The hypotonic environmental changes affect liposomal formulations for nose-to-brain targeted drug delivery

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Abstract

Systemic administration of drugs is ineffective in the treatment of central nervous system disorders due to the blood-brain barrier (BBB). Nasal administration has been suggested as an alternative administration route as drugs absorbed in the olfactory epithelium bypass the BBB and reach the brain within minutes. However, the nasal mucosa properties (e.g. tonicity, pH) are inconstant due to physiological and environmental factors and this might limit the therapeutic outcome of nanocarrier-based formulations. To shine light on the impact of environmental ionic strength on nanocarrier-based formulations, we have studied how liposomal formulations respond to the change of tonicity of the external environment. Large unilamellar vesicles (LUVs) loaded with six different drugs were exposed to different hypotonic environments, creating an osmotic gradient within the inner core and external environment of the liposomes up to 650 mOsm/kg. Both size and polydispersity of liposomes were significantly affected by tonicity changes. Moreover, the release kinetics of hydrophilic and lipophilic drugs were largely enhanced by hypotonic environments. These results clearly demonstrate that the environmental ionic strength has an impact on liposomal formulations stability and drug release kinetics and it should be considered when liposomal formulations for nose-to-brain targeted drug delivery are designed.

Keywords: liposomes; osmotic pressure; particle size; passive diffusion; controlled release; membrane resistance; drug transport; drug delivery system.

Abbreviations: CNS, central nervous system; EE, entrapment efficiency; LUVs, large unilamellar vesicles; PBS, phosphate buffered saline; PI, polydispersity index; R_B, resistance to drug transport through regenerated cellulose barrier; R_L, resistance to drug transport through liposomal bilayer; R_T, total resistance to drug transport; SD, standard deviation; SPC, soy-phosphatidylcholine; ZP, ζ-potential; ΔmOsm/kg, tonicity difference between the inner core and external environment of liposomes.

1. Introduction

Standard therapies for the treatment of the majority of central nervous system (CNS) disorders (i.e. Alzheimer's disease, multiple sclerosis, Parkinson's disease etc.) are based on daily systemic administration of drugs. The most serious limitation of systemic administration of drugs is that the blood-brain barrier (BBB) prevents drugs from reaching the CNS.[1-3] The BBB consists of tightly packed endothelial cells separating the systemic circulation from the neuronal cells. It is estimated that the BBB limits the access to the brain for 98 % of small molecules and 100 % of large ones.[4] To overcome these limitations, alternative routes of drug administration to the brain have lately emerged. One of the most promising routes of administration appears to be the nose-to-brain targeted drug administration.[5,6] The nasal epithelium is divided into the olfactory and respiratory region.[5-7] Drugs that are absorbed through the olfactory region have the potential to avoid systemic elimination (i.e. first-pass metabolism, renal clearance etc.), reach the cerebrospinal fluid (CSF) and accumulate in the brain bypassing the BBB.[5-8] This route of drug administration is unfortunately limited by low absorption through the olfactory epithelium because of the limited surface area, early enzymatic degradation and rapid ciliary clearance.[9] However, new research has shown that liposomes can optimize nose-to-brain targeted drug delivery.[10] Liposomes are spherical vesicles consisting of single or multiple phospholipid bilayers surrounding an aqueous core.[11,12] Liposomes for nose-to-brain targeted delivery have shown to protect drugs from early degradation and elimination due to their ability to entrap both hydrophilic and lipophilic compounds.[13,14] Recent *in vivo* studies in rats have shown that liposomal formulations administered via the nose reduce systemic side effects, improve apparent neurological functions and enhance cognitive functions for the treatment of Alzheimer's and Parkinson's disease.[10,15] Despite the promising results, liposome-based formulations intended for nose-to-brain targeted drug delivery seem to show inconsistent improvement in the therapeutic effects when compared to other nanoparticulate systems.[16,17] It has been suggested that one of the reasons might be the slow drug release kinetics (for both hydrophilic and lipophilic compounds) from the liposomal carrier.[18-20] Another important variable is related to physiological changes at nasal mucosal level. In fact, as the mucus is directly open to the external milieu, environmental factors such as air humidity or temperature can perturb the mucus properties such as viscosity, pH and most importantly, tonicity.[21,22] These alterations might also occur during the inflammation state.[23] It is well accepted that liposomal phospholipid bilayer allows small neutral molecules to pass through it to equalize the chemical activity gradient.[24-26] For instance, when the ionic strength of the liposomal core is higher than the outside environment, water molecules will diffuse through the lipid bilayer from the outside to the inside of the liposomes (following the chemical activity gradient). As a result of solvent movement, an osmotic pressure is generated on the liposomal surfaces and liposomes swell (water influx).[27-29] We have recently proved that the release of medium-sized hydrophilic marker (calcein) and lipophilic marker (rhodamine) from large unilamellar vesicles (LUVs) is influenced by osmotic stress.[30] Specifically, we proved that the release of a hydrophilic marker from LUVs was significantly more affected by the tonicity perturbations in comparison to a lipophilic marker. This suggests that the magnitude of these changes could be related to the interplay between the changes in liposomal size and the direction of water flux through the liposomal membrane (water influx or efflux). The aim of this study was therefore to verify how the changes in environmental ionic strength

might influence liposomal formulations designed for nasal administration. Specifically, we investigated how the exposure of LUVs to hypotonic environment affects the drug release kinetics of six active pharmaceutical ingredients (caffeine, hydrocortisone, ibuprofen, ketoprofen, methylprednisolone and theophylline). The drugs were chosen to cover a range of relevant physiochemical properties (different partition coefficients and ionization constants, see Table 1) within potential candidates in the treatment or prevention of the Alzheimer's disease.[31,32] The liposomal dispersions were characterized in terms of size, ζ-potential and entrapment efficiency, whereas drug release kinetics in uneven tonicities were studied by the classic Franz cell diffusion system equipped with regenerated cellulose barriers.

2. Materials and methods

2.1 Materials

Caffeine, hydrocortisone, ibuprofen, ketoprofen, methylprednisolone, theophylline, disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), sodium chloride (NaCl), sodium hydroxide (NaOH), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), chloroform and methanol were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Nordrhein-Westfalen, Germany). Lipoid S100 (SPC, soy-phosphatidylcholine >94 %) was kindly provided by Lipoid GmbH (Ludwigshafen, Rheinland-Pfalz, Germany).

2.2 Preparation of phosphate buffered saline (PBS)

PBS solutions were prepared following a method previously described.[30] In brief, a 300 mOsm/kg neutral (pH 7.4) buffer (PBS300) was obtained by dissolving $NaH_2PO_4 \cdot H_2O$, $Na_2HPO_4 \cdot 2H_2O$, NaOH and NaCl (4.5 g/L, 7.4 g/L, 0.8 g/L and 4.4 g/L, respectively) in distilled water. PBS300 was diluted 3:5 or 1:5 (v/v) with distilled water to achieve buffer solutions with reduced ionic strength (approx. 190 and 65 mOsm/kg, see Table 2). The ionic strength of PBS300 was increased by adding droplets of a 200 g/L NaCl solution (dissolved in PBS300) until a tonicity of 700 mOsm/kg tonicity was reached (PBS700). The measured osmolality (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) and pH (SensION™ +PH31 pH meter, Hach, Barcelona, Spain) of the different PBS solutions used in this study are represented in Table 2.

2.3 Preparation of LUVs

LUV dispersions were prepared following a method previously described.[30] A buffer solution (10 mL, PBS300 or PBS65) was gently added on top of an organic phase containing methanol (0.2 mL) and SPC/chloroform solution (200 mg/mL, 1 mL) in a 50 mL round bottom flask. LUV formulations containing caffeine, ibuprofen, ketoprofen or theophylline were prepared by dissolving the drug (2 mM concentration) in the aqueous phase (PBS300 or PBS65, respectively), whereas LUVs with hydrocortisone or methylprednisolone were prepared by dissolving the drug in the SPC/chloroform solution (drug-lipid ratio approx. 0.035 w/w). Unilamellar vesicles (containing 20 mg/ml lipid and 2 mM drug) were spontaneously formed after the removal of the organic phase by rotary evaporation (40 °C, 40 rpm, 0.1 bar, 90 min, Büchi R-124 rotavapor equipped with Büchi vacuum pump V-700 and Büchi B-480 water bath, Büchi Labortechnik AG, Flawil, Switzerland). Liposomal dispersions were subsequently extruded through polycarbonate membrane filters (5x800 nm and 10x400 nm, Nuclepore Track-Etched

Membranes, Whatman International Ltd., Maidstone, Kent, UK) at room temperature (23–25 °C) to obtain vesicles of homogeneous sizes.

2.4 Size characterization

LUVs' size distribution was measured by photon correlation spectroscopy (angle of 173°, 25 °C, Zetasizer Nano Zen 2600, Malvern, Worcestershire, UK). Prior analysis, each LUV dispersion was diluted 1:100 (v/v) with the same buffer used to prepare LUVs and filtered through polyether sulfone membrane filters (0.2 µm pore size, VWR International, Radnor, Pennsylvania, USA). Analysis were performed in four replicates (n=4), where each sample were measured thrice. For the investigation of LUV sizes in non-isotonic conditions, LUV dispersions (prepared from PBS300 with measured tonicity of approx. 710 mOsm/kg) were diluted 1:100 (v/v) with hypotonic buffers (PBS300 or PBS65, Table 2) and sizes were detected at intervals (approx. every 15 min) over a period of 90 min. Each experiment was repeated twice (n=2) and each sample was measured three times.

2.5 ζ-potential characterization

The electrokinetic potential (ζ-potential) of LUVs was measured by a Zetasizer Nano Zen 2600 (Malvern, Worcestershire, UK) following a procedure previously described.[30] LUV dispersions were diluted 1:20 (v/v) with filtrated deionized water (0.2 µm pore size, VWR International, Radnor, Pennsylvania, USA) prior measurements and analysis were conducted at room temperature (23-25 °C). Measurements were performed in four replicates (n=4), where each sample were measured thrice.

2.6 Entrapment efficiency of drugs

LUVs were separated from the supernatant (containing freely unentrapped drug) by ultracentrifugation (200 000 *g*, 10 °C, 30 min, Beckman model L8-70M with SW 60 Ti rotor, Beckman Instruments, Brea, California, USA). The pellet obtained after ultracentrifugation was dissolved in 1 mL methanol and drug concentration was quantified in the supernatant, as well as in the pellet solutions by UV-visible spectroscopy employing a Micro-titre plate reader (Spectra Max 190 Microplate, Spectrophotometer Molecular devices, Sunnyvale, California, USA) (see Table 1 for the specific detection wavelengths of each drug). Entrapment efficiency (EE) was calculated employing Eq. (1);

$$EE\ (\%) = \frac{M_{LUVs}}{M_{LUVs} + M_{free}} \cdot 100 \quad (1)$$

where M_{LUVs} represents the amount of liposomal entrapped drug (i.e. recovered in the pellet) and M_{free} represents the amount of freely unentrapped drug (i.e. detected in the supernatant). The drug recovery was determined from the total amount of drug (entrapped and unentrapped drug in LUVs) after centrifugation in comparison to the nominal amount of drug in the LUVs (i.e. initial total drug content before centrifugation). Analysis were performed in minimum duplicates (n≥2), whereas three samples of each batch were measured four times.

2.7 *In vitro* drug transport study

Drug transport studies were conducted employing Franz diffusion cell system (0.64 cm^2 diffusional area jacketed flat ground joint, PermeGear, Hellertown, Pennsylvania, USA) following a method previously employed.[30] In brief, the acceptor chamber was filled with 5 mL PBS (see Table 2). Regenerated

cellulose barriers (Visking dialysis tubing MWCO 12–14 kDa, Medicell Membranes Ltd., London, UK) were placed between acceptor and donor chamber. The experiment started by adding 0.8 mL of a liposomal dispersion (containing 20 mg/ml lipid, 2 mM total drug concentration) or, alternatively, drug solution (a.k.a. reference) to the donor chamber. In the case of soluble or poorly soluble compounds (caffeine, ibuprofen, ketoprofen, theophylline), 2 mM reference aqueous solution was employed, whereas for very poorly soluble drug (hydrocortisone and methylprednisolone) saturated suspension (1 mg/mL) was employed to maintain a consistent concentration gradient between donor and acceptor compartment. The thermodynamic solubility was predetermined to be 1 mM and 0.3 mM for hydrocortisone and methylprednisolone in PBS (both PBS65 and PBS300), respectively. Samplings (0.5 mL) from the acceptor chamber were carried out at intervals of 30 min over a period of 4 h. After withdrawal of samples, equal volumes of the respective PBS (with same tonicity) was reintroduced into the acceptor chamber in order to maintain sink condition. At the end of the experiment, drug concentrations in the acceptor and donor chambers were quantified by UV-visible spectroscopy (see section 2.6). The cumulative amount of diffused drug over time was calculated, and the linear part of the slope (representing steady state condition) was used to determine the apparent permeability coefficient (P, cm/sec) as shown in Eq. (2) rearranged from Brodin et al.;[33]

$$P = \frac{dm}{dt} \cdot \frac{1}{A} \cdot \frac{1}{c_d} \quad (2)$$

where dm/dt represents the rate of mass transfer of free drug molecules over time, A the diffusional area and c_d represents the initial total drug concentration in the formulation.

2.8 Resistance to drug transport through phospholipid bilayer calculation (R_L)

The resistance to drug transport of a compound through a barrier can be defined as the reciprocal function of P as shown in Eq. (3).[34,35]

$$R = \frac{1}{P} \quad (3)$$

In a permeation process where different layers need to be crossed, the total resistance to drug transport (R_T) can be calculated from the sum of the single resistances (of each of the barriers involved) to transport. In the case of LUV dispersion studies, drug molecules need to firstly cross the liposomal bilayer, representing the first resistance to drug transport (R_L, Figure 1). Secondly, drug molecules need to cross the regenerated cellulose (dialysis) barrier encountering a second resistance to drug transport, namely R_B (Figure 1). Based on this assumption, measuring the total resistance to drug transport (R_T,) and R_B (measured in the reference experiment with drug solutions) R_L can be calculated by Eq. (4) as;

$$R_L = R_T - R_B \quad (4)$$

2.9 Statistical data evaluation

Two-sample Student's *t*-test assuming unequal variances was used to determine the significant differences between the mean of two data sets. A value of *p* below or equal to 0.050 was considered as statistically significant.

3. Results

3.1 LUVs characterization

The most relevant physical characteristics of the different liposomal dispersions studied are summarized in Table 3.

LUV dispersions prepared in PBS300 exhibited a tonicity of approx. 710 mOsm/kg, whereas in PBS65 the tonicity of LUV dispersions was found to be between 425 and 455 mOsm/kg. In all dispersions, the liposome average sizes and PI were higher when prepared in PBS with lower ionic strength (65 mOsm/kg in respect to 300 mOsm/kg). The size differences were found significant for ketoprofen- (p=0.001) and methylprednisolone-LUVs (p=0.000). The same trend could be found for PI in addition to significant difference for caffeine- (p=0.042) and ibuprofen-LUVs (p=0.020). The ZP of all LUV dispersions prepared was close to neutral and significantly more negative (p≤0.026) for the dispersion prepared in PBS65 in comparison to PBS300. Entrapment efficiency was rather low for caffeine and theophylline (18-30 %) with significant enhanced entrapment for the LUVs prepared in PBS65 in comparison to PBS300 (p=0.040 and 0.014, respectively). We determined medium-high entrapment for ketoprofen and ibuprofen (41-56 %) and considerably higher entrapment for hydrocortisone and methylprednisolone (above 74 %).

3.2 Effect of the ionic strength on LUVs sizes

The changes in LUVs size distributions after the exposure to isotonic (A) and hypotonic environments (B-C) are reported in Figure 2 and 3. As it can be seen, LUVs were quite homogeneous in isotonic and low-hypotonic conditions (up to approx. 410 mOsm/kg differences, Figure 2A-B). When exposed to a higher tonicity gradient (approx. 650 mOsm/kg difference between initial LUV dispersion and external environment tonicity), the liposomal dispersions clearly indicated enlargement of the size. Similarly, the PI was relatively constant over time for LUVs in the isotonic and low-hypotonic conditions (Figure 3A-B). When the tonicity gradient between initial LUV dispersion and external environment of LUVs increased to approx. 650 mOsm/kg (Figure 3C), an increase in PI (as well as SD) was observed over time for all formulations tested.

3.3 *In vitro* transport study

3.3.1 Drug solutions

The initial drug concentration, tonicity and the resistance to drug transport through regenerated cellulose barrier (R_B) are presented in Table 4. As shown in Table 4, R_B was not significantly affected by the tonicity of the PBS employed to prepare the solutions. The lowest R_B were found for caffeine and theophylline (around $1.6 \cdot 10^4$ sec/cm), whereas for all other drugs (hydrocortisone, ibuprofen, ketoprofen, methylprednisolone), R_B were slightly higher and between $1.9 \cdot 10^4$ and $2.3 \cdot 10^4$ sec/cm (Table 4).

3.3.2 LUV dispersions

The phospholipid bilayer's resistance to drug transport (R_L) over the tonicity gradient is reported in Figure 4. As the tonicity gradient between initial LUV dispersion and external environment of liposomes increases (∆mOsm/kg), a decrease in the R_L was observed for all drugs to a different extent (Figure 4).

For caffeine and theophylline, a drastic shift in resistance was detected at a tonicity gradient of approx. 400 mOsm/kg, whereas for the other drugs the decrement in R_L seemed to be more gradual. A significant decrease in R_L ($p \leq 0.026$) could be found for all the LUV dispersions prepared at tonicity differences around 300 and 400 mOsm/kg with the exceptions of caffeine and methylprednisolone. Only in the case of caffeine, liposomal bilayer produced significantly higher resistance ($p \leq 0.009$) at low-hypotonic conditions (Figure 4, upper-left) but not in isotonic conditions. For methylprednisolone, a significant decrease in R_L ($p=0.026$) was already apparent at tonicity differences around 100 mOsm/kg. For all LUV dispersions prepared, the reduction in R_L at the highest concentration gradient (approx. 650 mOsm/kg) was found significantly different (p-value between 0.000 and 0.037) compared to the lowest concentration gradient (0 mOsm/kg, isotonic condition). The overall reduction in R_L was found to be between 75 and 114 % for the hydrophilic drugs (caffeine, theophylline), between 49 and 65 % for the lipophilic drugs (ibuprofen, ketoprofen) and approx. 27 % for the hydrophobic drugs (methylprednisolone, hydrocortisone).

4. Discussion

4.1 LUVs characterization

The LUVs were prepared using natural lipid (SPC) and PBS (adjusted to physiological pH and tonicities) to achieve LUV dispersions suitable for nasal administration.[21,22] In relation to liposomal sizes, PI and ZP, the prepared LUV formulations exhibited suitable profiles when compared with other liposomal formulations intended for nasal administration.[13,14,36,37] In agreement with our previous findings,[30] the ZP was found to be slightly more negative for the LUVs prepared in PBS65 compared to PBS300 (Table 3). Although the neutral ZP at higher ionic strength can be expected due to the formation of a thicker ion shell surrounding the liposomes,[38] the larger sizes of LUVs prepared in PBS65 in comparison to PBS300 are difficult to explain. It could be argued that this discrepancy is related to small changes in elasticity of the phospholipid bilayers in environments of different ionic strengths. The prepared LUVs were also found to be suitable carriers to entrap all the drugs with different magnitude of loading. In accordance with their distribution coefficients at pH 7.4 ($logD_{7.4}$, Table 1) and the literature, hydrophobic drugs (hydrocortisone and methylprednisolone) reached the highest entrapment efficiency into liposomes (between 74 and 85 %, respectively), whereas the entrapment was much lower for hydrophilic drugs (close to 25 % for caffeine and theophylline).[39] The lipophilic drugs (ibuprofen and ketoprofen) showed a medium-high entrapment efficiency ranging between 41 and 56 % (similarly to what has been reported previously by Nii and Ishii.[40] The entrapment was significantly enhanced for LUVs with hydrophilic drugs prepared in PBS65 when compared to PBS300 (caffeine $p=0.040$, theophylline $p=0.014$, respectively). This might be related to the increased size of liposomal carriers when prepared in different PBS (Table 3). In our previous study we assumed, due to the lack of available literature on the topic, that the total tonicity of the liposomal formulation should have been primarily influenced by the ionic strength of the solution.[30] In the present work, we measured the tonicity of each of the LUV dispersions (Table 3), and surprisingly, a significant discrepancy in tonicity for LUV dispersions in comparison to plain buffer (Table 2) was found for all formulation tested. As the drug alone did not affect the buffer's tonicity at the experimental condition (Table 4), assuming that at the equilibrium, the tonicity of the inner core of liposomes is equal to the measured tonicity of the LUV dispersion (i.e. external environment), it appears

that liposomes themselves acted as strong tonicity agents (the influence on total tonicity accounts for more than 300 mOsm/kg. A very similar trend was observed for empty liposomes (measured tonicity of 718 ± 34 mOsm/kg and 448 ± 22 mOsm/kg when prepared in PBS300 or PBS65, respectively). To the best of our knowledge, this phenomenon has not been described earlier and it might be of extreme importance in liposomal drug research.

4.2 Effect of the ionic strength on LUVs sizes

It has been reported previously that LUVs sizes can be affected by changes in tonicity of the surrounding environment.[30,41] If the environment surrounding liposomes is hypotonic in comparison to the liposomal core, liposomes have a tendency to increase in size as a result of water influx into the liposomes.[27-29] Photon correlation spectroscopy is a powerful technique applied to quantify liposomal sizes and PI in a dispersion. However, in these experiments, it was difficult to determine an accurate size of the LUVs under the influence of tonicity perturbations. In order to have a better and clear picture of the effect that hypotonic surrounding environments had on the formulations, LUV dispersions (approx. 710 mOsm/kg) were exposed to two buffers of different ionic strengths (300 mOsm/kg and 65 mOsm/kg) and sizes of the liposomes were measured at approx. 15 min intervals for a total period of 90 min. The size distribution of all formulations was rather homogenous (Figure 2). Interestingly, the dispersions were more homogeneous in the low-hypotonic conditions rather than isotonic conditions (Figure 2A-B). Since ions are capable of neutralizing liposomal surface charges due to ion-shell formation,[38,42-45] it is reasonable to assume that liposomal aggregation is more significant in isotonic conditions than in low-hypotonic conditions due to the surface charge neutralization of liposomes (see also Table 3). When the surrounding liposomal environment was highly hypotonic (ΔmOsm/kg of 648 ± 19 mOsm/kg, Figure 2C) the size distribution became very heterogeneous. The same trend could be observed for the PI that was significantly increased over time just at high tonicity gradient (Figure 3C). The combination of these results clearly indicates that LUVs grew in sizes when exposed to hypotonic environments at differences of approx. 648 mOsm/kg, whereas smaller differences were not apparent due to the disturbances on the liposomal surfaces which might have affected the LUVs behaviour and the size measurements.

4.3 Resistance to drug transport through regenerated cellulose barrier (R_B)

In this work, the kinetics of transport for the investigated drugs through barrier(s) were described by calculating the resistance of each single barrier involved. This was done to better differentiate the role of each barrier involved in the total net transport of drug (Eq. (3)) and is essential for a correct interpretation of transport studies that involves liposomes. The R_B was determined by measuring the drug's permeability (Eq. (2)) from drug solutions, or in the case of very poorly soluble drugs (hydrocortisone and methylprednisolone), employing aqueous drug suspensions (no liposomes present). As can be seen in Table 4, the lowest R_B were found for caffeine and theophylline, whereas the highest were found for hydrocortisone and methylprednisolone. The reason for the significant discrepancy could to be attributed to the different size (i.e. molecular weight, Table 1) of the molecules. In fact, Eq. (5) (adaptation of Fick's first law) can describe the permeability of a drug through a regenerated cellulose barrier as;

$$P = \frac{D}{C_d} \cdot \frac{dc}{dx} \quad (5)$$

where D represents the diffusion coefficient and dc/dx the gradient of concentration between donor and acceptor compartment. From this equation it is evident that, normalizing the concentration and assuming same thickness of the barrier in all experiments, the differences in permeability (and therefore in resistance to transport) within different drugs through cellulose barriers are solely given by the different diffusion coefficients of each drug. Indeed, D is higher for small compound such as caffeine (measured diffusivity in similar conditions, $9 \cdot 10^{-6}$ cm^2/sec[46]) and lower for larger compounds such as ketoprofen (measured diffusivity in similar conditions, $6 \cdot 10^{-6}$ cm^2/sec[46]).

4.4 Resistance to passive transport through liposomal barrier (R_L)

The liposomal bilayer of LUVs represents an additive barrier that drug molecules need to cross to reach the acceptor compartment (Figure 1). In order to calculate the resistance to drug transport through the phospholipid bilayer (R_L), the permeability of drugs through regenerated cellulose barrier of LUVs loaded with drug (R_T) needed to be measured and subtracted from R_B (Eq. (4)). In isotonic conditions (Figure 4, ΔmOsm/kg of approx. 0 mOsm/kg), hydrophilic compounds (caffeine and theophylline) exhibit a R_L of approx. 0.4 to $0.7 \cdot 10^4$ sec/cm and this resistance rises with the lipophilicity of the compounds (Table 1) up to $14.6 \cdot 10^4$ sec/cm for very hydrophobic compound, methylprednisolone. The higher resistance to transport through the lipid bilayer expressed by hydrophobic compounds in comparison to hydrophilic is not surprising and is due to the fact that the lipophilic compounds are tightly embedded in the lipid bilayers and cannot escape (be released) easily. These results are in agreement with previous findings.[18] Interestingly, for the hydrophilic compounds (caffeine and theophylline) and to a minor but substantial extent, lipophilic acidic drugs (ketoprofen and ibuprofen), a strong reduction in R_L was measured with reduced external ionic strength (increased ΔmOsm/kg, Figure 4). This is a clear evidence that exposure of drug-loaded LUVs to hypotonic environment is a powerful trigger of drug release. This can be attributed to the stretching of the phospholipid bilayers induced by LUVs size enlargements that makes the barrier leakier and drugs can permeate more easily.[29,47] This phenomenon is in agreement with previous findings.[48-50] An alternative hypothesis to explain the increased drug release in hypotonic conditions is the pore formation during liposomes swelling which can cause a pulsating release of entrapped content.[41,51-53] For caffeine and theophylline, the R_L become practically zero (i.e. no resistance to drug transport caused by phospholipid bilayer) when the tonicity differences between the inner core and external environment of LUVs reached around 350 mOsm/kg. Interestingly, R_L increases at the lowest tonicity gradients for caffeine (below 300 mOsm/kg, Figure 4 upper-left). It can be argued that at low tonicity gradients, the stretching of phospholipid bilayers might be compensated (if not overdriven in the case of caffeine) by the water flux directed inwards (i.e. against drug flux), causing therefore an increasing in R_L. For ibuprofen and ketoprofen, the reduction in R_L seemed to be more proportional and reaching a minimum of approx. half of the initial R_L at the highest tonicity gradient (ΔmOsm/kg above 600 mOsm/kg). Interestingly, the release of hydrophobic compounds (hydrocortisone and methylprednisolone) was also positively affected by the hypotonic surrounding environment, however to a minor extent in comparison to the other compounds tested. At a tonicity gradient above

600 mOsm/kg, the R_L for hydrophobic hydrocortisone and methylprednisolone is reduced by approx. 27% in comparison to isotonic condition. These results are in agreement with our previous findings where we demonstrated that the kinetic of calcein release (hydrophilic marker) from LUVs was more affected by tonicity perturbation in comparison to the lipophilic marker (rhodamine).[30] It is clear that the effect of the environmental tonicity on the release of liposomal drugs needs to be studied to assist in optimization of liposomal formulations destined for nasal administration.

5. Conclusions

In this work, we have proven that the tonicity of the environment surrounding liposomes plays a crucial role in LUVs' physical characteristics (i.e. size, polydispersity and surface charge) as well as drug release profiles. Firstly, we have showed that liposomes themselves significantly affect the total tonicity of the dispersions. Secondly, we have demonstrated that LUVs size as well as polydispersity increase after exposing liposomes to hypotonic environment proven them osmotically active. Finally, we have proven that the exposure of drug-loaded LUVs to hypotonic environments reduces R_L and therefore enhances drug release kinetics of both hydrophilic and lipophilic/hydrophobic drugs. The findings have clear implications in the development and optimization of liposomal formulations targeting nasal administration. Moreover, the observed effects can be utilized to tailor the release of liposomal drugs within nasal environment.

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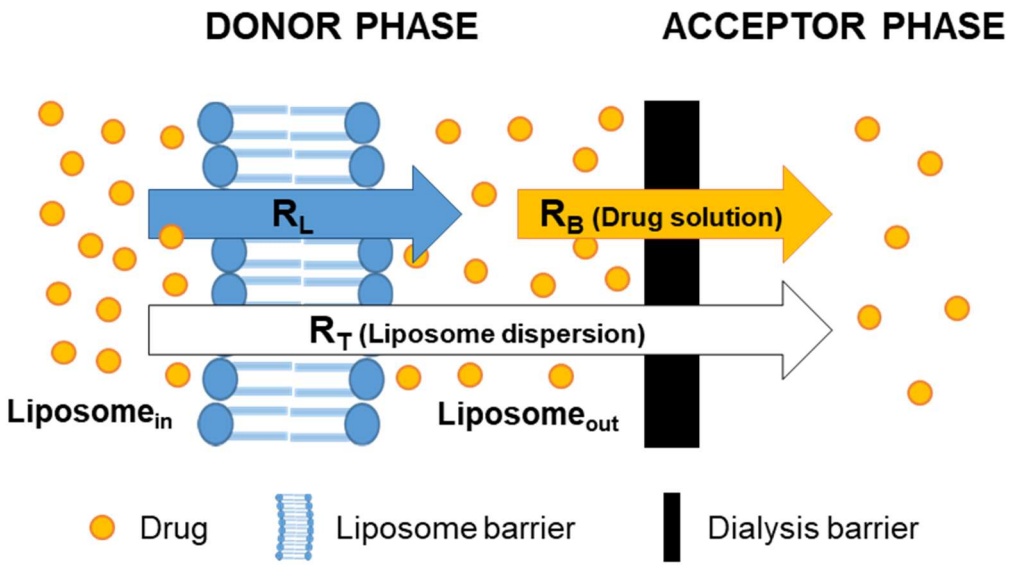

Figure 1. Schematic representation of the passive diffusion setup. R_B represents the resistance to drug transport through regenerated cellulose barrier (measured for drug in solution), whereas R_T represents the total resistance to drug transport (measured for liposome dispersion) and R_L represents the resistance to drug transport through liposomal bilayer (calculated with Eq. (4)).

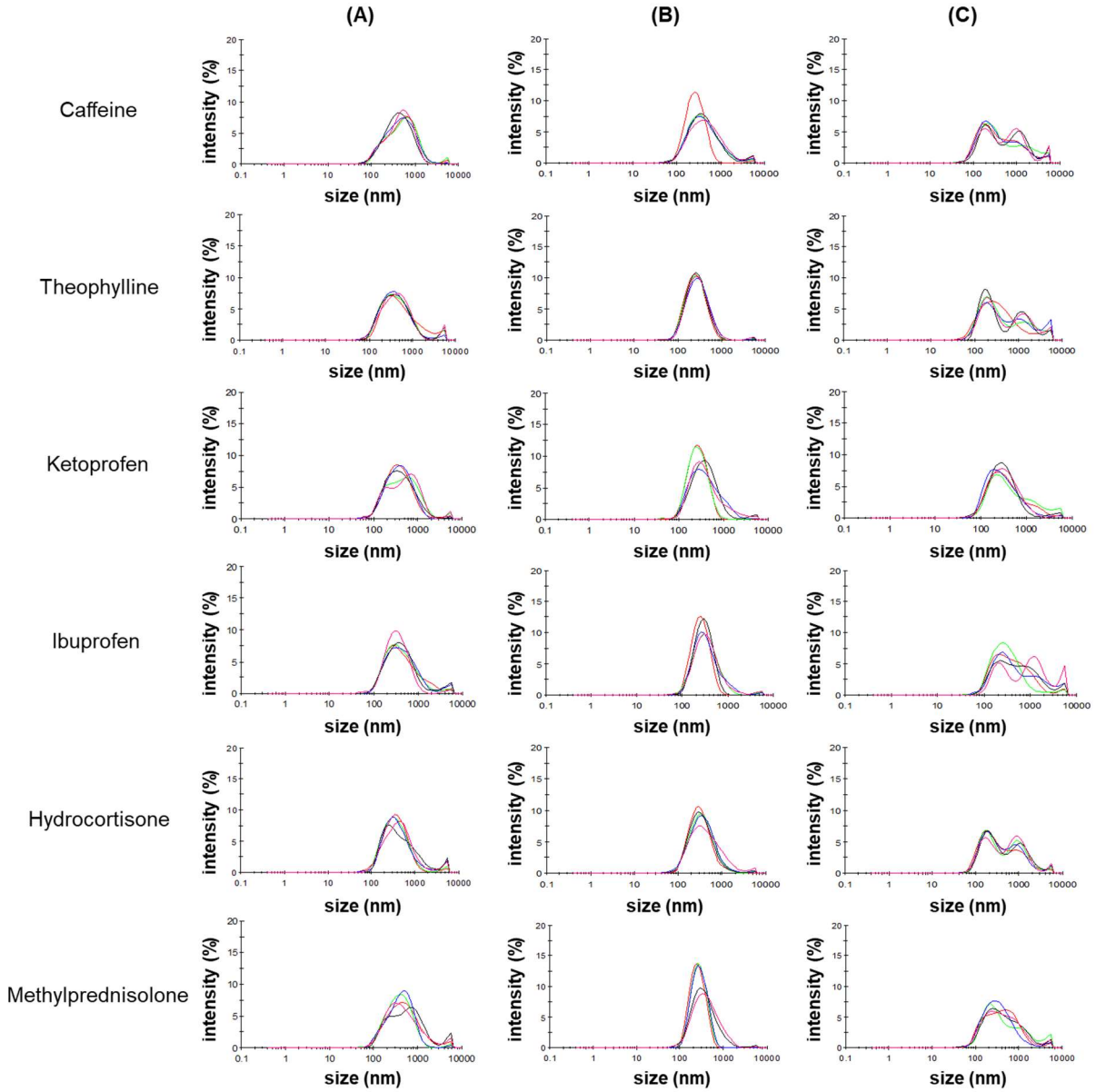

Figure 2: Size distributional changes for drug-loaded LUVs in a) isotonic condition (ΔmOsm/kg of 3 ± 2 mOsm/kg), b) low-hypotonic condition (ΔmOsm/kg of 414 ± 19 mOsm/kg) and c) hypotonic condition (ΔmOsm/kg of 648 ± 19 mOsm/kg). Each line represents the mean size distribution (n=2) measured at five different time points within 90 min.

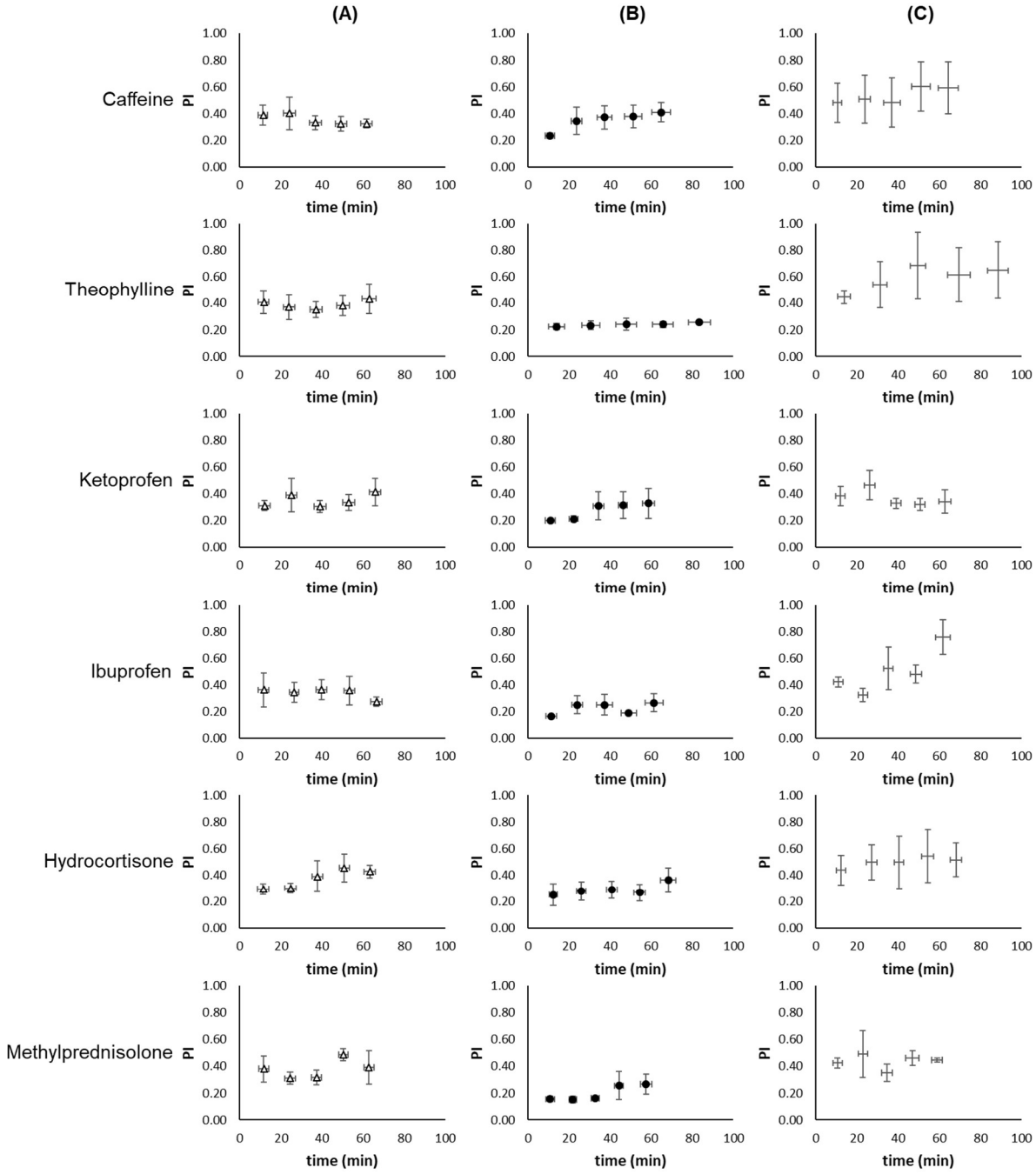

Figure 3. Polydispersity index (PI) changes for drug-loaded LUVs in a) isotonic condition (ΔmOsm/kg of 3 ± 2 mOsm/kg), b) low-hypotonic condition (ΔmOsm/kg of 414 ± 19 mOsm/kg) and c) hypotonic condition (ΔmOsm/kg of 648 ± 19 mOsm/kg). Results represents mean ± SD (n=2).

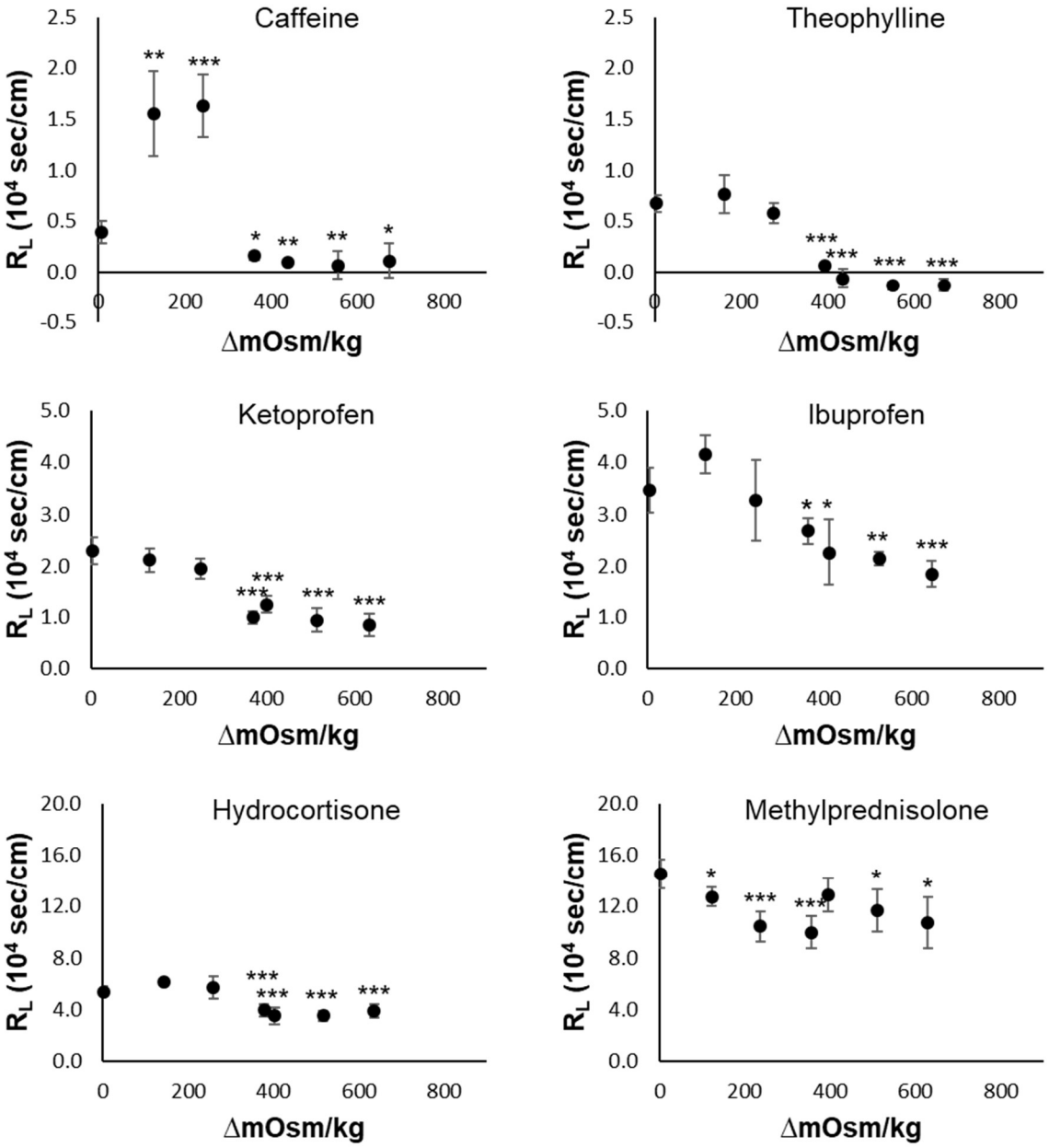

Figure 4: Liposomal bilayer resistance (R_L) to drug transport under the influence of hypotonic environmental changes. The tonicity differences between the inner core and external environment of liposomes are shown as ΔmOsm/kg. Results represents mean ± SD (n=4) and significant difference (*p≤0.050, **p≤0.010, ***p≤0.001) in R_L is determined between the hypotonic compared to isotonic condition.1000

Table 1. Molecular weight (MW), dissociation constant (pKa), distribution coefficient at pH 7.4 ($logD_{7.4}$) and wavelength of maximum absorbance (λ_{max}) of the investigated drugs.

Drug	MW (g/mole)	pKa	$logD_{7.4}$	λ_{max} (nm)
Caffeine	194.2	10.4[54]	0.0[58]	273
Theophylline	180.2	8.8[55]	-0.1[58]	272
Ketoprofen	254.3	4.5[56]	0.2[59]	261
Ibuprofen	206.3	4.9[57]	1.0[59]	222
Hydrocortisone	362.5	Not relevant	1.5[58]	247
Methylprednisolone	374.5	Not relevant	2.1[58]	248

Table 2. Experimentally determined osmolality, pH and calculated phosphate concentration for each of the PBS solutions employed. Results represents mean ± SD (n=5).

Buffer solution	Osmolality (mOsm/kg)	pH	Phosphate (mM)
PBS700	707 ± 6	7.21 ± 0.01	74 ± 0
PBS300	298 ± 12	7.39 ± 0.03	74 ± 0
PBS190	183 ± 2	7.49 ± 0.04	44 ± 0
PBS65	64 ± 3	7.60 ± 0.04	15 ± 0

Table 3. Measured tonicity, size, polydispersity index (PI), ζ-potential (ZP), entrapment efficiency (EE) and drug recovery for all formulations investigated. Results represents mean ± SD (n≥2).

Drug	Buffer solution	Tonicity (mOsm/kg)	Size (nm)	PI	ZP (mV)	EE (%)	Drug recovery (%)
Caffeine	PBS65	430 ± 17	288 ± 53	0.34 ± 0.03	-2.99 ± 0.85	22 ± 4	97 ± 3
	PBS300	719 ± 18	262 ± 42	0.27 ± 0.03*	-0.87 ± 0.95***	18 ± 3*	99 ± 1
Theophylline	PBS65	455 ± 6	341 ± 72	0.37 ± 0.04	-1.99 ± 0.93	30 ± 0	100 ± 3
	PBS300	719 ± 22	327 ± 62	0.31 ± 0.04	-0.08 ± 1.28***	23 ± 6*	103± 3
Ketoprofen	PBS65	429 ± 4	368 ± 68	0.34 ± 0.03	-5.40 ± 0.98	42 ± 1	97 ± 2
	PBS300	718 ± 28	249 ± 36***	0.22 ± 0.02**	-3.97 ± 0.98	41 ± 4	98 ± 1
Ibuprofen	PBS65	429 ± 1	252 ± 36	0.22 ± 0.02	-8.68 ± 1.05	56 ± 4	99 ± 3
	PBS300	686 ± 23	246 ± 33	0.20 ± 0.02*	-6.26 ± 1.01**	46 ± 9*	101 ± 1
Hydro-cortisone	PBS65	437 ± 6	332 ± 60	0.31 ± 0.03	-5.86 ± 1.10	77 ± 3	94 ± 5
	PBS300	718 ± 14	314 ± 58	0.32 ± 0.03	-2.49 ± 0.95*	74 ± 7	97 ± 4
Methyl-prednisolone	PBS65	425 ± 7	285 ± 46	0.29 ± 0.03	-3.12 ± 0.91	85 ± 3	97 ± 3
	PBS300	701 ± 9	256 ± 35***	0.20 ± 0.02***	-0.75 ± 0.94***	84 ± 5	97 ± 3

Significant difference (*p≤0.050, **p≤0.010, ***p≤0.001) between the LUVs prepared in PBS300 in comparison to PBS65.

Table 4. Regenerated cellulose barrier's resistance to drug transport (R_B) of drug solutions in phosphate buffered saline. Results represents mean ± SD (n=4).

Drugs	Buffer solution	Drug concentration (mM)	Tonicity (mOsm/kg)	R_B (10^4 sec/cm)
Caffeine	PBS65	2.04 ± 0.03	65 ± 1	1.64 ± 0.03
	PBS300	2.00 ± 0.00	300 ± 2	1.64 ± 0.05
Theophylline	PBS65	1.92 ± 0.03	65 ± 1	1.58 ± 0.05
	PBS300	1.99 ± 0.02	297 ± 1	1.60 ± 0.07
Ketoprofen	PBS65	2.00 ± 0.04	66 ± 2	2.26 ± 0.08
	PBS300	2.00 ± 0.00	298 ± 0	2.08 ± 0.11
Ibuprofen	PBS65	2.03 ± 0.00	68 ± 4	2.14 ± 0.14
	PBS300	2.01 ± 0.00	308 ± 10	2.18 ± 0.15
Hydrocortisone	PBS65	1.02 ± 0.01	66 ± 1	1.92 ± 0.23
	PBS300	1.03 ± 0.02	298 ± 0	2.22 ± 0.17
Methylprednisolone	PBS65	0.26 ± 0.00	64 ± 0	2.03 ± 0.18
	PBS300	0.25 ± 0.00	301 ± 5	2.27 ± 0.34