other biomolecules,[33,34] encapsulated into vesicles, which show a considerable increase of their

therapeutic activities. These systems are generally obtained using methodologies that require further post-formation steps to achieve the desired size and to reduce the multilamellarity, steps that sometimes affect the final protein-to-lipid ratio and reduce the drug activity in the resulting formulation.[35] To evaluate the performance of the DELOS-SUSP methodology for encapsulating proteins ensuring that their biological activity is preserved, we integrated two different model proteins into SUVs: a modified GFP protein, the GFP-H6 (~27 KDa) tagged with six histidine residues (Supporting Information), and the commercially available BSA protein (66.5 KDa). We chose GFP because is usually employed as a natural marker for gene expression[36,37] and has been incorporated into micelles,[38] polymeric particles[39] or protein capsids.[40] On the other hand BSA is the most abundant protein in the blood and has been extensively used as a model protein in studies such as protein-membrane association,[41] protein-surfactants interaction[42] and for entrapping into liposomes.[43]

The preparation of Liposome-GFP conjugates was performed by depressurizing a CO_2-expanded ethanolic solution of DPPC and cholesterol over a Tris buffer saline solution containing the GFP-H6 protein. DLS measurements showed a size distribution centered at 228 nm (Table 1) and Cryo-TEM images revealed a homogeneous morphology (Figure 3f). The Z potential was low, near zero, but the formulation remained macroscopically stable for more than one week. The fluorescence of the GFP-H6 protein after processing, along with the confirmation of its entrapment in the liposomes was assessed using dual colour total internal reflection fluorescence (TIRF)-Epi microscopy. For this purpose DiD was also chosen as a membrane marker for labelling the protein-loaded liposomes at a concentration of 0.6 nM (Supporting Information Figure S7). A sample of 200 µl of DiD-labeled Liposome-GFP conjugate was deposited on a glass coverslip mounted into a microscope chamber. EPI-TIRF images were

collected in two different channels, red and green, to allow the simultaneous monitoring of the

signals from DiD and GFP, respectively. The spatial colocalization of both signals confirmed

that the GFP-H6 was successfully incorporated into liposomes and that its fluorescence was not

affected upon processing (Supporting Information Figure S11). To determine the amount of

protein incorporated, the free GFP-H6 was first separated from the loaded liposomes using

centrifugal filter devices of 100 KDa. The loaded vesicles were analyzed by SDS-PAGE and

further Western-blot (Supporting Methods) and the entrapment efficiency (%EE) was calculated

by dividing the mass of integrated active between the total initial mass. A 44 ± 7 % of protein

encapsulation within the liposomes was obtained for a 0.4 μmol protein/mmol total lipid in the

final formulation. This encapsulation efficiency value resulted fairly high considering that

hydrosoluble proteins generally presents low encapsulations efficiencies, especially in small

vesicles with diameters ranging from 50 to 150 nm.[35]

To perform the entrapment of BSA (66.5 KDa) we chose Quatsomes, since these vesicular

structures are promising nanocarriers for the topical delivery of therapeutic biomolecules, like

enzymes, becoming a real alternative to phospholipid liposomes and non-ionic surfactant

niosomes.[44] Following the procedure schematized in Figure 2, an expanded organic solution

containing cholesterol was depressurized over an aqueous phase containing CTAB and the

protein. A macroscopically stable disperse system, with a very narrow size distribution centered

in 122 nm, was achieved (Table 1).The Z potential value was high and positive in agreement

with the membrane composition and the fact that the system remains stable over a period larger

than 5 months (Supporting Information). The Cryo-TEM images (Figure 3g) showed a

homogeneous system with nanoscopic, spherical and unilamelar vesicular structures. To

determine the encapsulation efficiency the BSA-loaded vesicles were separated from the non-

incorporated protein using centrifugal filter devices of 100 kDa. Free BSA in the supernatant was quantified using a colorimetric method (Supporting Information). The entrapment efficiency in the vesicles was calculated subtracting the mass of free protein to the initial mass and dividing the result between the initial protein mass. A 96 ± 1.3 % of protein entrapment in the Quatsomes was found which resulted in an extremely high value, considering that BSA is hydrosoluble and that the vesicles are of nanoscopic size. A reasonable explanation for this high degree of encapsulation is related to the presence of protein-membrane interactions.Indeed, BSA has a negative charge under the entrapment conditions (isoelectric point of 4.7 in water), and therefore could form a complex with the cationic nanovesicles. This complex together with the entrapment of the protein inside the aqueous core of the vesicles explains the high encapsulation efficiency.Thus, the use of DELOS-SUSP methodology for encapsulating BSA in Quatsomes gave rise to high protein loadings, long stabilities in time and very homogeneous morphological characteristics meliorating the association efficiencies achieved (20-75 %) for the encapsulation of BSA in liposomes with conventional methodologies.[45] Additionally, this production platform allows the preparation of nanovesicle-protein conjugates without damaging the activity of the protein and with high final/raw protein ratios.

We also extended the DELOS-SUSP method to the simultaneous PEGylation and protein loading of Quatsomes. A CO_2-expanded ethanolic solution containing cholesterol and cholesterol_PEG_{1000} was depressurized over an aqueous solution containing CTAB and BSA at same concentrations used in the previous experiments. Multifunctional conjugates with nanoscopic size, homogeneous morphology and great stability in time were obtained together with an 84 ± 3 % of BSA entrapment efficiency (Table 1, Figure 3h). These results show that the

reported methodology allows the preparation of multifunctional vesicles with high structural homogeneity in terms of size and lamelarity, and high protein loadings.

Scaling-up production of nanovesicle-bioactive conjugates

Finally, bench-scale to clinical-scale reproducibility was check in order to evaluate the potentiality of this new platform for the production of nanovesicle-bioactive conjugates with sufficient quantities for clinical studies. The encapsulation of BSA in Quatsomes, as a model formulation, was repeated under the same experimental conditions but in a 40 times larger high pressure vessel (from 7.5 mL to 315 mL) using the same equipment configuration with minor modifications in the automation procedure (Supporting Information Figure S2 and S12). With this scale-up the batch volume of vesicle suspension was increased from milliliter up to liter scale, which could allow the production of nanomedicine batches to be used in pre-clinical and even clinical studies. The influence of DELOS-SUSP scale-up on the physicochemical characteristics of the BSA-loaded Quatsomes was analyzed in terms of size, morphology and entrapment efficiency. The resulting vesicles presented diameters around 123 nm with narrower particle size distributions (Figure 5), indicating that even a more homogeneous system is obtained at large scale. The differences in homogeneity are most probably due to the variance of the configuration between the two equipments in the depressurization stage. Thus, in the case of the large vessel, the manual depressurization valve was substituted for an automatic depressurization valve that allows a better control and hence higher vesicle homogeneity and batch-to-batch reproducibility. Cryo-TEM images depicted unilamellar and spherical nanovesicles confirming the great degree of homogeneity achieved (Figure 5). According to MicroBCA protein assay, 99 % of BSA was entrapped into the Quatsomes prepared with the larger reactor, similar to the values obtained when using the smaller one. Moreover it is

important to highlight here that DELOS-SUSP operates under sterile conditions due to the use of compressed CO_2, which is another important issue in the manufacturing of vesicles for human and animal use.[46] The good reproducibility in terms of encapsulation percentages and physicochemical characteristics between batches produced with the two reactors, demonstrate the feasibility of scaling-up the method for the encapsulation of hydrosoluble proteins.

In conclusion, we have demonstrated that DELOS-SUSP methodology is a platform that enables an easy and direct preparation of different SUV-biomolecule conjugates with nanoscopic size and great degree of unilamelarity. Moreover, this platform shows "batch-to-batch" consistency and allows the preparation of sufficient quantities of nanotherapeutics for clinical testing. This one-step process allows the preparation of nanovesicles loaded with hydrosoluble proteins, vesicular conjugates functionalized with targeting peptides or stealth polymers with excellent perspectives as drug delivery platforms, and if desired/needed the incorporation of two biomolecules simultaneously. Bioactivity of the integrated molecules is unaffected under the processing conditions with CO_2-expanded solvents. The method overcomes major limitations related with conventional methodologies and offers the possibility to synthesize a great variety of nanovesicle based formulations, in a simpler, less time consuming and more environmentally friendly way. Finally this method may be easily scaled-up following the Good Manufacturing Practices requirements, becoming an attractive methodology for accelerating the clinical translation of nanomedicines based on nanovesicles.

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FIGURES
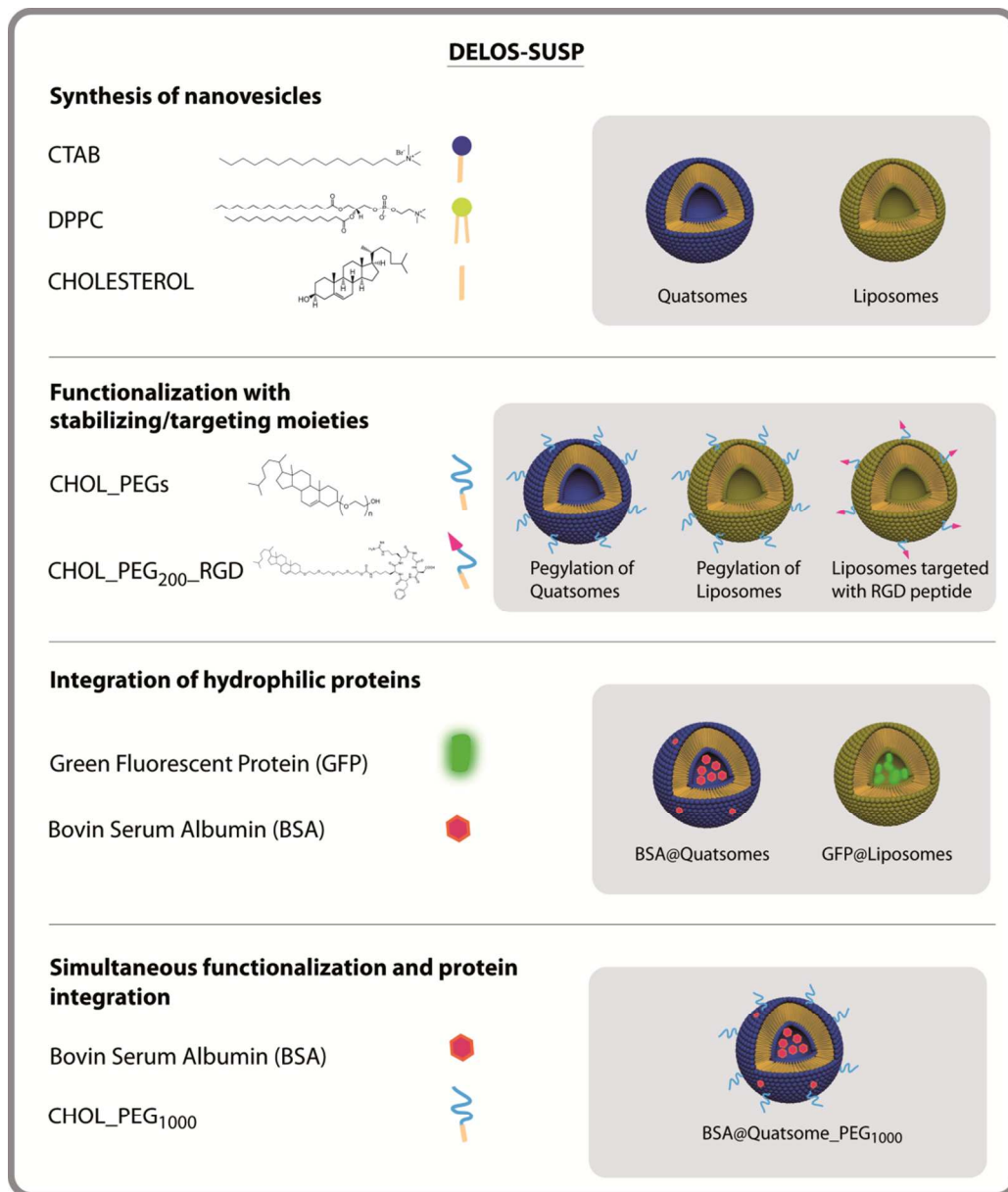
Figure 1. Schematic representation of multifunctional nanovesicle-bioactive conjugates prepared

by DELOS-SUSP method and the molecular structure of their components.

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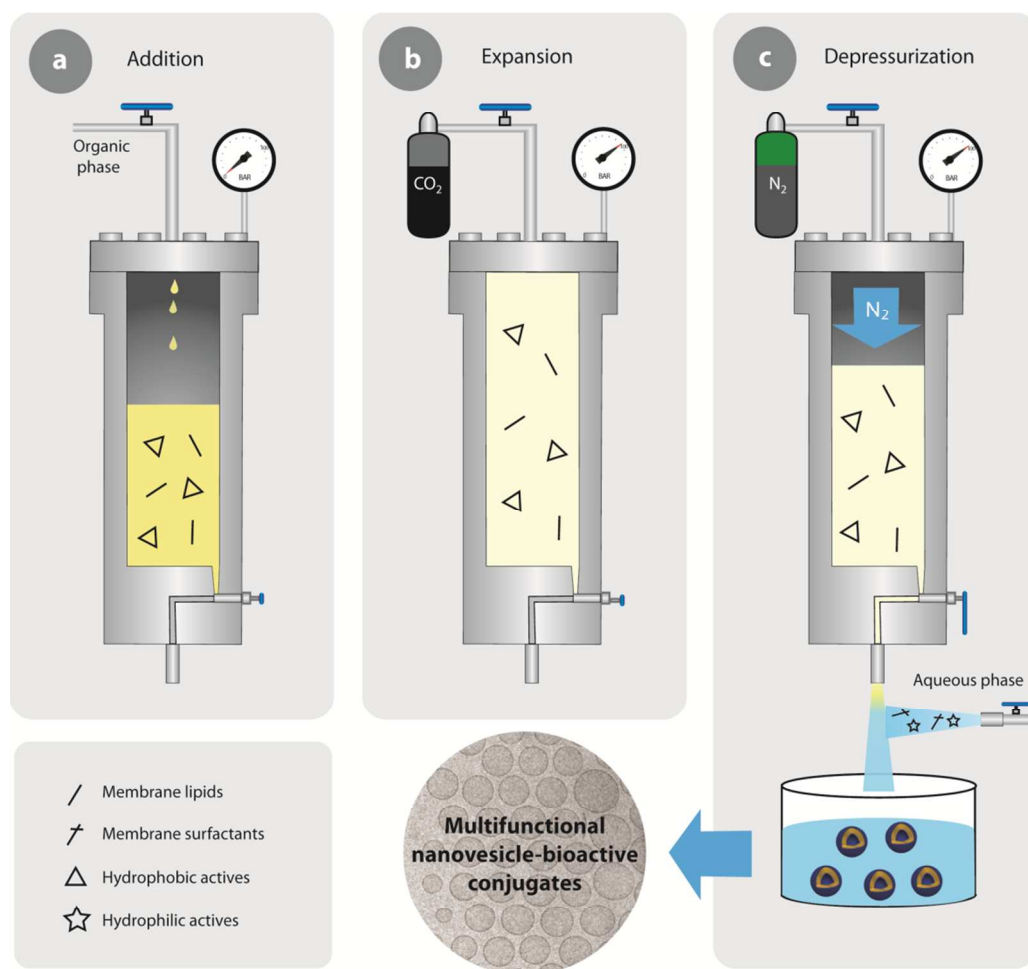
Figure 2. Schematic representation of the DELOS-SUSP method for efficient preparation of multifunctional nanovesicle-bioactive conjugates**.** The whole procedure includes the loading (a) of an organic solution of the lipidic membrane components and the desired hydrophobic active compounds/molecules into an autoclave at a working temperature (T_w) and atmospheric pressure. The addition of CO_2 (b) to produce a CO_2-expanded solution, at a given X_{CO2}, working pressure (P_w) and T_w, where the hydrophobic actives and membrane components remain dissolved. Finally, the depressurization (c) of the expanded solution over an aqueous solution, which might contain membrane surfactants and hydrophilic biomolecules, to produce an aqueous dispersion

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of the nanovesicle-bioactive(s) conjugates with vesicle-to-vesicle homogeneity regarding size

and morphology.
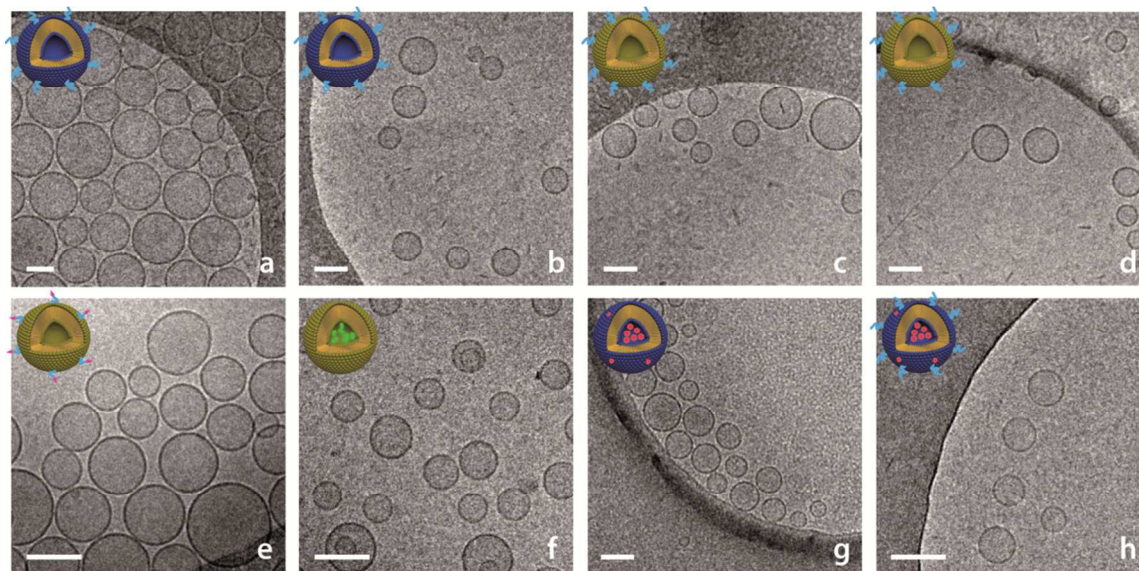
Figure 3. Cryo-Transmission Electron Microscopy images of nanovesicle-bioactive conjugates

produce by DELOS-SUSP. a, PEGgylatedQuatsomes with cholesterol_PEG1000. b,

PEGgylatedQuatsomes with cholesterol_PEG2000. c, PEGylated Liposomes with

cholesterol_PEG1000. d, PEGylated Liposomes with cholesterol_PEG2000. e, Functionalized

Liposomes with targeting units of cholesterol_PEG200_RGD. f, Liposomes with integrated GFP

protein. g, Quatsomes with integrated BSA protein. h, Quatsomes functionalized with a

stabilizing cholesterol_PEG1000 unit and integrated BSA protein. Scale bars are 100 nm.

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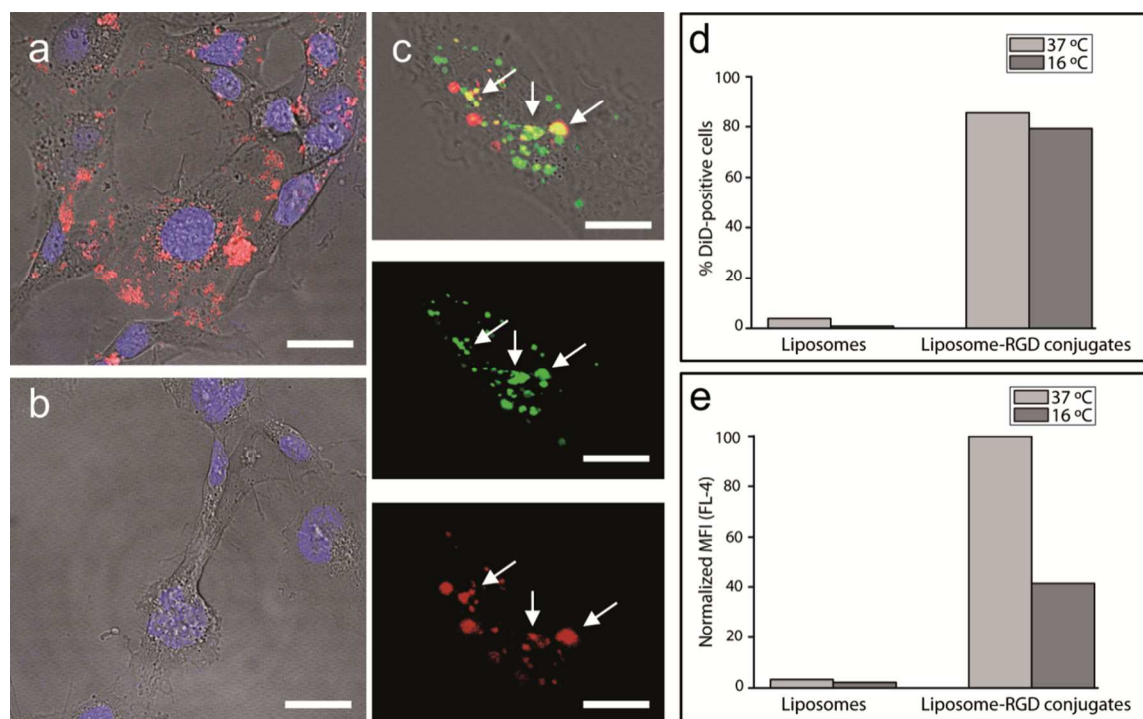
Figure 4. Internalization of nanovesicles on endothelial cells assessed by fluorescence. Confocal

images of HMEC-1 cells incubated during 3 hours at 37°C in a 5% of CO2 atmosphere with a,

Liposome-RGD conjugates (red) at 0.3 mg/mL and b, plain Liposomes at 0.3 mg/mL. Cells

nuclei were stained with Hoechst 33342 (blue). Scale bars are 10 μm. c, Colocalization of

Liposome-RGD conjugates (red) and the lysotracker (green) as observed by confocal

microscopy. The upper image shows merging of both signals, where arrows highlight the co-

localization of Liposome-RGD conjugates with lysosomal compartments. Independent signals

are shown in the lower panels. Scale bars are 10 μm. d, Flow cytometry quantification of the

fraction of cells that had bound or internalized plain Liposomes and Liposome-RGD conjugates

as the percentage (%) of DiD-positive cells among the total number of cells counted. e, Mean

fluorescence intensity (MFI) of DiD in the cells normalized to the maximum fluorescence

intensity. Cells were incubated with plain and functionalized Liposomes for 3 hours at 16°C or

37°C .

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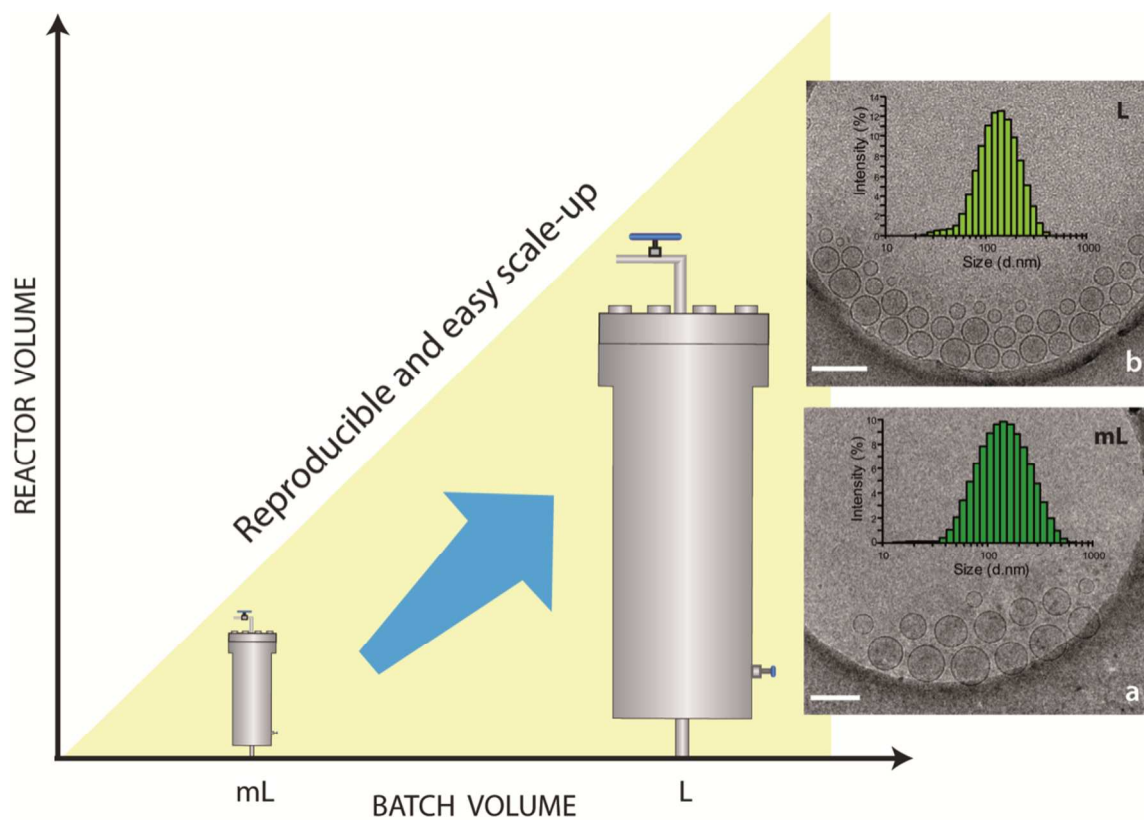
Figure 5. Scale-up of DELOS-SUSP method. BSA protein was integrated into Quatsomes at a concentration of 16 µM in water using both a small (7.5 mL) and a large (315 mL) high pressure reactor that produced 27 mL and 1.3 L of an aqueous dispersion of the nanovesicle-bioactive conjugates, respectively. a, Cryo-TEM image and size distribution of BSA protein loaded Quatsomes obtained at small scale. b, Cryo-TEM image and size distribution of the protein loaded Quatsomes obtained at large scale. After scaling-up the nanovesicles maintain similar physicochemical and morphological characteristics. Scale bars are 200 nm.

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TABLES

Table 1. Physicochemical characteristics of the different vesicular formulations obtained by DELOS-SUSP method

System (Number of batches)	Size		Z potential
	Mean (nm)	PdI	(mV)
Quatsome_PEG$_{1000}$ (3)	67 ± 6	0.15 ± 0.07	69 ± 8
Quatsome_PEG$_{2000}$ (3)	84 ± 0.5	0.20 ± 0.03	38 ±1
Liposome_PEG$_{1000}$ (3)	138 ± 10	0.4 ± 0.1	13 ± 2
Liposome_PEG$_{2000}$ (3)	135 ± 9	0.47 ± 0.04	8 ± 2
Liposome_RGD (4)	144 ± 12	0.19 ± 0.01	31 ± 1
GFP loaded-Liposomes (2)	228 ± 8	0.42 ± 0.02	-1.24 ± 0.06
BSA loaded-Quatsomes (3)	149 ± 12	0.26 ± 0.1	75 ± 7
BSA loaded-Quatsome_PEG$_{1000}$ (2)	82 ± 8	0.23 ± 0.01	52 ± 4

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Table 2. Compositions used for the preparation of the different vesicular formulations by DELOS-SUSP method

System	Organic phase	Aqueous phase	Biom/lipid Ratio (μmol/mmol)	Lipidic conc.* (mg/mL)
Quatsome_PEG$_{1000}$	Cholesterol (48 mM) + CHOL_PEG$_{1000}$ (8 mM)	CTAB (7.8 mM) in water	66	5
Quatsome_PEG$_{2000}$	Cholesterol (38 mM) + CHOL_PEG$_{2000}$ (6.3 mM)	CTAB (7.8 mM) in water	58	5
Liposome_PEG$_{1000}$	Cholesterol (18 mM) + DPPC (27 mM) + CHOL_PEG$_{1000}$ (3 mM)	water	64	1.4
Liposome_PEG$_{2000}$	Cholesterol (14mM) + DPPC (27 mM) + CHOL_PEG$_{2000}$ (2.4 mM)	water	54	1.4
Liposome_RGD	Cholesterol (17 mM) + DPPC (27 mM) + CHOL_PEG$_{200}$_RGD (2.8 mM)	water	59	1.4
GFP loaded-Liposomes	Cholesterol (26 mM) + DPPC (27 mM)	GFP-H6 (1μM) in TRIS buffer (pH =7.5)	0.4	1.4
BSA loaded-Quatsomes	Cholesterol (68 mM)	CTAB (7.8 mM) + BSA (16 μM) in water	1	5
BSA loaded-Quatsome_PEG$_{1000}$	Cholesterol (48 mM) + Cholesterol_PEG$_{1000}$ (8 mM)	CTAB (7.8 mM) + BSA (16 μM) in water	1.2	5

*The lipidic concentration is defined as the total mass of lipids comprising the vesicles divided by the total volume of vesicular suspension.

ASSOCIATED CONTENT

Supporting Information. The Supporting information contains a description of the materials used, the physicochemical characterization of the vesicles, the production of bioactive compounds and the determination of the degree of loading/functionalization in the conjugates. Description of the DELOS-susp method and details on the equipment configurations of the two reactors used is reported. Internalization experiments details and images of the nanovesicles functionalization procedure are shown. Citotoxicity and Hemocompatibility assays are described. Lipid thin-film hydration methodology to produce Liposome-RGD conjugates is reported. Differences in homogeneity of Liposome-RGD conjugates prepared using different methodologies are demonstrated by Cryo-TEM images and DLS measurements. Differences in homogeneity between Liposomes and Liposome-RGD conjugates are demonstrated through SAXS measurements, DLS measurements, and Cryo-TEM images. Dual color EPI-TIRF microscopy images of Liposome-GFP conjugates labelled with DiD are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

I.C. carried out DELOS-SUSP experiments, the physicochemical characterization of the systems, analyzed the data and wrote the manuscript; E.E. participated in the design and engineering of the small scale reactor and assisted in the manuscript preparation; O.E. carried out the TIRF-EPI

and confocal microscopy measurements, the flow cytometry experiments and the cellular uptake experiments; J.L.C. performed the SDS-page and Western-Blot experiments; M.M. and D.P. synthesized the cholesterol_PEG_{200}_RGD molecule and quantified its integration percentage into the liposomes; A.C. participated in the design and engineering of the small scale reactor; E.M. performed the synchrotron SAXS measurements; U.U. and E.V. synthesized the GFP-H6 protein; F.A. and M.R. designed the experiments related to Cholesteol:PEG_{200}:RGD molecule synthesis and quantification; E.V. and A.V. designed the experiments related to florescent protein manipulation; M.G-P. conceived TIRF-EPI, confocal microscopy, cellular uptake experiments and contributed to the correction of the manuscript ; I.A. designed and performed the cytotoxicity and hemocompatibility assays and S.S.Jr. reviewed the obtained results; N.V conceived the DELOS-SUSP process, provided overall scientific guidance and carried out the final edition of the manuscript; J.V. conceived some experiments, provides scientific guidance and contributed to the correction and final edition of the manuscript.

REFERENCES

(1)   Sawant, R. R.; Torchilin, V. P. *Soft Matter* **2010**, *6*, 4026.

(2)   Reflection paper on the data requirements for intraveneous liposomal products developed with reference to an innovation liposomal product. European MedicinesAgency, http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_00056 4.jsp&mid=WC0b01ac05806403e0

(3)   Peer, D.; Karp, J. M.; Hong, S.; Farokhzad, O. C.; Margalit, R.; Langer, R. *Nature Nanotechnology* **2007**, *2*, 751.

(4)   Duncan, R.; Gaspar, R. *Mol. Pharm.* **2011**, *8*, 2101.

(5)   Farokhzad, O. C.; Langer, R. *ACS Nano* **2009**, *3*, 16.

(6)   Hussain, S.; Pluckthun, A.; Allen, T. M.; Zangemeister-Wittke, U. *Mol. Cancer Ther.* **2007**, *6*, 3019.

(7)   Colletier, J. P.; Chaize, B.; Winterhalter, M.; Fournier, D. *BMC Biotechnology* **2002**, *2*.

(8)   Glavas-Dodov, M.; Fredro-Kumbaradzi, E.; Goracinova, K.; Calis, S.; Simonoska, M.; Hincal, A. A. *Acta Pharmaceutica* **2003**, *53*, 241.

(9)   Szoka, F.; Olson, F.; Heath, T.; Vail, W.; Mayhew, E.; Papahadjopoulos, D. *BBA - Biomembranes* **1980**, *601*, 559.

(10)   Kubo, T.; Sugita, T.; Shimose, S.; Nitta, Y.; Ikuta, Y.; Murakami, T. *International journal of oncology* **2000**, *17*, 309.

(11)   Templeton, N. S.; Lasic, D. D.; Frederik, P. M.; Strey, H. H.; Roberts, D. D.; Pavlakis, G. N. *Nature Biotechnology* **1997**, *15*, 647.

(12)   Yatvin, M. B.; Weinstein, J. N.; Dennis, W. H.; Blumenthal, R. *Science* **1978**, *202*, 1290.

(13)   Mayer, L. D.; Hope, M. J.; Cullis, P. R. *BBA - Biomembranes* **1986**, *858*, 161.

(14)   Olson, F.; Hunt, C. A.; Szoka, F. C.; Vail, W. J.; Papahadjopoulos, D. *Biochimica Et Biophysica Acta* **1979**, *557*, 9.

(15)   Eaton, M. A. W. *Journal of controlled release : official journal of the Controlled Release Society* **2012**, *164*, 370.

(16)   Perrut, M.; Clavier, J. Y. *Ind. Eng. Chem. Res.* **2003**, *42*, 6375.

(17)   Hakuta, Y.; Hayashi, H.; Arai, K. *Curr. Opin. Solid State Mat. Sci.* **2003**, *7*, 341.

(18)   Eckert, C. A.; Knutson, B. L.; Debenedetti, P. G. *Nature* **1996**, *383*, 313.

(19)   Jessop, P. G.; Subramaniam, B. *Chem. Rev.* **2007**, *107*, 2666.

(20)   Cano-Sarabia, M.; Ventosa, N.; Sala, S.; Patiño, C.; Arranz, R.; Veciana, J. *Langmuir* **2008**, *24*, 2433.

(21)   Elizondo, E.; Larsen, J.; Hatzakis, N. S.; Cabrera, I.; Bjornholm, T.; Veciana, J.; Stamou, D.; Ventosa, N. *Journal of the American Chemical Society* **2012**, *134*, 1918.

(22)   L. Ferrer-Tasies, E. M.-C., M. Cano-Sarabia, M. Aguilella-Arzo, A. Angelova, S.; Lesieur, S. R., J. Faraudo, N. Ventosa, J. Veciana *(10.1021/la4003803)*

(23)   Cromie, S. R. T.; Ballone, P. *The Journal of chemical physics* **2009**, *131*, 034906.

(24)   Klibanov, A. L.; Maruyama, K.; Torchilin, V. P.; Huang, L. *FEBS Lett.* **1990**, *268*, 235.

(25)   Moghimi, S. M.; Hunter, A. C.; Murray, J. C. *Pharmacological Reviews* **2001**, *53*, 283.

(26)   Dubey, P. K.; Mishra, V.; Jain, S.; Mahor, S.; Vyas, S. P. *Journal of drug targeting* **2004**, *12*, 257.

(27)   Temming, K.; Schiffelers, R. M.; Molema, G.; Kok, R. J. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* **2005**, *8*, 381.

(28)   Schiffelers, R. M.; Koning, G. A.; Ten Hagen, T. L. M.; Fens, M. H. A. M.; Schraa, A. J.; Janssen, A. P. C. A.; Kok, R. J.; Molema, G.; Storm, G. *Journal of Controlled Release* **2003**, *91*, 115.

(29)   Xu, Y. L.; Swerlick, R. A.; Sepp, N.; Bosse, D.; Ades, E. W.; Lawley, T. J. *Journal of Investigative Dermatology* **1994**, *102*, 833.

(30)   Ventosa-Rull, N. C., I.; Elizondo, E.; Veciana, J.;   Sala, S.; Melgarejo, M.; Royo, M.; Albericio. F.; Pulido, D. *Spanish Patent Appl. P201231020* **2012**.

(31)   Pisal, D. S.; Kosloski, M. P.; Balu-Iyer, S. V. *Journal of pharmaceutical sciences* **2010**, *99*, 2557.

(32)   Hubbell, J. A. *Science* **2003**, *300*, 595.

(33)   Luisa Corvo, M.; Jorge, J. C. S.; Van't Hof, R.; Cruz, M. E. M.; Crommelin, D. J. A.; Storm, G. *Biochimica et Biophysica Acta - Biomembranes* **2002**, *1564*, 227.

(34)   Kisel, M. A.; Kulik, L. N.; Tsybovsky, I. S.; Vlasov, A. P.; Vorob'yov, M. S.; Kholodova, E. A.; Zabarovskaya, Z. V. *International Journal of Pharmaceutics* **2001**, *216*, 105.

(35)   Xu, X.; Costa, A.; Burgess, D. J. *Pharmaceutical research* **2012**, *29*, 1919.

(36)   Chalfie, M.; Tu, Y.; Euskirchen, G.; Ward, W. W.; Prasher, D. C. *Science* **1994**, *263*, 802.

(37)   Nasevicius, A.; Ekker, S. C. *Nature Genetics* **2000**, *26*, 216.

(38)   Uskova, M. A.; Borst, J. W.; Hink, M. A.; Van Hoek, A.; Schots, A.; Klyachko, N. L.; Visser, A. J. W. G. *Biophysical Chemistry* **2000**, *87*, 73.

(39)   Holgado, M. A.; Cozar-Bernal, M. J.; Salas, S.; Arias, J. L.; Alvarez-Fuentes, J.; Fernandez-Arevalo, M. *Int J Pharm* **2009**, *380*, 147.

(40)   Worsdorfer, B.; Pianowski, Z.; Hilvert, D. *Journal of the American Chemical Society* **2012**, *134*, 909.

(41)   Yokouchi, Y.; Tsunoda, T.; Imura, T.; Yamauchi, H.; Yokoyama, S.; Sakai, H.; Abe, M. *Colloids and Surfaces B: Biointerfaces* **2001**, *20*, 95.

(42)   Maulik, S.; Dutta, P.; Chattoraj, D. K.; Moulik, S. P. *Colloids and Surfaces B: Biointerfaces* **1998**, *11*, 1.

(43)   Dai, C.; Wang, B.; Zhao, H.; Li, B.; Wang, J. *Colloids and surfaces. B, Biointerfaces* **2006**, *47*, 205.

(44)   Ventosa-Rull, L. C., I.; Veciana, J.; Santana, H.; Martinez, E., Berlanga, J. A. *Cuban Patent Appl. CU2012-0112* **2012**.

(45)   Martins, S.; Sarmento, B.; Ferreira, D. C.; Souto, E. B. *International journal of nanomedicine* **2007**, *2*, 595.

(46)   Lesoin, L.; Boutin, O.; Crampon, C.; Badens, E. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2011**, *377*, 1.
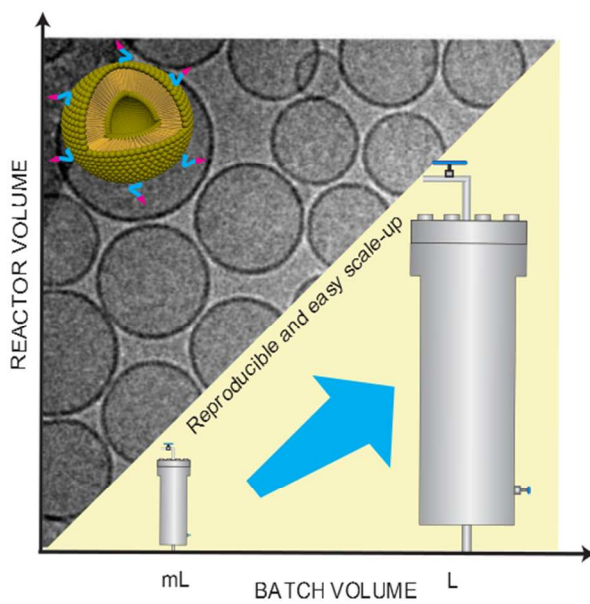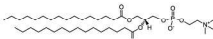
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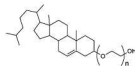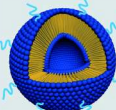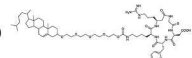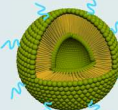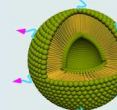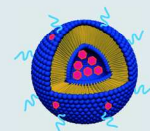
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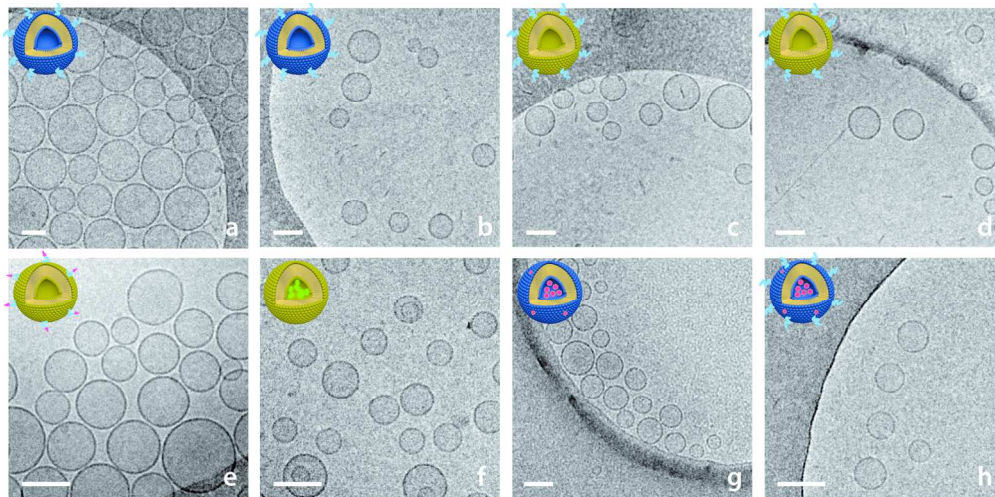
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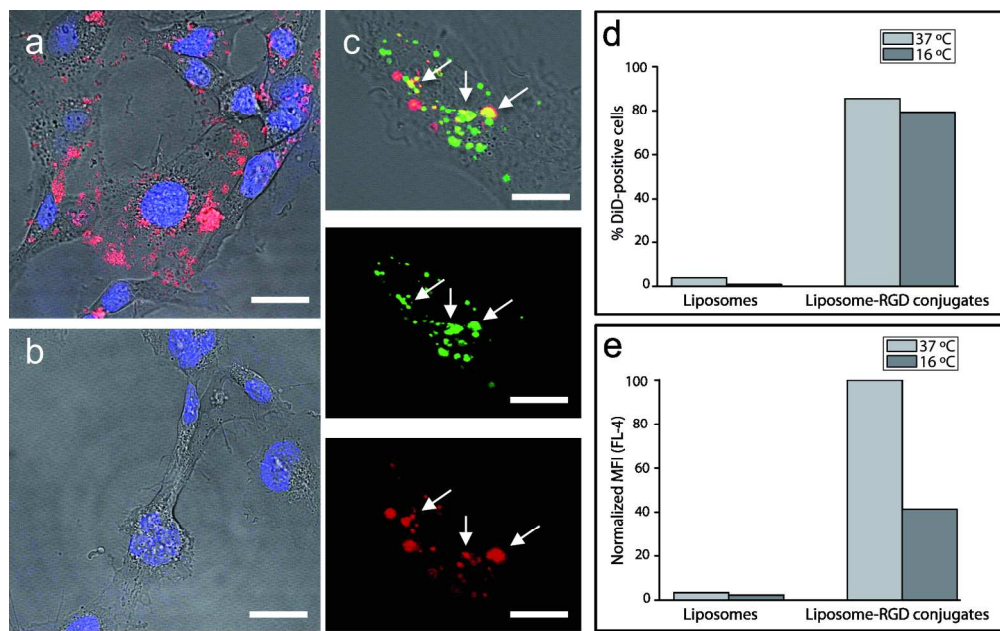
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