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Conception of nanosized hybrid liposome/poloxamer particles to thicken the interior core of liposomes and delay hydrophilic drug delivery

Shayan Ahmed^{1,2,3}, Yohann Corvis^{1,2,3}, Rabah Gahoual^{1,2,3}, Arlen Euan^{1,2,3}, Rene Lai-Kuen⁴, Brice Martin Couillaud^{1,2,3}, Johanne Seguin^{1,2,3}, Khair Alhareth^{1,2,3}, Nathalie Mignet^{1,2,3}.

1 Chemical and Biological Technologies for Health Unit (UTCBS), CNRS UMR8258, 75006 Paris, France

2 UTCBS, INSERM U1267, 75006 Paris, France

3 Faculté de Pharmacie, Université Paris Descartes, Université de Paris, 75006 Paris, France

4 Plateau Technique Imagerie Cellulaire et Moléculaire, CNRS UMS3612, INSERM US25, Université de Paris, 75006 Paris, France

Corresponding author: Nathalie.Mignet@parisdescartes.fr

Abstract

Liposomes are nanocarriers composed of phospholipids, especially designed to potentially carry drugs. However, liposomes suffer in terms of leakage of small hydrophilic drugs. To control the release, a system with lipid shell and polymeric viscous core, namely Hybrid liposome/polymer inside (HLP_{in}), has been designed. For this purpose, we setup a syringe pump apparatus equipped with homemade tubing system. HLP_{in} formulation consisting of poloxamer (5% w/v) was found to be optimal when produced at injection rates of 5 mL.min⁻¹. Then, we tend to characterize the HLP_{in} with DLS, TEM, TRPS, thermal analysis and densitometry in comparison with a polymer added after formation of the liposomes. The optimal formulation was evaluated for its stability and cytotoxicity. The selected conditions and composition resulted in nanocarriers which are highly reproducible with mono-disperse size distribution with an average size of 206 ± 4.8 nm and a polydispersity index of 0.15 ± 0.015. Densitometry and thermal analysis results confirmed the formation of HLP_{in}. Interestingly, HLP_{in} were stable over 2 months, produced no cytotoxicity and exhibited slow release of rhodamine and Doxorubicin in comparison to liposome formulation. Our homemade tubing system coupled with syringe pump apparatus achieved reproducible, precisely controlled production for the HLP_{in} formulation which can be scale up.

Keywords: Hybrid liposome/polymer; Syringe pump; Gelliposomes; Rhodamine; Poloxamer.

Abbreviations

DOPC, 1,2 Dioleoyl-*sn*-glycero-3-phosphocholine; P407, Poloxamer 407; CL, Conventional liposome; HLP_{in}, Hybrid liposome/polymer inside; HLP_{out}, Hybrid liposome/polymer outside; TEM, Transmission electron microscopy; Rhod, Rhodamine B; DLS, Dynamic light scattering; DSC, Differential scanning calorimetry; TRPS, Tunable resistive pulse sensing; CT, Cell toxicity; Gelliposomes, Gl

1. Introduction

Liposomes are vesicles composed of phospholipid molecules enclosing an aqueous droplet. Initially designed to mimic cell membranes *in vitro*, they were consequently proposed as drug delivery system. Due to high loading capacity as well as similarity with cell membrane, liposomes have been implemented to serve in several applications in the field of pharmaceuticals, such as cancer diagnosis and therapy (Torchilin, 2005). Potential application of liposomes as therapeutic tools is still being challenged by their physical and chemical instability, which may result in increased bilayer permeability and drug leakage (Guan et al., 2015). In order to improve the stability of liposomes, many approaches have been suggested like liposomes coated with PEG polymer. However, the use of an external coating could reduce liposomes cellular uptake (Wollina et al., 2003; Zhang et al., 2013). More recently, a new approach has been investigated in order to alleviate the limitations of liposomes; a system composed of a polymeric-core and a lipid shell have been introduced, in this nanosystem both lipid and polymer are unified to yield theoretically stable particles, enhanced encapsulation, controlled and modified release drug delivery system (Mandal et al., 2013).

Polymers form 3D structures in contact with water. Hydrophilic polymers swells when hydrated and expand by the absorption of water (Peppas, 2000). Poloxamers are non-ionic copolymers arranged in triblock of EOx-POy-EOx consisting of ethylene oxide (EO) and propylene oxide (PO) parts, x and y values can have modulated producing a collection of poloxamer derivatives. Poloxamers exhibit low critical solution temperature (LCST). The poloxamer based nanogels have the particularity to be temperature responsive. Small nanogel particles around 120 nm swelled drastically to over 400 nm when temperature rapidly decreased below LCST (Lee et al., 2008).

In the literature hybrid structures of liposomes with polymer or gel are termed differently such as gelliposome or lipogels. These structures design different compositions which possess various characteristics. To control the release of drug, Kim et al formulated hybrid liposomal lipid bilayers with a Poly(Poly(*N*-Isopropylacrylamide-co-acrylic Acid) core (Kim et al., 1997). In 2015, Guan et al prepared gelliposomes composed of liposomes with a gelatin core to enhance the stability and the structure of liposomes (Guan et al., 2015).

In our case, we were interested in developing interiorly viscous core liposomes with no additional charges or chemical component to avoid the risk of toxicity. The most similar works in the literature were carried by Chandaroy and his team who proposed the hybrid Liposomes and very low concentration of Pluronic, the another similar work in the literature was done by Zhang et al. His team proposed interiorly thickened liposomes with high poloxamer concentration of poloxamer (Chandaroy et al., 2001; Zhang et al., 2013). This approach lead to an increase of liposomes stability and provided controlled release of encapsulated drugs. The obtained micro particles having an average size of 1.2 μm and exhibiting an important polydispersity (Zhang et al., 2013). In order to enhance the encapsulation of drug and slow their release while keeping a good pharmacokinetics profile, it would be preferable to obtain monodisperse nanoparticles with controlled preparation process. In this work, we aimed to obtain nanosized hybrid liposome/polymer particles with enhanced inner core viscosity using an optimum concentration of poloxamer, that should enhance the encapsulation and delay the release of drug. For this purpose, an appropriate preparation method with controlled experimental conditions had to be set-up.

Several techniques were described for the preparation of liposomes, the conventional ethanol injection technique provides the advantage of being simple to form vesicles without the application of a lot of energy and the possibility of scale up (Akbarzadeh et al., 2013; Batzri and Korn, 1973; Wagner et al., 2002). Automatization of the process by the merger of ethanolic injection technique with a syringe pump apparatus enhanced the reproducibility while reducing the variability related to the experimenter. Additionally it allows to achieve a large scale production of liposomes (Pham et al., 2012; Pradhan et al., 2008).

In this paper, we developed a hybrid liposome/poloxamer inside (HLP_{in}) formulation using a syringe pump apparatus coupled with a homemade tubing system. We first optimized the composition and the experimental parameters in order to obtain small size and monodispersed particles. The optimal formulation was extensively characterized in term of physico-chemical properties. The cytotoxicity, encapsulation and release kinetics of **two hydrophilic drug models** were then evaluated.

2. Materials

DOPC (1,2 Dioleoyl-sn-glycero-3-phosphocholine) was purchased from Avanti polar Lipids (Alabaster, Alabama, USA), Poloxamer 407 (P407), also known by its trade name as Kolliphor P407 was provided by BASF (Ludwigshafen, Germany), **Doxorubicin Hydrochloride (Sigma Aldrich, France)**, **Rhodamine B (Sigma Aldrich, France)**, Iodine and Potassium Iodine were provided by Prolab (Briare, France). Water was purified on Milli-Q system from Millipore (Fontenay-sous-bois, France), Ethanol 96% was purchased from Carlo-Erba reagents (Val-de-Reuil, France). Ultra-filtration units Vivaspin[®]20 were purchased from Sartorius (Geottingen, Germany), ultra-filtration units NANOSEP 300k was purchased from PALL filtron (Massachusetts, USA), Dialysis device Microfloat-A-lyzer 100 KD was purchased from spectrum laboratories (CA, USA). Polyether ether ketone (PEEK) tubes, T-shaped connectors and ferrules were purchased from IDEX Health & Science (Oak Harbor, WA, USA).

3. Methods

3.1. Preparation of the formulations

In this work, three formulations were designed: Conventional non-ionic liposomes, Hybrid liposome/poloxamer inside (HLP_{in}) and Hybrid liposome/poloxamer outside (HLP_{out}).

Conventional liposomes (CLs) were prepared by the ethanolic injection method using a syringe pump apparatus (Harvard apparatus PHD 2000). One syringe (1 mL) was filled with ethanolic solution of DOPC (20 mg.mL⁻¹, 25.4 μmol.mL⁻¹) and another syringe (10 mL) was filled with Milli-Q[®] water. Both syringes were fixed on the syringe pump apparatus, injection speed was set to 0.5 mL.min⁻¹ for the lipid solution and 5 mL.min⁻¹ for the aqueous solution. Apparatus ran for 1 min giving final volume of 5.5 mL, yielding a final concentration of DOPC (2 mg/mL, 2.54 mmol.mL⁻¹).

Hybrid liposome/poloxamer inside (HLP_{in}) was prepared using the same procedure as liposomes preparation. The aqueous phase was replaced by poloxamer 407 (P407) solution at different concentration (0.5, 5 and 10 % w/v). Different flow rates of P407 solution injection (0.1, 1, 5, 10 and 37 mL.min⁻¹) were tested in order to choose the optimal value. Ratio of the injection flow rates between the lipid solution and the P407 solution was kept at 1:10 for all experiments.

Liposomes with gel outside termed as Hybrid liposome/poloxamer outside (HLP_{out}). HLP_{out} was prepared by first preparing conventional liposomes with concentration of DOPC (4mg.mL⁻¹, 5.08 mmol. mL⁻¹). A poloxamer solution (10 % w/v) was prepared. Liposome suspension and poloxamer solution were then mixed with 1:1 volume ratio to obtain final concentration of lipid and poloxamer similar to HLP_{in}.

In case of rhodamine or Doxorubicin HCL (DOX) encapsulation, the formulations were prepared by following the same procedure discussed above for each formulation. The only difference was the addition of rhodamine or DOX (0.5 mg.mL⁻¹ and 5 mg.mL⁻¹, respectively) in aqueous phase or in poloxamer solution during preparation.

The theoretical calculation of Reynolds Number to determine the laminar flow in the tubes was computed using the equation,

$$Re = \frac{\rho V D}{\mu}$$

Where ρ represents the fluid density, V is the linear velocity of the solution, D the tube diameter and μ the dynamic viscosity of the fluid. The density of a 5% P407 solution was experimentally determined by densitometry (DMATM 4500 M, Anton Paar) performed at 20 °C.

Where $\rho = 1.00612$ kg/L, V is the linear velocity which depends on the injection rate, $D = 0.25$ mm, the viscosity of poloxamer (P407 0.5%) $\mu = 2.29$ cP.

3.2. Evaporation of ethanol

The removal of ethanol was performed using a rotary evaporator. The formulation was collected in an evaporation flask and treated by using a rotavapor (Rotavapor R-144, Büchi, Switzerland) at the following conditions (rotating rate 60 rpm, 35 °C, pressure 10 mbar). These conditions applied for 15-20 min to achieve 25-35% evaporation and 30-40 minutes to achieve 60-65% evaporation of formulation measured by weight which were later adjusted with by adding Milli-Q[®] water.

3.3. Densitometry

The density was measured by using a Densitometer (Anton Paar DMATM 4500 M). Densitometry analysis is based on the principle of oscillating U-tube to determine the density of samples based on an electronic measurement of the frequency of oscillation, from which the density value is calculated. Experiments were performed at temperature 25 and 37 °C. The U-tube was washed with Milli-Q[®] water then with ethanol 96% following by drying with air, before and after each utilization. The oscillating U-tube was filled with about 1.5 mL of sample with a syringe. The U-View TM camera function shows and records live images of the oscillating U-tube to ensure that there should be no bubbles and that the U-tube is entirely filled with the sample.

3.4. Ultrafiltration by Centrifugation

Ultrafiltration column (Pall Filtron, USA) with 300 kDa molecular weight cut-off (MWCO) were used to separate free poloxamer and non-encapsulated rhodamine B from liposomal suspension. The filtration was carried out by centrifugation at 13000 rpm for 30 min in a refrigerated bench-top centrifuge (Hettich Rotanta 460 RF, Germany) at 4 °C.

3.5. Physicochemical characterization

Dynamic light scattering (DLS)

The liposome, HLP_{in} and HLP_{out} diameters were determined at 25 °C by quasi-elastic light scattering using a Zetasizer Nanoseries Nano-ZS (Malvern Instruments, France). The scattering was measured at a 173° fixed angle and the position at 4.65. Each sample was run for 3 measurements. Results were expressed as the average hydrodynamic diameter and the standard deviation of the triplicate.

Transmission Electron Microscopy (TEM)

The liposome, HLP_{in} and HLP_{out} formulations were observed by Transmission Electron Microscopy (TEM). Briefly, 10 µL of the sample was deposited on a carbon-coated copper grids, excess solution was removed after 2 min by a filter paper. This was followed by the application of 5 µL of uranyl acetate 1% to the same grid for 2min, excess stain was then removed with filter paper. Sample is subsequently air-dried at room temperature. The grid placed on a slide was

inserted in the microscope. The sample analysis was performed with a JEOL JEM 100 S (JEOL Ltd Tokyo, Japan) TEM operating at 80 kV. TEM images were captured using an Orius Sc 200 digital Camera (Gatan-Roper Scientific, Evry, France).

Tunable Resistive Pulse Sensing (TRPS)

Measurements were made using a qNano system from IZON Science equipped with an upper and a lower fluid cell, each one containing an Ag/AgCl electrode. The measurements were done using electrolyte (standard provided with Izon kit) solution and a pressure of 0 mbar to allow the passage of the particles through the pore. The membrane stretch was comprised between 45 and 47 mm and the voltage were adjusted in order to have a baseline current between 120 and 145 nA. Data were acquired using Izon Control Suite V3.1. Each acquisition presented is the result of the passage of at least 500 liposomes. The liposome size and concentration were calculated after the measurement of the calibrated particles solutions with Nano-pore (NP250) allowing a measurement of a total size range between 100 and 400 nm according to manufacturer. The results were determined using the IZON Control Suite software.

Thermal analysis

The Differential Scanning Calorimetry (DSC) experiments were performed on the differential scanning calorimeter 822e (Mettler-Toledo, Switzerland) previously calibrated with high purity indium ($T_{\text{fus}} = 156.6\text{ }^{\circ}\text{C}$, $\Delta_{\text{fus}}H = 28.45\text{ J.g}^{-1}$) and zinc ($T_{\text{fus}} = 419.6\text{ }^{\circ}\text{C}$, $\Delta_{\text{fus}}H = 107.5\text{ Jg}^{-1}$). DSC experiments were performed at temperature starting from 5 to 60 $^{\circ}\text{C}$ at 1 K.min^{-1} scan rate under dry air (60 mL.min^{-1}). Empty aluminum pan of 100 μL was hermetically sealed and holed from the top by means of a small pin of controlled size (0.7 mm) and used as a reference.

3.6. Calorimetric assay (Baleux assay)

Poloxamer quantification was carried out by a colorimetric assay as previously described by Baleux et al (Baleux and Champetier, 1972). Briefly, 12.5 μL Iodine solution (iodine solution at 10 mg/mL and potassium iodide solution at 20 mg/mL) was added to 1 mL of diluted sample. The absorbance of the complex was measured after 5 min at 540 nm using UV-Vis spectrophotometer (Varian CARY[®] 100). linearity was checked for standard solutions with

concentration ranging from 0.05 to 100 $\mu\text{g.mL}^{-1}$. Poloxamer concentration of samples were calculated using calibration curve with r^2 of 0.99.

3.7. Stability study

Stability tests were carried out for CLs, HLP_{in} and HLP_{out} formulations for 2 months. The three formulations were prepared, filtered and kept at 4 °C. The liposome and HLP diameters were determined at 25 °C by quasi-elastic light scattering using a Zetasizer Nanoseries Nano-ZS (Malvern Instruments, France).

3.8. Cytotoxicity tests

The murine fibroblast NIH/3T3 (ATCC[®] CRL1658[™]) and liver normal epithelial BNL 1ME A.7R.1 (ATCC[®] TIB75[™]) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, USA). The cell line NIH/3T3 was grown at 37 °C and 5% CO₂ in DMEM containing 2 mM L-glutamine, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin, 10% bovine serum, whereas cell line BNL was grown in the same medium just replacing the bovine serum by a fetal one. Cells were plated onto 96-well plates at 20000 cells per well in 100 μL of culture medium. Twenty-four hours after plating, 100 μL of medium containing the compound of interest (final concentrations ranging from 0.06-2 mg/mL in terms of lipid or 1.5-50 mg/mL in terms of P407, in 2-fold dilutions) was added to the wells. After 24h of exposure, the cell viability was evaluated using the MTT test. Then, the absorbance was read at 560 nm in a microplate reader (Infinite F200 Pro - TECAN). Results are expressed in percent of viability compared to the same concentration of solvent.

3.9. Loading content and encapsulation efficiency

The liposome and HLP_{in} formulations encapsulating rhodamine B (0.5 mg.mL⁻¹) or doxorubicin (5 mg.mL⁻¹) were filtered as described in section 3.3. The sample remained on the filter was re-suspended with Milli-Q[®] water in order to make up to the initial volume and quantified by spectrofluorometry using a Varian CARY[®] spectrofluorometer at **excitation 490 nm and emission 580 nm for rhodamine and at excitation 490 nm and emission 595 nm for doxorubicin**. linearity was checked for standard solutions with concentration ranging from 0.075 to 1 $\mu\text{g.mL}^{-1}$ for rhodamine and 1 to 25 $\mu\text{g.mL}^{-1}$ for doxorubicin. Rhodamine and doxorubicin concentration of

samples were calculated using calibration curve with r^2 of 0.98 and 0.97, respectively. The solutions of rhodamine B and rhodamine B with P407 5% were taken as controls. Loading content and encapsulation efficiency were calculated by using the following equations:

$$\text{Loading Content (\%)} = \left(\frac{\text{weight of loaded drug}}{\text{weight of drug loaded particles}} \right) \times 100$$

$$\text{Encapsulation Efficiency (\%)} = \left(\frac{\text{weight of loaded drug}}{\text{weight of drug in feed}} \right) \times 100$$

3.10. *In vitro* release

A stock solution of rhodamine B (0.5 mg.mL^{-1}) was used to prepare two different samples: solution of rhodamine B encapsulated within liposomes and rhodamine B encapsulated within HLP_{in} formulation. Dialysis device (Micro Float-A-Lyzer, France) was filled with the required volume of each sample and kept floating in a beaker with PBS at 37°C using an agitation rate of 60 rpm, volume of medium to volume of sample ratio was fixed at 1:1000. At defined points, 1 mL of samples were removed from the release medium and replaced with fresh PBS to maintain the volume constant. The amount of rhodamine B released was monitored by spectrofluorometer (Varian CARY®) at 580 nm. *In vitro* release test was performed in triplicates. A similar procedure was followed for Doxorubicin Phosphate (5 mg.mL^{-1}) release study.

3.11. Statistical analysis

For the size measurement and the Stability test, the data was presented as the mean \pm SD. The graph Pad Prism software was used to analyses the data and determine statistical significance between groups for cytotoxicity and release test. Cytotoxicity experiment data was treated with Gaussian nonlinear regression fit and two-way ANOVA was applied $p < 0.001$, ***. The release test graphs presented one phase decay nonlinear regression fit and student t test was applied $p = 0.0001$, ***. All the experiments were performed in triplicates.

4. Results and Discussion

4.1. Conception of the formulations

In this study, a hybrid composition of lipid and polymer was selected to achieve a formulation that offers an improved encapsulation and controlled release of the encapsulated drug. The lipid used for this formulation was DOPC and the polymer used was poloxamer 407 (P407). The designed formulation is referred as Hybrid liposome/poloxamer inside (HLP_{in}). Regarding physicochemical properties, the HLP_{in} system is expected to increase the viscosity and the density of the liposome core by incorporating poloxamer into the aqueous core of the liposomes. This approach should enhance the entrapment of drug and avoid its fast release.

4.1.1. Optimization of the experimental conditions

All formulations were prepared according to the principle of ethanolic injection with the help of a syringe pump device and tubing system that we developed (methods section 2.1). The preparation of formulations with the syringe pump apparatus combines two main principles, the ethanolic injection and microfluidics. The instrumental setting was designed to provide fully controlled and reproducible experimental conditions due to minimum experimenter influence, in addition to offer the possibility to scale up the production (Charcosset et al., 2015). From a formulation standing point, the main advantage is to produce a narrow size distribution of liposomes in a single step, without extrusion or sonication. However, to generate a reproducible formulation having relevant characteristics, several parameters needed to be setup, i) optimization of experimental setup, ii) optimization of formulation regarding the lipid and poloxamer ratio, and iii) optimization of formulation regarding the solvent content.

4.1.1.1. Optimization of experimental setup

In order to optimize the conditions for formulations preparation, the influence of two instrumental parameters on the final preparation properties was studied, a) the tubing internal diameter and b) the injection flow rate. The HLP_{in} formulation with poloxamer concentration (0.5% w/v) was used for the optimization of the experimental conditions. For this experiment, two types of tubing systems with different compositions and internal diameters were considered in order to select the most suitable to prepare the formulations as emphasized in Figure 1. Both preparation systems are comprised of 3 distinctive tubes connected to each other by the intermediate of T-shaped connector (Figure 1A). T-1 tube system was composed of two polyethylene inlet tubes having a 1 mm internal diameter followed after the junction by one outlet tube of 250 μ m internal diameter composed of polyether ether ketone (PEEK) which is high

performance engineering thermoplastic inert to water and the most common chemical solvents (Haleem and Javaid, 2019). Whereas, T-2 preparation system composed of three PEEK tubes with the same internal diameter (250 μm). HLP_{in} formulation was prepared using both preparation systems according to the procedure described in preparation of the formulations (section: 2.2.1).

The second parameter requiring optimization was the injection flow rate. For that purpose, HLP_{in} formulation was prepared using different injection flow rates (0.1 $\text{mL}\cdot\text{min}^{-1}$, 1 $\text{mL}\cdot\text{min}^{-1}$, 5 $\text{mL}\cdot\text{min}^{-1}$, 10 $\text{mL}\cdot\text{min}^{-1}$, 37 $\text{mL}\cdot\text{min}^{-1}$). The optimal injection flow rate was selected according to the DLS characterization of the final preparation (Figure 1B: left and right, respectively). First, we verified that both preparation systems would generate to laminar flow which is necessary to obtain liposomes (Yu et al., 2009). For this purpose, we calculated the Reynolds (Re) number for both preparation systems depending on the injection flow rate. As described in the literature, a Reynold number lower than 2000 is representative of laminar flow conditions, favorable for liquid mixing and to the formation of smaller and uniform size liposomes with low PDI (Bessoth et al., 1999; Jahn et al., 2004; Yu et al., 2009). The Reynold number was calculated for each tube (Table 1). Regardless of the flow rates employed, Re number was systematically well within the range of 2000 and lower for the T-2 tube than the T-1 tube demonstrating the formation of a laminar flow and (Table 1). Therefore, the different configurations T-1 and T-2 were suitable for the formation of liposomes.

Table 1. Re numbers obtained by T-1 and T-2 tube system obtained with different flow rate

Flow Rate ($\text{mL}\cdot\text{min}^{-1}$)	Reynolds number T-1 tube	Reynolds number T-2 tube
0.1	0.06	0.01
1	0.56	0.07
5	2.80	0.35
10	5.60	0.70
37	20.7	2.59

All preparations produced had acceptable range of average hydrodynamic size and PDI except in the case of the lowest infusion flow rate (0.1 $\text{mL}\cdot\text{min}^{-1}$) with Tube-1 system. The comparison showed that the polydispersity (PDI) of the liposomes obtained from T-1 tube was significantly higher than the PDI obtained from T-2 preparation system, as the average PDI for all flow rates was 0.232 and 0.098 respectively. As the T-2 preparation system exhibited lower Re number than

T-1 system at each flow rate (see Table 1), this could explain the formation of formulations with slightly better PDI obtained with the T-2 tube system (Figure 1B: left and right).

The two preparation systems designed to be used in conjunction with the syringe pump demonstrated to be suitable for the production of CLs and HLP formulations in a robust manner. This instrumental approach has the advantage to provide an optimal usage of the volume of the different solutions by minimizing the dead volume of the preparation system. In addition, because it relies on the use of PEEK tubing readily available, it appears as particularly cost-effective approach which can be easily implemented. On the basis of Re numbers and PDI of HLP_{in}, Tube-2 system was considered further for optimizing lipid/poloxamer ratio according to the injection flow rate.

4.1.1.2. Optimization of the formulation regarding lipid and polymer ratio

The lipid and polymer ratio adjustment was the next phase to attain the intended formulation. The lipid to poloxamer ratio was optimized at fixed lipid concentration and varying poloxamer concentration using various flow rates. We observed that the average hydrodynamic size and PDI values increased with the rise of P407 percentage, nearly at all flow rates (Figure 2 A and B). HLP_{in} with poloxamer 0.5% had the smallest size and PDI but at this concentration, it was thought difficult to increase the liposome inner core viscosity. HLP_{in} with poloxamer 5% was found optimal as it exhibits an average size within acceptable range and a slightly better PDI than HLP_{in} with poloxamer 10%.

Finally, the formulation with the optimal average size and PDI was obtained with the T-2 tube system at a flow rate of 5 ml.min⁻¹ with 5% of P407 in HLP_{in}. These parameters were also set for the other two formulations taken as controls, Conventional liposomes (CL) and Hybrid liposome/poloxamer outside (HLP_{out}) in which the poloxamer was added after the formation of liposomes.

4.1.1.3. Optimization of the formulation regarding the solvent content

Density analysis was performed in order i) to optimize the evaporation procedure with respect to HLP integrity, and ii) to evidence the internalization of the polymer within the liposomes. As far as the validation of the evaporation procedure is concerned, it has been demonstrated that the control of ethanol/water quantity in the formulation is necessary to prevent liposomes from

dissolving (in case of too high quantity of ethanol, *e.g.* 10% in the present study) or aggregating (in case of water over-evaporation). To optimize the evaporation procedure, the obtained formulations were evaporated up to 25-35% and 60-75% at controlled temperature and pressure. As it can be seen in Table 2, volumetric mass of HLP_{in} and HLP_{out} was differentiated only for formulation evaporated up to 25-35%. In the case of non-evaporated formulations, a slight differentiation was noticed by comparing conventional liposome formulations and water/ethanol 90/10 v/v solutions volumetric masses. Similarly, volumetric mass was quite comparable for the HLP_{in} and HLP_{out} formulations with 60-75% evaporation + q.s. Milli-Q[®] water. These results could be explained by relative disruption of the liposomes membrane leading to the same P407/lipid system.

Table 2. Volumetric mass of different formulations as function of the ethanol/water ratio at 25 °C

Formulation	Volumetric mass (g.cm ⁻³) 25 °C		
	Without evap.	25-35% evap. + q.s.	60-75% evap. + q.s.
Milli-Q water	0.99705	0.99705	0.99705
Water/ethanol (90/10, v/v)	0.98462	0.99637	0.99702
Conventional liposomes	0.98644	0.99634	0.99685
HLP _{out}	0.99743	1.00214	1.00236
HLP _{in}	0.99171	0.99881	1.00325

Interestingly, densitometry analysis has also demonstrated that with 25-35% of evaporation of the ethanol/water medium, less than 0.3% of ethanol was remaining in the final formulations. Such ratio makes the formulation more stable and suitable for *in vivo* administration.

Furthermore, once the overall formulation procedure has been optimized (*i.e.* 5% P407, 25-35% evaporation + q.s. Milli-Q[®] water), densitometry experiments help to have a first insight into HLP_{in} and HLP_{out} properties differentiation. For that purpose, a poloxamer density calibration curve has been firstly established (data not shown) and highlights the fact that volumetric mass of aqueous solution of P407 is directly proportional to the polymer concentration. This could be helpful to estimate the poloxamer amount in the HLP formulations. As far as the present results are concerned, a 5% P407 solution have a volumetric mass of 1.00253 g.cm⁻³ at 25 °C. This density value is similar to that of the optimized HLP_{out} formulation, possibly due to the fact that P407 in the HLP_{out} formulation is present in the continuous medium. For the HLP_{in} formulation,

the density is lower compared to the 5% P407 solution but higher compared to the conventional liposome prepared the same way. This suggests that the polymer is encapsulated within the liposomes.

To apprehend the HLP_{in} and HLP_{out} behavior at body temperature, densitometry experiments were also performed at 37 °C (Table 3). A similar behavior is observed, indicating that the liposome as well as the poloxamer entities undergo no change at that temperature. We assume that, at the body temperature, the encapsulation efficiency should be the same as at room temperature. These results will comfort the further *in vitro* and *in vivo* experiments. As far as the current results are concerned, a 5% P407 solution have a volumetric mass of 0.99816 g.cm⁻³ at 37 °C. To resume, the densitometry experiment demonstrated that 25-35% of evaporation of the continuous medium was the best range to optimize the HLP formulations by maintaining its required characteristics.

Table 3. Volumetric mass of different formulations after ethanol/water evaporation up to 25-35% + q.s. Milli-Q® water 25 and 37 °C

Formulation	Volumetric mass (g.cm ⁻³) 25°C	Volumetric mass (g.cm ⁻³) 37°C
Milli-Q water	0.99705	0.99328
Conventional liposomes	0.99634	0.99263
HLP _{out}	1.00214	0.99766
HLP _{in}	0.99881	0.99431

Concurrently, this procedure allows removing the maximum of ethanol to make the HLP formulations biocompatible. The formulations retained with the help of the densitometry experiments were then filtrated and considered for further characterization studies such as physicochemical experiments, within the objective of accessing the poloxamer encapsulated inside liposome for the HLP_{in} formulations.

4.2. Physicochemical characterization

4.2.1. Dynamic light scattering (DLS)

The results of the particle sizing of our formulations are summarized in table 4. The table shows the z-average diameters, the polydispersity index obtained for the various liposome, HLP_{in} and HLP_{out} formulations. The z-average diameter values are the mean of measurements repeated on 5

samples of each formulation of CL, HLP_{in} and HLP_{out}. The data shows that the measurements are reproducible for each formulation. In addition, the results show that the size is dependent of the formulation composition. We obtained nanoscale particle size within a range suitable for systemic delivery. This was definitely an advances as regard to the microscale particles gelliposomes reported in the literature (Guan et al., 2015; Zhang et al., 2013)

Table 4. DLS results of size and PDI for CL, HLP_{out} and HLP_{in}

Formulation	Size (nm)	PdI
CL	91 ± 12.8	0.22 ± 0.019
HLP _{out}	183 ± 5.5	0.17 ± 0.016
HLP _{in}	206 ± 4.8	0.15 ± 0.015

The physical characterization of liposomes, HLP_{in} and HLP_{out} is of great importance in understanding their stability and suitability for the variety of applications. After size determination, the TEM imaging was carried out to examine the morphological difference between the formulations.

4.2.2. Transmission electron microscopy (TEM) and Tunable resistive pulse sensing (TRPS)

TEM images of liposome, HLP_{in} and HLP_{out} revealed the structure and morphology of each system. The morphology of these formulations was different from each other (as shown in fig: 3 left panel). The liposome exhibits various morphologies lose their defined shape related to the effect of grid drying. HLP_{out} exhibited more defined structures with few clusters. The deformation of the HLP_{out} vesicles can be explained by a significant amount of free poloxamer in the continuous medium. Interestingly, HLP_{in} showed regular structures under TEM observations (Figure 3A, B, and C, left panel).

To complete the DLS and TEM data, the number of nanoparticles obtained were determined using tunable resistive pulse-sensing. The liposomes and HLP_{out} tend to exhibit a main size distribution between 150 to 200 nm with concentration 1.4×10^9 particles/mL and from 170 to 250 nm with concentration 1.5×10^9 particles/mL respectively (figure 3 A and B graph at right).

HLP_{in} tends to exhibit a broader range from 150 to 200 nm with concentration 7.0×10^8 particles/mL (figure 3 C graph at right). These interpretations indicate that the HLP_{in} formulation may contain encapsulated poloxamer as it contains a smaller number of particles, which can be

Formulation	Experimental T _{mic} (°C)	Experimental ΔT _{mic} (°C)	P407 %	Extrapolated T _{mic} (°C)	Effective ΔT _{mic} (°C)	due to mor e hom oge neo
Free P407	23.6	–	5.0%	–	–	
HLP _{in}	23.6	–	5.0%	–	–	
HLP _{out}	23.6	–	5.0%	–	–	
filtrated HLP _{in}	29.1	5.5	3.4%	24.2	4.9	
filtrated HLP _{out}	27.2	3.6	2.3%	24.8	2.4	

us shape and size as observed in TEM images and a better PDI obtained with DLS. The poloxamer inside the liposome might provide stability, strength and viscosity to the core of liposomes (Zhang et al., 2013). In order to verify this hypothesis, thermal analysis experiments were performed from

Table 5. Experimental and recalculated temperature of micellization

5 to 60 °C, as the poloxamer suspension presents a micellization transition in this range of temperature, and since the poloxamer environment may impact such a transition.

4.3. Differential scanning calorimetry (DSC)

Poloxamer behavior as a function of temperature in aqueous medium is well known and discussed in numerous publications (Chen et al., 2013; Dumortier et al., 2006; Pembouong et al., 2011). Indeed, one can have access to the poloxamer micellization transition by deriving the heat flow vs. temperature signal obtained from the DSC analysis of the poloxamer solution. An endothermic signal is then obtained with a peak position corresponding to the micellization temperature. This transition corresponds to the removal of water (likely dehydration process) from PPO of the PEO-PPO-PEO chains of the poloxamer during the micelles formation (Alexandridis et al., 1994; Ur-Rehman et al., 2010). Since the micellization temperature decreases with concentration, and since the signal area is proportional to the concentration, the micellization temperature is inversely proportional to peak height. No difference in micellization was noticed between P407 5% aqueous solution, unfiltered HLP_{in} and HLP_{out} formulations (Fig.

8 A, B, and C). This is due to the fact that free poloxamer influences the thermal transition of the overall lipid/polymer systems. But once HLP_{in} and HLP_{out} are filtrated, one can easily distinguish both formulations since their temperatures of micellization are shifted to higher temperatures (Figure 4 D and E). Interestingly, P407 temperature of micellization (T_{mic}) for filtered HLP_{in} is 5.5 °C higher than that of both unfiltered HLP_{in} and free P407 5% formulations. As far as filtered HLP_{out} is concerned, P407, the micellization temperature is 3.6 °C higher than that of both unfiltered HLP_{out} and free P407 5% formulations (see experimental ΔT_{mic} data in Table 5).

This result should support our former hypothesis of encapsulated poloxamer in the HLP_{in} vesicles only if we can certify that decrease in P407 quantity after the filtration process is not the only factor responsible of such a shift in the temperature of micellization. To evaluate the concentration contribution to T_{mic} data for filtrated HLP_{in} and HLP_{out} formulations, a T_{mic} vs. concentration curve calibration has been established (results not shown), and then correlated to calorimetric assay of poloxamer in filtrated in and out formulations. The results of the latter experiment are presented hereafter in part 4.4 and are also considered for some data in Table 5. By measuring temperature of micellization for several P407 aqueous solutions in the 5% to 20% range of concentrations, the relation between T_{mic} and P407 concentration ($C\%$) allowed us to obtain a linear correlation that is fitting by the following equation, $T_{mic} = -0.53 \times C\% + 26.0$ ($r^2 = 0.99999$). P407 concentration will be later determine by calorimetric assay, allowing to recalculate the temperature of micellization (extrapolated T_{mic}) that would correspond to phase transition temperature taking into consideration the polymer concentration decrease due to the filtration. Finally, the extrapolated T_{mic} allows determining the effective variation of temperature of micellization (effective ΔT_{mic}) between the filtereted and unfiltereted formulations, independent to P407 variation of concentration. The latter result clearly indicates that the effective increase of 4.9 °C for filtrated HLP_{in} T_{mic} is due to the poloxamer encapsulation within the liposomes. By similarity, the effective increase of 2.4 °C for filtrated HLP_{out} T_{mic} can be explain by i) a percentage of poloxamer entrapped in the liposomes, and/or ii) a specific interaction between poloxamer and the DOPC. It is noteworthy to mention here that mixtures of unorganized DOPC and poloxamer do not modify the temperature of micellization of poloxamer compared to that of free poloxamer (results not shown).

4.4. Calorimetric assay (Baleux assay)

To determine the quantity of poloxamer entrapped inside the liposomal shell, calorimetric assay was carried out by means of poloxamer/KI₃ interactions. This has been made possible since we demonstrated that DOPC presence does not influence the quantification of poloxamer by such a technic (results not shown). The estimated percentages of entrapped poloxamer for the filtrated HLP_{in} and HLP_{out} formulations were 67% and 45%, respectively. The distinguishable data between these two formulations confirm the TEM images previously described. Indeed, HLP_{in} have higher amount of entrapped poloxamer than HLP_{out}. Additionally, the results obtained allow us to interpret the DSC data.

4.5. Stability

The liposome stability is the limit to which a drug substance or product retains the same properties and characteristics, throughout its period of storage and use (Winterhalter and Lasic, 1993). Stability study of liposome, HLP_{out} and HLP_{in} formulations carried in terms of measurement of average size and PDI. The samples were kept at 4 °C during the whole experiment of 2 months. The results were compared between day 0 and day 58 in terms of average size and PDI. In case of conventional liposomes and HLP_{in} no significant difference was observed in terms of their average size and PDI. Whereas in HLP_{out} formulation, the change in average size and PDI was observed that may be due to presence of substantial amount of P407 which leads to liposomal membrane disruption. The stability study was also discussed previously by Guan et al showing that the stability of liposomes was definitely improved in gel core liposomes (Guan et al., 2015)

The stability of our formulation was notable in comparison of HLP_{out} formulation used as control. This experiment confirms that our main formulation HLP_{in} was stable enough and should be considered for next experiments. Therefore, we first assessed its cytotoxicity in two different cell lines.

4.6. Cytotoxicity

To investigate the cytotoxicity of our formulations *in vitro*, the study was carried out on two different cell lines, NIH 3T3 and BNL. Both the cell lines were treated with each formulation (CL, HLP_{out}, HLP_{in}, and P407 10%). The viability results for each cell lines are presented in Figure 6 A and B, as a function of lipid (left) and P407 concentration (right). The mean inhibition

concentration 50 (IC50) values determined for each curve is shown in Table 6. Cytotoxicity results obtained for HLP_{in} and HLP_{out} formulations in both cell lines were similar to the cytotoxicity of conventional liposomes and poloxamer P407.

Table 6. IC50 values as function of Lipid and P407 on NIH/3T3 and BNL cells

Cell lines		CL	HLP _{out}	HLP _{in}	P407
NIH 3T3 (IC50)	Lipid	>2	1.4 ± 0.1	1.5 ± 0.2	--
	P407	--	37.9 ± 5.5	35.2 ± 5.8	28.1 ± 7.6
BNL (IC50)	Lipid	1.3 ± 0.2	1.5 ± 0.4	1.3 ± 0.2	--
	P407	--	36.6 ± 12.8	32.7 ± 7.0	38.5 ± 4.7

In BNL cell line the IC50 value was approximately 1.5 mg/mL in terms of lipid and 35 mg/mL in terms of P407. However, on NIH/3T3 cells, liposome showed low toxicity with 80% of viability at 2 mg.mL⁻¹ of lipids (p<0.001, ***). Hybrid lipid/poloxamer formulations induced a little toxicity in NIH3T3 cell line, probably due to P407 because IC50 was around 35 mg.mL⁻¹. The MTT assay revealed the no cytotoxicity over the concentration of poloxamer in our preparations (approximately 30 mg.mL⁻¹ for HLP_{in} and 25 mg.mL⁻¹ for HLP_{out}, measured after filtration by calorimetric assay). This can be ascribed to the biocompatible nature of lipid and poloxamer in our formulations.

4.7. Encapsulation efficiency and loading content.

The main idea to design this lipid/polymer hybrid a carrier was to improve the encapsulation and/or release kinetic of hydro-soluble drugs. For the evaluation of encapsulation ability, Rhodamine B was used as a model drug and doxorubicin as a drug. Three different concentrations of drug solution were used to evaluate the effect of initial drug solution concentration on encapsulation efficiency and loading content. Rhodamine or doxorubicin were added in H₂O or P407 solution and encapsulated during the preparation of CL and HLP_{in}, respectively. The encapsulation efficiency and loading content of HLP_{in} was compared to those of liposomes (Figure 7 A for rhodamine and B for doxorubicin). For both nanosystems, EE %

and LC % were increased for both drugs when increasing the initial concentration. Differences in encapsulation values between CLs and HLP_{in} were significant for the low initial concentrations of drugs. It was previously reported that the interior content of liposomes is a key factor for increasing encapsulation (Guan et al., 2015). This implies that the incorporation of rhodamine in thickened liposomes should have been greater than encapsulating rhodamine in empty liposome. However, no difference was observed at the highest concentrations, 0.5 mg.mL⁻¹ and 5 mg.mL⁻¹ for rhodamine and doxorubicin, respectively.

To examine if these two formulations could exhibit a different release kinetics, a release study was carried out on these two formulations. The high concentrations for both drugs was used for the release study, this choice was made considering the volume of release medium and the limit of detection of the spectrofluorometer.

4.8. *In vitro* release kinetics

In vitro release studies were conducted to determine the difference between drug solutions (rhodamine or doxorubicin), encapsulated drug in liposomes and HLP_{in} from 1 to 24 hours (Figure 8 and 9, respectively). All the experiments were performed in triplicates at 37 °C. The half-life of rhodamine solution, rhodamine release from CL and HLP_{in} were close around 1.83, 1.34 and 2.18 hours, respectively. However, the final release from these formulations after 24 h was significantly different. It reached 94% for the rhodamine solution, 91% for CL and only 73% for HLP_{in}. This significant variance is probably due to the viscous core of HLP_{in} which lead to a slower release of rhodamine.

In vitro kinetics were also carried out with doxorubicin. The final release of doxorubicin from these formulations after 30 h was significantly different which was 96% for the doxorubicin solution, 56% for CL and 89% for HLP_{in}. Interestingly, the release of doxorubicin from HLP_{in} displayed delayed release of drug comparing to CLs. The amount of doxorubicin released during the first hour was relatively lower as compared to the one of CLs. Noteworthy, HLP_{in} exhibited a release half-life of doxorubicin 3 times higher than the one with CL. This substantial variation between the CLs and HLP_{in} might probably be attributed to the inner core viscosity of HLP_{in} which led to delayed and slower release of doxorubicin.

The release of doxorubicin from CLs reached to a plateau around 4 hours whereas a large part of drug payload remained associated to the nanosystem. In contrast, a continuous release throughout

the experiment was observed in the case of HLP_{in}, this can be explained by the presence of poloxamer in the nanosystem which released into the release medium that improving the Dox solubility. The characteristic of poloxamer to keep the drugs solubilized and stable in term of their size has been described in previous literature. (Bodratti and Alexandridis, 2018; Couillaud et al., 2019; Devi et al., 2013).

If we compare HLP_{in} to Gelliposomes (GLs) formulation described in the literature by Zhang et al, the kinetic profile of our formulation was in first order and not biphasic. This could be explained by the fact that the HLP_{in} core was viscous but not gelified like GLs. Moreover, the sustained release of the rhodamine and Dox in HLP_{in} was faster than GLs which have a relatively bigger particle size (1.2µm) and higher amount of poloxamer.

5. Conclusion

HLP_{in} nanoparticles formulation was effectively produced by using syringe pump system. The poloxamer provides viscosity to aqueous core of liposomes which prevents the leakage of small hydrophilic drug and increase the entrapment of drugs. The physicochemical characterization of our HLP systems opens a path for better understanding of formulating vesicles encapsulating polymers. The formation of HLP_{in} was confirmed by DSC and density analysis. *In vitro* release of HLP_{in} showed slow release in comparison of CL in 24 hours for rhodamine and 30 hours for doxorubicin. HLP_{in} formulation found to be stable for 2 months of storage and shown no cytotoxicity. In conclusion, HLP_{in} present different physiognomies to be used as controlled drug delivery systems. Our homemade designed syringe pump tubing achieved reproducible, economical, precisely controlled fabrication for the liposomes, HLP_{in} formulations which can be scale up with a minimal influence of experimenter.

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Figure captions

Figure 1. Comparison between two Tube System T-1 (left) and T-2 (right), (a) Pictorial illustration, (b) Graphical representation of size and PDI of liposomes obtained with different flow rate of each tube system (n=3).

Figure 2. Tube system and formulation optimization (a) Graphical presentation of hydrodynamic average size of HLP_{in} obtained with different poloxamer (P407) percentages at different flow rates (0.5, 1, 5, 10, and 37 mL.min⁻¹), (b) Graphical presentation

Figure 3. (A) Conventional liposomes as spherical unilaminar vesicles of variable sizes (24-329 nm), (B) HLP_{out} as heterogenous mixture with non-definite shape particles with size range (30-445), and (C) HLP_{in} as unilaminar vesicles of variable sizes (50-188 nm) .

Figure 4. Derived DSC curves obtained (A) P407 5%, (B) HLP_{out}, before filtration, (C) HLP_{in}, before filtration, (D) HLP_{out}, after filtration, and (E) HLP_{in} after filtration.

Figure 5. Stability test graph corresponds to the difference in size and PDI of conventional liposomes, HLP_{in}, and HLP_{out} with in a period of 2 months.

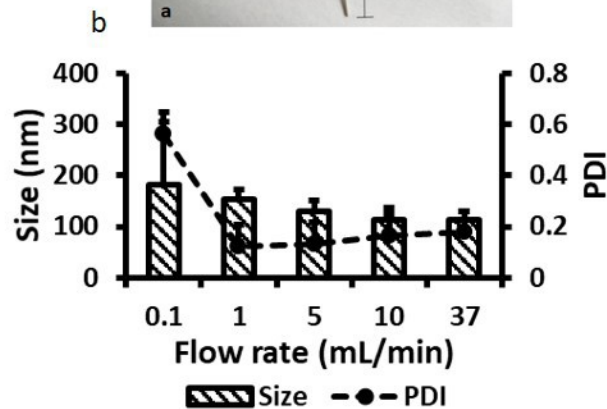
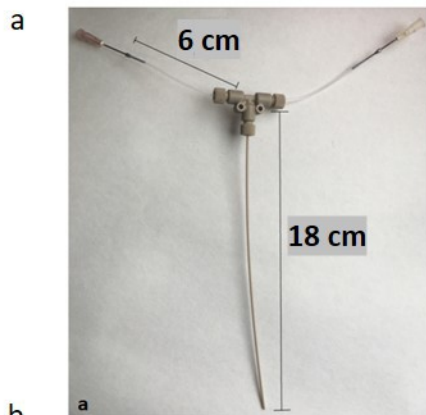
Figure 6. *In vitro* cell viability curves A) Graphs for cell line NIH/3T3 cell viability in relation of Lipid and P407 concentration, left and right respectively B) Graphs for cell line TIB75 cell viability in relation of Lipid and P407 concentration, left and right respectively. Gaussian nonlinear regression fit and two-way ANOVA was applied p<0.05.

Figure 7. A) Encapsulation efficiency and loading content of rhodamine B inside CIs and HLP_{in}. B) Encapsulation efficiency and loading content of doxorubicin inside CIs and HLP_{in}. Two way ANOVA p=0.01, **.

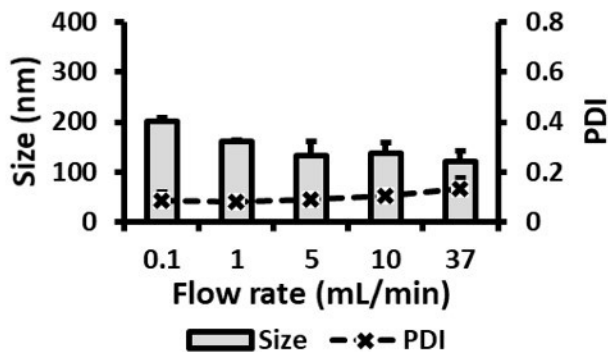
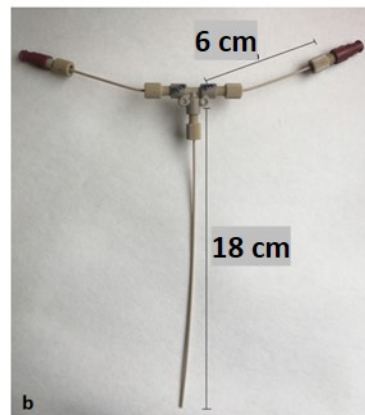
Figure 8. Release of Rhodamine B from nonionic lipids vesicles with and without poloxamer. Formulation composition: (●) Rhodamine solution, (□) Liposome encapsulating Rhodamine, (■) HLP_{in} encapsulating Rhodamine. One phase decay nonlinear regression fit and student t test p=0.0001, ***.

Figure 9. Release of Doxorubicin from nonionic lipids vesicles with and without poloxamer. Formulation composition: (●) Doxorubicin solution, (□) Liposome encapsulating Doxorubicin, (■) HLP_{in} encapsulating Doxorubicin. One phase decay nonlinear regression fit and student t test p=0.0001, ***.

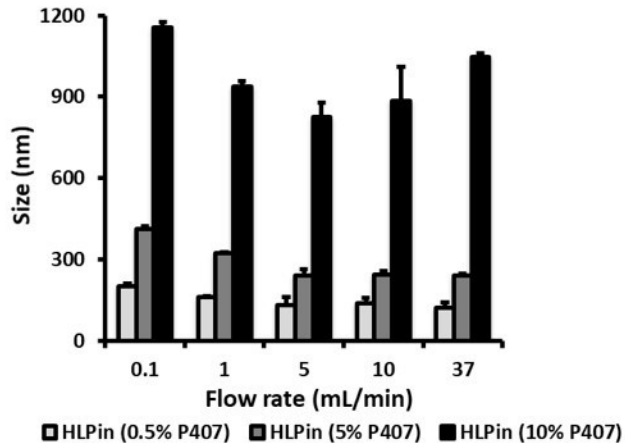
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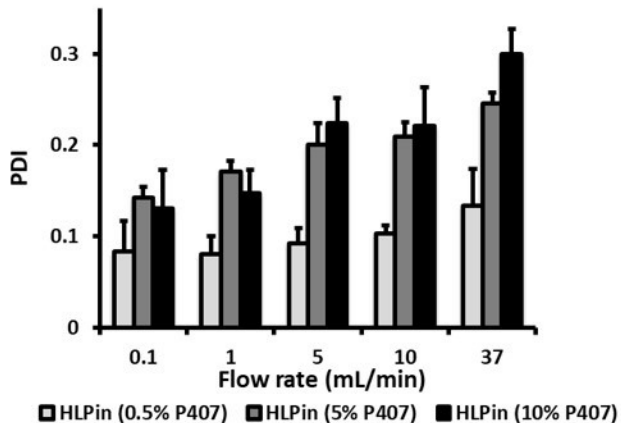
T-2 Tube system

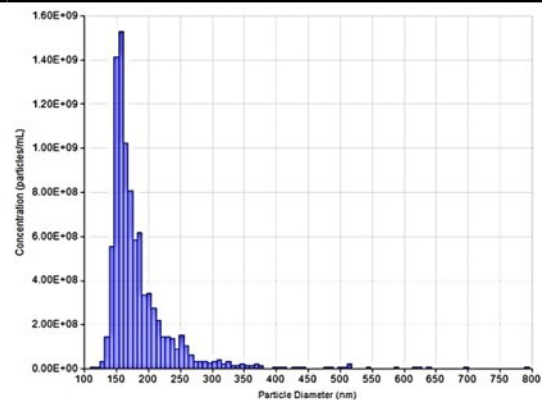
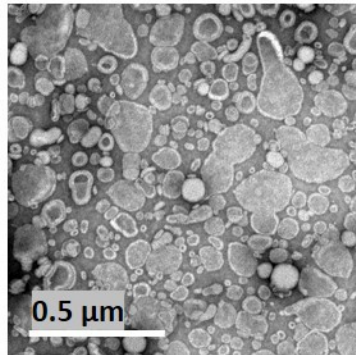
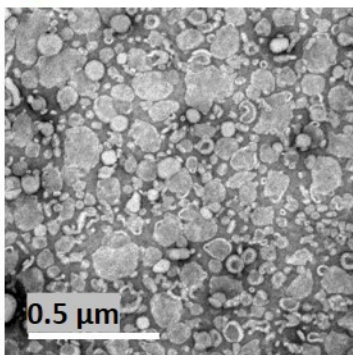
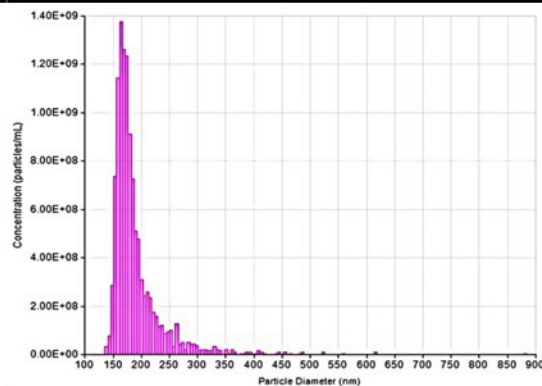
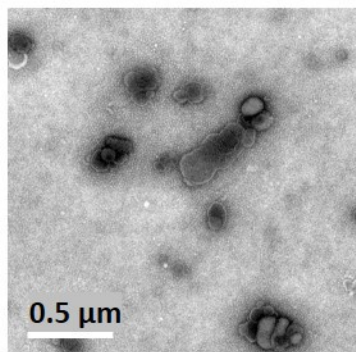
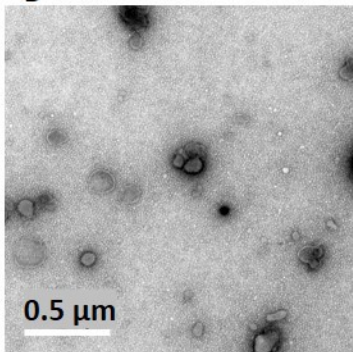
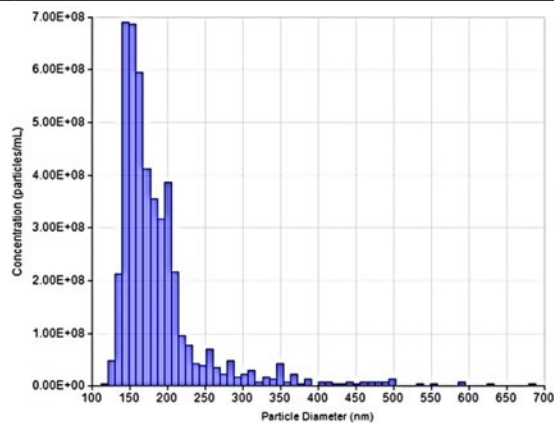
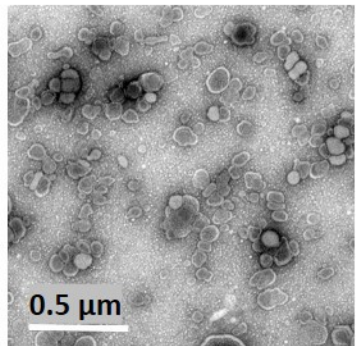
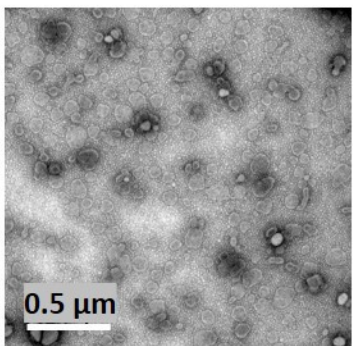


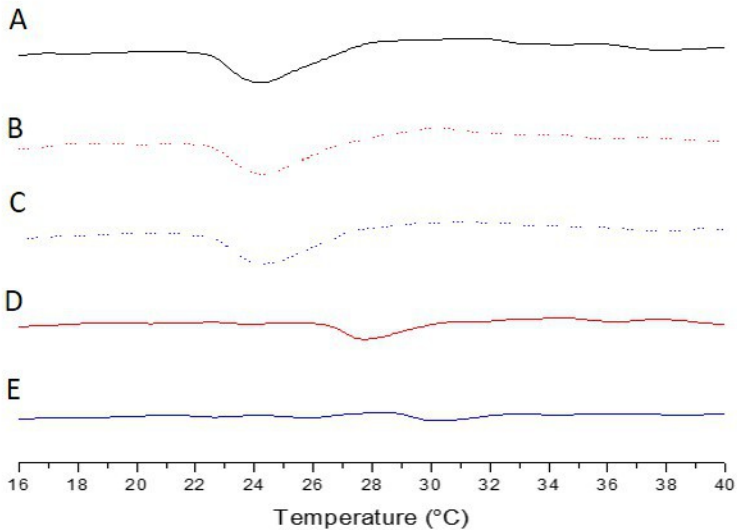
A

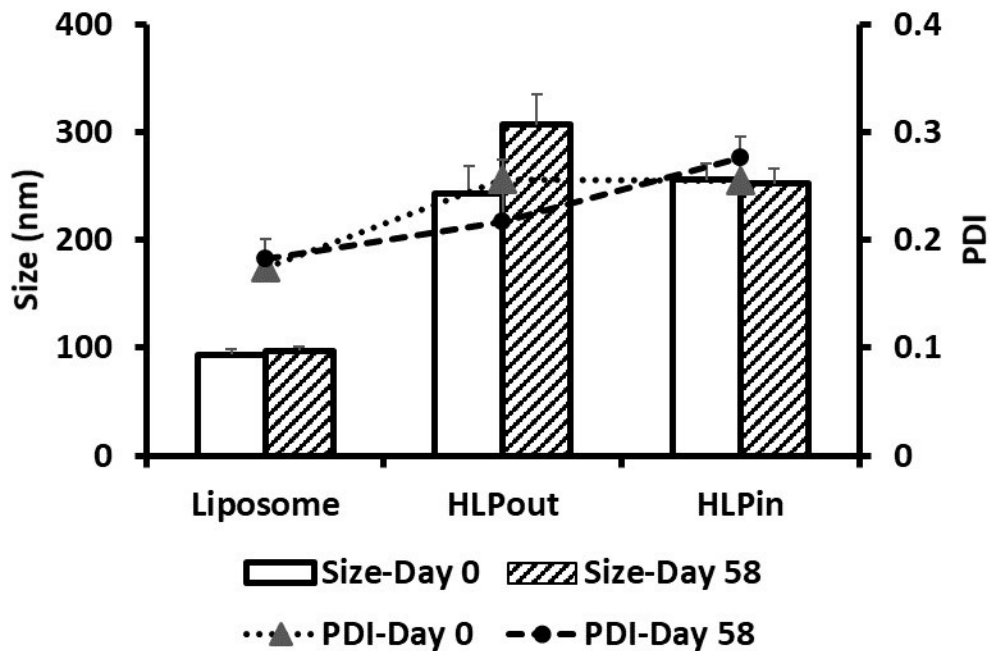


B



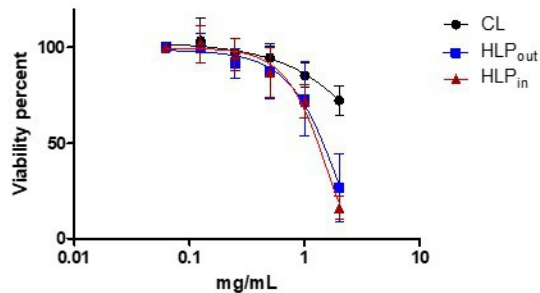
A**B****C**



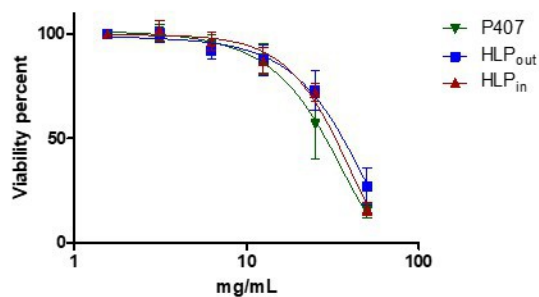


A

Lipid concentration

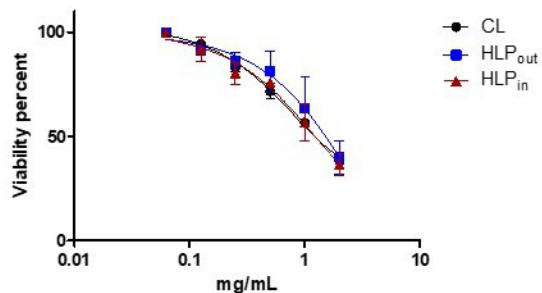


P407 concentration

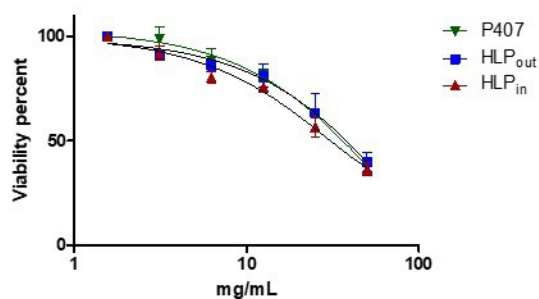


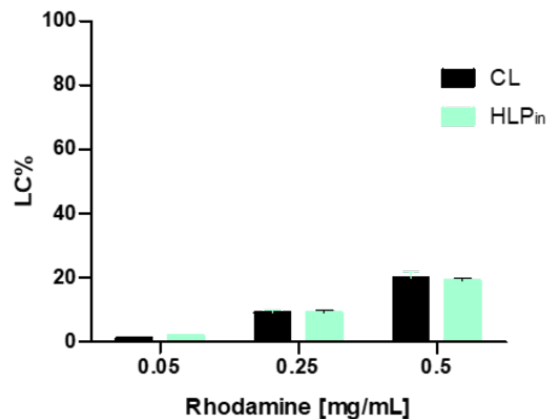
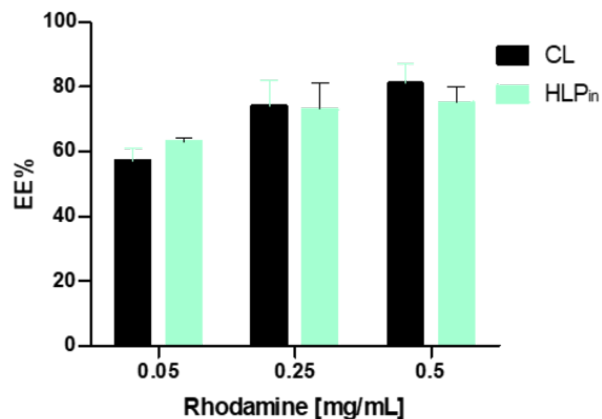
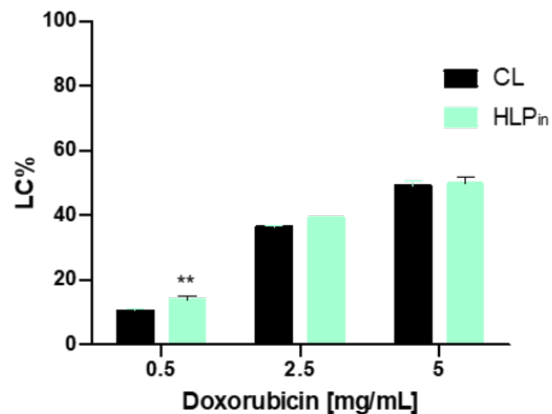
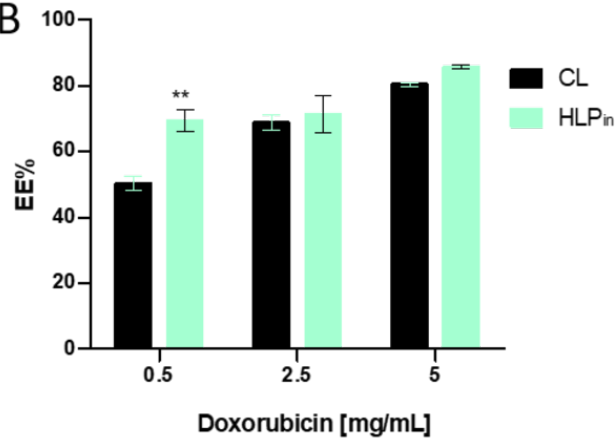
B

Lipid concentration

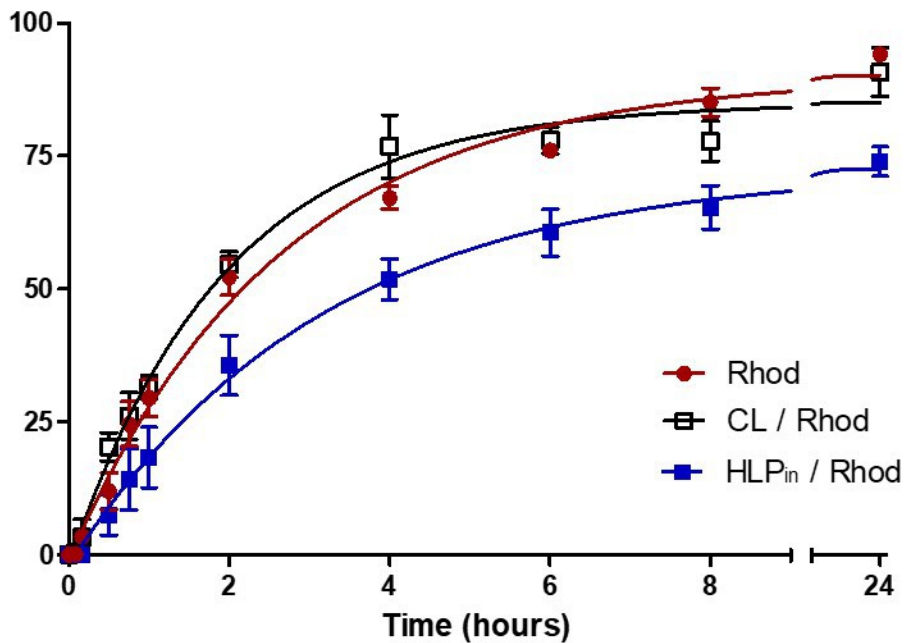


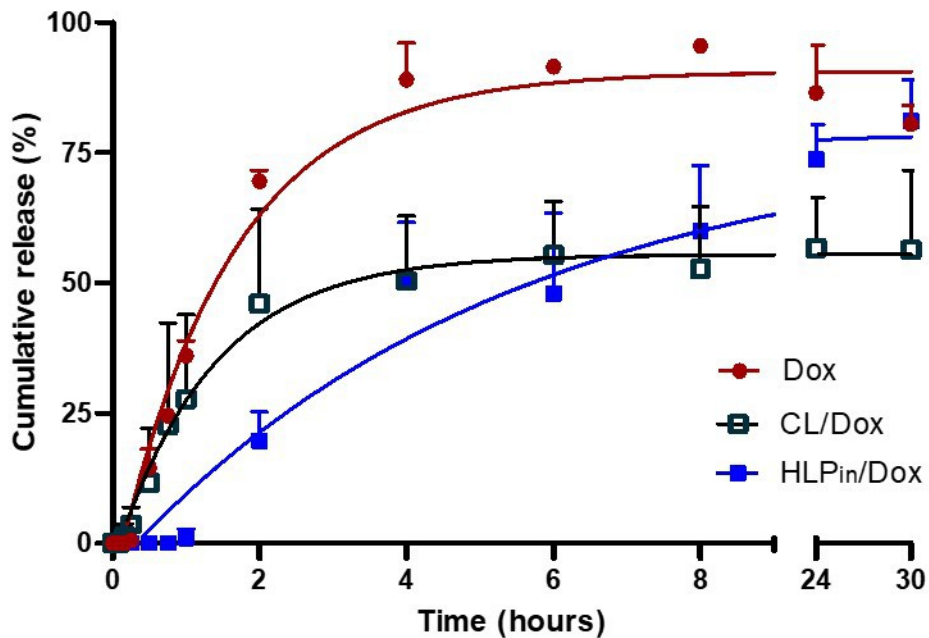
P407 concentration

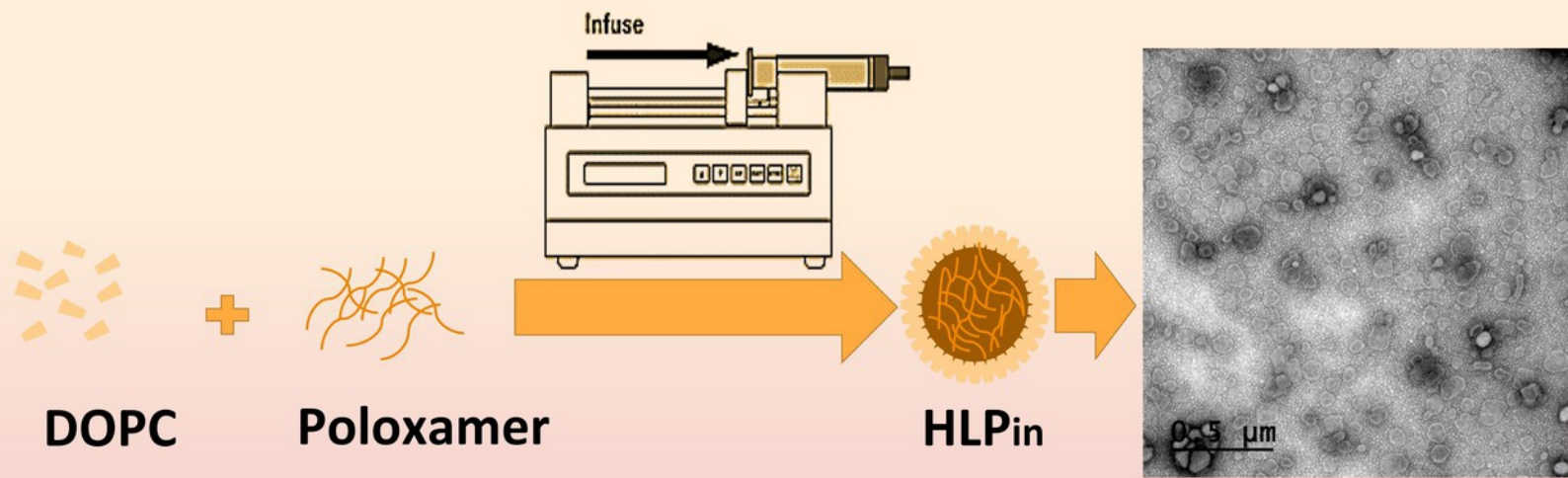


A**B**

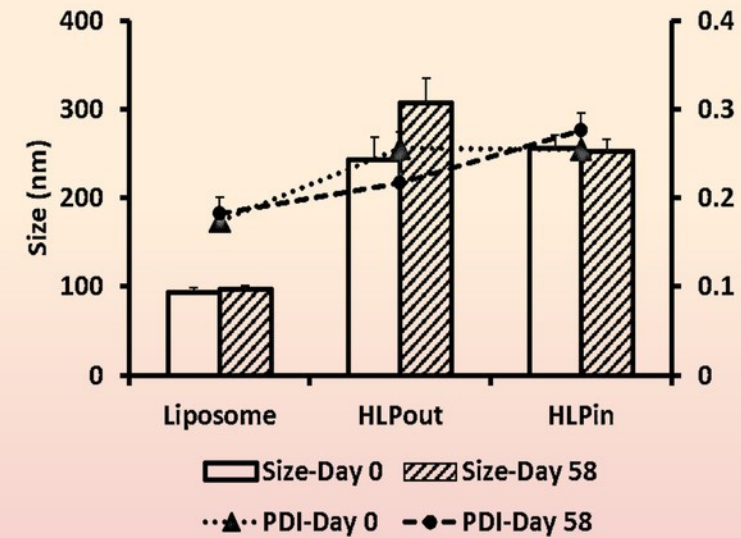
Cumulative release (%)



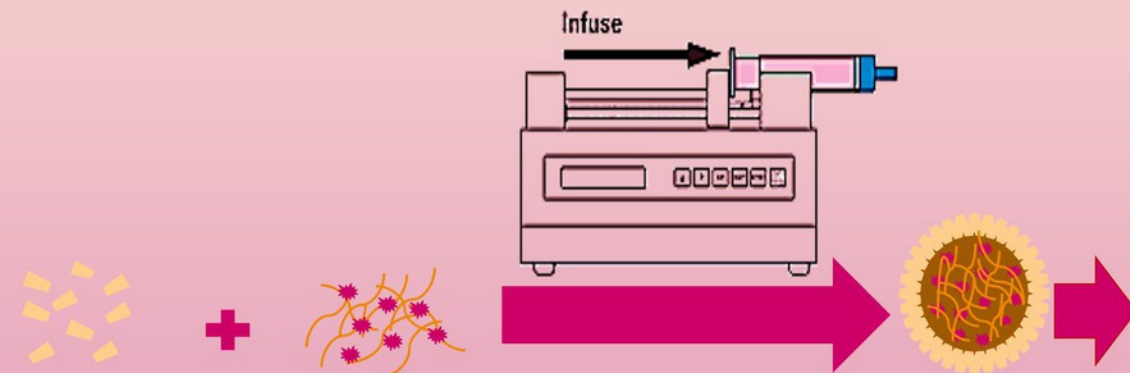




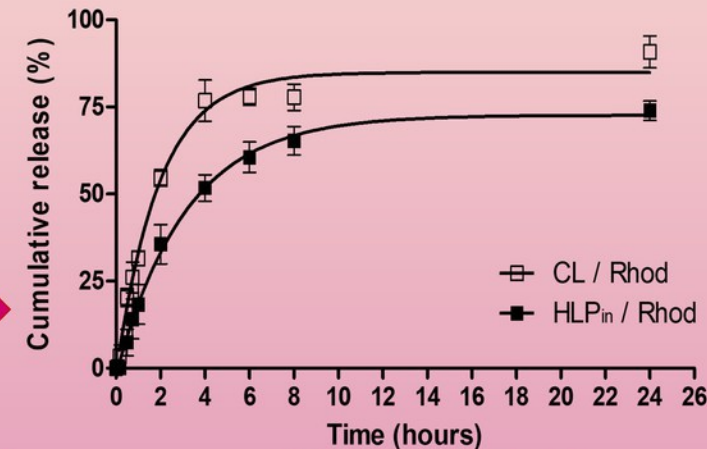
TEM



Stability



Increase in size, E.E and LC%



Slow release