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Physicochemical characterization and cytotoxic studies of nonionic surfactant vesicles using sucrose esters as oral delivery systems

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ABSTRACT

Several nanotechnological solutions for mucosal immunization have been proposed, such as nanoparticles, liposomes, solid lipidic particles, micelles, and surfactant vesicles. In recent years, surfactant vesicles have gained increasing scientific attention as an alternative potential drug delivery system to the conventional liposome. This type of vesicle known as niosomes or nonionic surfactant vesicles (NSVs) has a structure and properties similar to those of liposomes. Both of them can transport hydrophilic drugs by encapsulation in the aqueous inner pool or hydrophobic drugs by intercalation into hydrophobic domains. The aim of this study was to prepare and characterize vesicles formed by sucrose esters as protective systems of bioactive molecules for oral administration. Vesicles were prepared using two commercial products formed by mixtures of mono and diesters S-570 and S-770, respectively. Determined parameters were size and zeta potential; the stability of formulations was tested in presence of increasing concentrations of a surfactant, and at several pH values observed in the gastrointestinal tract. Solubilization experiences showed an initial decrease in size for vesicles of both ester mixtures, samples showed detergent resistance at higher Triton X-100 concentrations. Vesicles showed stability at pH 5–7.4 up to 90 min; however, both formulations showed colloidal instability at pH = 2, which corresponds to the isoelectric point of these vesicles. To evaluate the cytotoxicity of both vesicle formulations and separately each pure ester, Caco-2 cells were used. Cytotoxic evaluation indicated that both types of vesicles and free sucrose distearate were safe for Caco-2 viability; however, free sucrose monostearate was toxic for the cells.

As a conclusion of these preliminary studies, it can be stated that vesicles formed with mixtures of sucrose esters showed a size in the range of 200 nm maintaining their size when exposed to the action of a surfactant, but showing aggregation at acidic pH.

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1. Introduction

Mucosal vaccination is an attractive alternative for parenteral vaccination offering several advantages, among them it increases immunity in mucosal compartments, the prime portal of entry for majority of pathogens; in contrast, immunity generated via parenteral administration cannot be transferred to mucosal surface [1]. One key element for successful oral vaccination is an efficient delivery of antigen to the gut-associated lymphoid tissue (GALT), the inductive site in the intestine where antigen-specific immune responses are initiated [2]. However, this approach has some biopharmaceutical drawbacks, being the lack of protection of antigens, labile compounds such as peptides, from the hostile environment of the gastrointestinal tract one of them. Incorporation of antigens into different nanocarriers such as nanoparticles, liposomes, solid lipid particles, micelles, and surfactant vesicles [3] have been used for antigen protection and delivery to reach the Peyer's patches in the intestinal epithelium. Peyer's patches are aggregates of lymphoid tissue involved in the initiation of intestinal immunoglobulin A (Ig A) responses [4].

Orally administered nanocarriers must overcome large changes in pH, high concentrations of surfactants, and hydrolytic enzymes to reach the Peyer's patches. Physicochemical properties such as size, zeta potential, surface hydrophobicity, and the presence of ligands are important for stability and for interaction with the Peyer's patches. Particle size has been reported to be a critical parameter; vesicles larger than 1 μm are trapped within the Peyer's patches and do not migrate to the lymph nodes, whereas vesicles with sizes close to 200 nm are more efficiently processed [5–10].

In recent years, surfactant vesicles gained increasing scientific attention as an alternative potential drug delivery system to

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conventional liposomes. This type of vesicle known as niosomes or nonionic surfactant vesicles (NSVs) [11,12] has a structure and properties similar to those of liposomes. Both of them can carry hydrophilic drugs by encapsulation in the aqueous inner pool or hydrophobic drugs by intercalation into hydrophobic domains [13,14]. The chemical stability as well as the relatively low cost of the materials used to prepare NSVs makes this vesicle more attractive than conventional liposomes for industrial productions both in pharmaceutical and cosmetic applications [15–23]. There are several nonionic surfactants able to form NSVs [15,24–26], among them we are interested in sucrose esters (SEs), compounds having a sugar substituent, sucrose, as polar head group and long chain fatty acids as nonpolar moiety. The length of fatty acid chain and the degree of esterification determine properties like the hydrophilic lipophilic balance (HLB from 1 to 16) value and the melting point. These surfactants are prone to be employed alone or in complex systems like tablets or vesicles, because they have low toxicity to the ocular surface, are biocompatible, are less hemolytic and irritating than other surfactants, and finally they have excellent biodegradability [27]. Their absorption, distribution and metabolism have been thoroughly evaluated [28,29]. The SEs are hydrolyzed to sucrose and fatty acids prior to intestinal absorption, the extent of the hydrolysis is directly related with the degree of esterification. Daniel et al. [30] studied the metabolism of sucrose mono and diesters of beef tallow in rats and showed that these esters were hydrolyzed to sucrose and fatty acids before absorption and did not accumulate in the tissues following repeated exposure. Some SEs have a Drug Master File, and sucrose stearate and sucrose palmitate are featured in the European Pharmacopoeia (Ph. Eur) and in the United States Pharmacopeia/National Formulary (USP NF), confirming their human applicability. As sucrose stearate and sucrose palmitate are registered in Pharmacopoeias, these SEs are increasingly used in pharmaceutical products. The FDA Inactive Ingredients Database lists sucrose stearate and sucrose palmitate in oral dosage forms, and sucrose distearate and sucrose polyesters for administration by the topical route [28].

The objective of this study was to characterize two formulations of vesicles using commercial sucrose esters with different HLB and evaluate their vesicles as possible vehicles for oral administration of antigens. The vesicles were characterized determining size, surface charge, stability at physiological pH. Cytotoxicity of both the vehicle and its independent components was determined using Caco-2 cell culture.

2. Materials and methods

2.1. Materials

Two grades of commercial sucrose stearate were kindly donated by Mitsubishi-Kagaku Foods Corporation (RYOTO® sugar ester, Mitsubishi-Kagaku Foods Corporation, Japan), S-570 (HLB = 5), S-770 (HLB = 7) and pure mono-stearate and di-stearate sucrose esters were donated by Laboratorio de Cinética y Fotoquímica, Sodium 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton X-100, cholesterol 95%, and dicetylphosphate (DCP) were purchased from Sigma-Aldrich (Sigma-Aldrich, Chile). Streptomycin/penicillin/amphotericin, sodium pyruvate, and minimum essential medium (MEM) with nonessential amino acids (MEM-NEAA) were purchased from Invitrogen (Invitrogen Corporation, Argentina). CytoTox 96® Non-radioactive Cytotoxicity Assay Kit (Promega, Argentina). Fetal calf serum (FBS) was purchased from Bioser Gen SA (Bioser Gen SA, Argentina). L-Glutamine, trypsin and ethylenediaminetetraacetic acid (EDTA) were provided by PAA Laboratories GmbH (PAA Laboratories GmbH, Austria). All other products and reagents were of analytical grade.

2.2. Preparation of nonionic surfactant vesicles

Vesicles were prepared using the thin layer evaporation method [31]. Briefly, solutions of sucrose stearate S-570 or S-770 (60 mM), cholesterol (75 mM), and DCP (15 mM) in chloroform/methanol (3:1) were mixed in the proportion 4:5:1, respectively, in order to obtain a homogeneous solution, which was subsequently vacuum evaporated. The resulting dried film was hydrated with phosphate buffered saline (PBS pH 7.4) at 60 °C. The dispersion obtained was vortexed (5 min) and then sonicated for 20, 40, or 60 s in a ultrasonic homogenizer Cole-Parmer CP-501 at 30% of its nominal power (500 W).

2.3. Transmission electron microscopy (TEM)

The previously prepared vesicles were morphologically characterized using TEM (Philips Tecnai 12 Bio Twin, Japan).

2.4. Size and zeta potential measurements

Size (μm) and zeta potential (mV) of the prepared NSVs (diluted 1:10 in PBS) were determined by dynamic light scattering (DLS) and phase analysis light scattering (PALS), respectively, using a nanoZ-sizer (ZEN 3600, Malvern, UK). Polydispersity index (p.i.) was also determined.

2.5. Zeta potential as a function of pH

Determinations of zeta potential and electrophoretic mobility were conducted using a Zeta Meter model 3.0 (Zeta Meter Inc. USA), with an automatic transfer unit sample. For measurement, the sample was suspended in 1×10^{-3} M KCl and pH was adjusted with 0.1 M HCl or KOH.

2.6. Stability of vesicles

2.6.1. Turbidity measurement

Colloidal stability of vesicle solutions was studied following changes in the turbidity of suspension in a Jasco V530 spectrophotometer (absorbance at 520 nm).

2.6.2. Stability of vesicles in the presence of Triton X-100

An aliquot of the vesicles was diluted with PBS at a concentration of 1.3 mM of the total formulation (0.5 mM sucrose stearate). Subsequently, amounts of Triton X-100 were added to give concentrations of 8.2, 16.4, and 24.6 mM. After each addition, the sample was incubated for 30 min at 37 °C.

2.6.3. Stability of vesicles at different pH

An aliquot of the vesicles was diluted to reach a concentration of 1.3 mM in the following solution pH 2, pH 5 (buffer solutions were prepared according USP XXII protocols), or PBS pH 7.4. Samples were incubated for 30, 60, and 90 min at 37 °C. Changes of vesicles size at different pH was also monitored by DLS.

2.7. Cytotoxicity of vesicles

Caco-2 cells were routinely cultured in MEM-NEAA with 2 mM L-glutamine and 1% pyruvate; medium was supplemented with 10% fetal calf serum and 1% streptomycin/penicillin/amphotericin, at 37 °C in 5% CO_2 and 95% humidity. Cell viability upon treatment with vesicles was measured by MTT assay and lactate dehydrogenase (LDH) leakage in culture supernatants. Caco-2 cells were seeded in 96-well plates at a density of 5×10^4 cells/well and allowed to attach overnight. Medium was then replaced with fresh

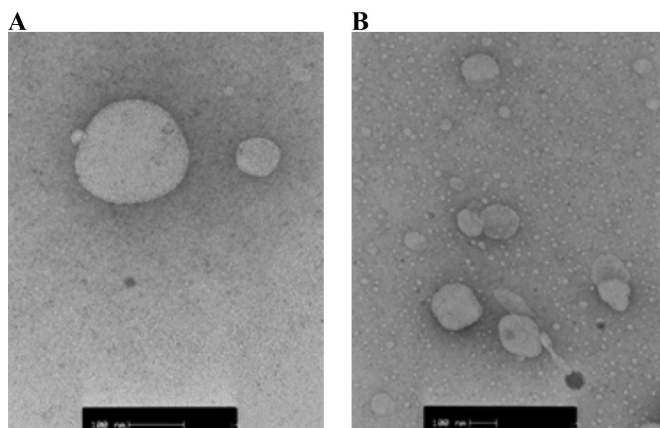

Fig. 1. Transmission electron micrographs after 60 s of sonication for S-570 vesicles (A) and S-770 vesicles (B). The bar corresponds to 100 nm.

medium with 5% FBS containing increasing concentrations of vesicle solution, corresponding to 100, 500, and 1000 μg/mL of sucrose esters. Upon 24-h incubation at 37 °C, supernatants were transferred to fresh tubes, centrifuged at 250*g* for 4 min, and LDH content was measured using lactate dehydrogenase CytoTox Kit [33]. LDH concentration was expressed as percentage LDH release relative to treatment with the detergent Triton-X 100; percentage of viability was calculated considering the LDH leakage of cells grown in medium. Cells attached to plates were processed for MTT assay [34]. For the assay, 100 μl of 0.5 mg/ml MTT solution in medium was added. After 3-h incubation, MTT solution was removed, the insoluble formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader.

Additionally experiences to determine toxicity of isolated sucrose esters dissolved in dimethylsulfoxide (DMSO) were carried out, dispersed into culture medium at concentrations mentioned above. It must be indicated that final DMSO concentration in contact with cells was only 2% v/v; therefore cell viability is not affected by the presence of this solvent [32]. Additionally, a vesicle clean-up process was performed, in order to remove any component not included in vesicle structure, the sonicated dispersion was centrifuged and supernatant discarded, immediately vesicles were re-suspended in PBS. This operation was repeated twice and the samples obtained were directly employed to test cytotoxicity.

All cytotoxicity tests were carried out five times. Viability of cells was expressed as percentage of the viability of cells grown in medium.

2.8. Statistical analysis

Results correspond to the mean of three independent experiments ±SD (standard deviation). Statistical data analysis was performed using the *t*-test. To evaluate statistical significance of results, $p \leq 0.05$ was used as criterion.

3. Results and discussion

3.1. Characterization of nonionic surfactant vesicles

Nonionic surfactant vesicles were prepared by the film method. Fig. 1(A) and (B) shows TEM photographs of vesicles prepared with S-570 and S-770, respectively, being both spherical with defined smooth edges. This observation indicates that vesicles' morphology is independent on mono and diester proportions.

Sonication of 60 s was determined as the optimal time to minimize polydispersity, and all vesicle solutions employed correspond to this time of sonication. Table 1 shows the average size of vesicles

Table 1
Characterization of NSVs: size (nm), zeta potential (mV) and polydispersity index (p.i.) of analyzed sample ($n = 3$; ±SD).

Vesicles	Size (nm)	Z-potential (mV)	p.i.
S-570	167.48 ± 26.28	−79.80 ± 25.31	0.32 ± 0.09
S-770	153.10 ± 5.59	−53.05 ± 4.03	0.25 ± 0.02

expressed as diameter of a sphere volume equivalent; both formulations showed homogenous and monodisperse size distributions (polydispersity index was close to 0.3) with an average size around 200 nm and no significant differences were found between both commercial esters. The commercial sucrose esters used to prepare the vesicles correspond to mixtures of different proportions of mono and diesters; the amount of each one modulates the HLB of the sample. Mollee et al. characterized exhaustively the bilayer of reversed vesicles formed with sucrose ester of stearic and palmitic acid, with and without the presence of cholesterol. They state that esters in the vesicles would be arranged differently depending on its substitution; in systems or domains where monostearate prevails, complete interdigitation of alkyl chains would occur, being bilayers narrower, in contrast with ones where distearate predominates, where bilayer is more than 1 nm wider. Authors propose that cholesterol is located in di-ester rich regions, preventing chain interdigitation [35]. This proposal is compatible with our results for vesicles formed with S-570, which have a slightly larger particle size and are more heterogeneous.

One of our objectives was to obtain vesicles with less than 200 nm of diameter because Peyer's patches only caught particles smaller than this critical size [5–9,11,35–37].

According to surface charge results given in Table 1, both formulations show a strong negative surface charge and there are no significant differences between their zeta potentials, −79.80 ± 25.31 for S-570 and −53.05 ± 4.03 for S-770 formulations. These values indicate coloidal stability; according to literature, a physically stable nanosuspension solely stabilized by electrostatic repulsion will have a minimum zeta potential of −30 mV [25].

3.2. Stability

As mentioned previously, changes in turbidity in presence of increasing concentrations of surfactant or at different pH are usually employed as measure of membrane stability [38–42]. The changes in absorbance (at 520 nm) are interpreted as a change in vesicle integrity, decrease in scattered light intensity corresponds to a reduction in vesicle size (solubilization and loss of material in membrane) whereas any increase in absorbance can be interpreted as an increase on average size, for example agglomeration processes [43].

3.2.1. Stability of vesicles in the presence of Triton X-100

Triton X-100, a nonionic detergent, has many applications in biological research, particularly in the solubilization of phospholipid bilayers membranes [44]. It has a critical micelle concentration (CMC) of 0.22 mM, well below the CMC of the bile salts [45], and is widely employed as solubilizing agents [46]. Turbidity of vesicles upon incubation with increasing concentrations of Triton X-100 is shown in Fig. 2. For both S-570 and S-770-vesicles, there was a 40% decrease in absorbance of the dispersions after the first addition of Triton (8.5 mM). These results indicate that vesicles were disrupted in the presence of surfactant and smaller structures such as mixed micelles were formed [46]. Although both formulations were sensitive to surfactant presence, the impact of Triton X-100 at higher concentration (24.6 mM) does not drop absorbance to zero, whereas for conventional liposomes (e.g. phosphatidylcholine) solubilization is complete. [46].

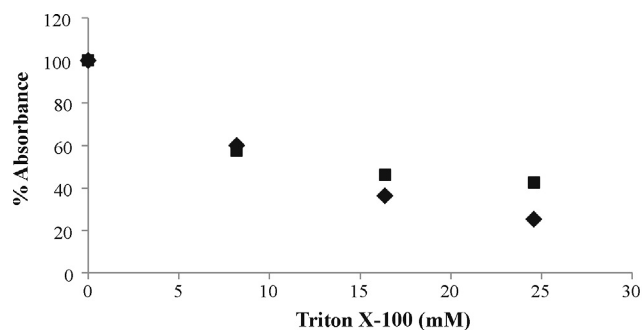

Fig. 2. Stability of NSVs' in the presence of increasing concentrations of Triton X-100. Absorbance percentage change in static light-scattering of vesicles suspended at pH 7.4 in the presence of increasing amounts of Triton. S-570 (♦) and S-770(■) (mean $n = 3$, error bars are omitted because SD is smaller in size than symbols).

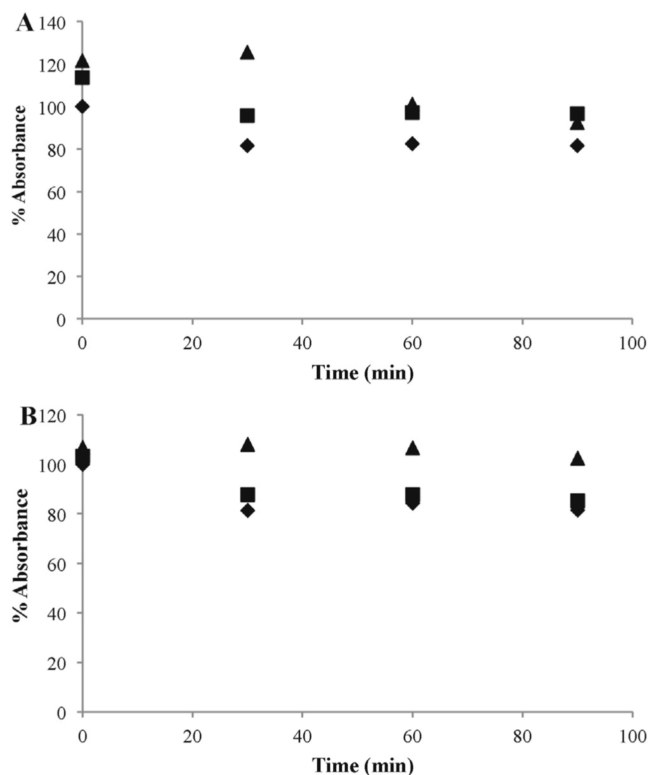

Fig. 3. In vitro stability of NSVs at gastrointestinal tract pH ranges. Absorbance percentage changes in static light-scattering of vesicles suspended at pH 2 (▲), pH 5 (■) and pH 7.4 (♦). A: S-570 vesicles; B:S-770 vesicles (mean $n = 3$, error bars are omitted because SD is smaller in size than symbols).

3.2.2. Stability of vesicles at different pH

Being the main objective of our study to design a vesicular drug delivery system for oral administration of proteins, NSVs stability at different pH was determined.

Turbidity measurements for vesicle dispersions incubated at pH 2, 5, and 7.4 are shown in Fig. 3(A) and (B). Both systems suspended in media at pH 5 and 7.4 show a small decrease of absorbance after 30 min, with no additional change until 90 min. A different response was observed at pH 2, where an important increase in turbidity is observed.

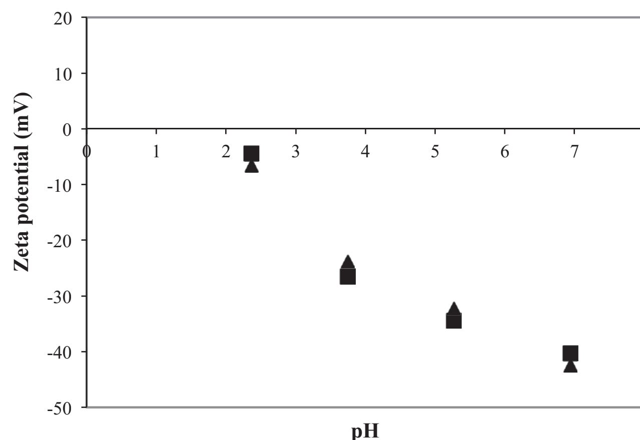

Fig. 4. Values of zeta potential as a function of pH, for S-570 vesicles (▲) and S-770 vesicles (■) (error bars are omitted because SD is smaller in size than symbols).

As can be seen in Fig. 4, at pH 2 zeta potential is zero for both systems, and agglomeration is readily observed (the lack or reduction of formal charge at pH 2 would facilitate conditions for the agglomeration of vesicles). The DCP is an anionic lipid and it is 85% protonated at pH 1.2 (with 15% of anion form) and at pH 4 DCP ionization reaches a 99%, maintaining disaggregation of the vesicles. These results were confirmed by dynamic light scattering as can be seen in Table 2, with both formulations showing a sharp increase in the particle size in an acidic medium. Average size reach a value of about 3 μm in diameter and a p.i. of 0.5, reflecting a very heterogeneous population. In contrast, when vesicles are dispersed at pH 5 and pH 7 they maintain their integrity, and no differences between both samples ($p > 0.05$) were observed.

3.3. Cytotoxity

Safety is an important aspect to evaluate in an innovative drug delivery system. Caco-2 cells are used extensively as an in vitro model for the rapid screening of intestinal absorption and cytotoxicity.

The effect of vesicles of commercial and pure sucrose esters on viability of Caco-2 cells was measured by the MTT assay and or by LDH leakage. S-570 vesicles did not reduce viability of Caco-2 cells upon 24-h incubation over the tested concentration range, (Fig. 5A). On the other hand, when cells were exposed to the action of S-770 vesicles, cellular viability decreases about 50% at the highest concentration studied (1000 μg/mL) (Fig. 5B). MTT test results showed to be concentration dependent while LDH reach a plateau at the higher concentrations tested (500 and 1000 μg/mL).

The apparently different response obtained for both assays can be originated on the different cellular damage sensed. LDH assay give satisfactory responses with cell membrane damaging agents, and MTT assay allows to detect alterations on the metabolic activity of the mitochondria; despite the difference, both methods correlate well [47].

A similar result was found by Marianecci et al. [15], when polysorbate 20 vesicles were evaluated with the MTT assay in

Table 2
Size of NSVs as a function of pH: size (nm) and polydispersity index (p.i.) of the vesicles exposed to different pH of analyzed sample ($n = 3$; ±SD) $^*p \leq 0.05$.

pH	Size of S-570 vesicles (nm)	p.i.	Size of S-770 vesicles (nm)	p.i.
2	6949.80 ± 39.63*	0.48 ± 0.21	3798.20 ± 584.29*	0.52 ± 0.45
5	137.06 ± 1.11	0.17 ± 0.01	138.56 ± 3.56	0.10 ± 0.02
7	133.02 ± 1.25	0.21 ± 0.01	124.56 ± 1.18	0.20 ± 0.02

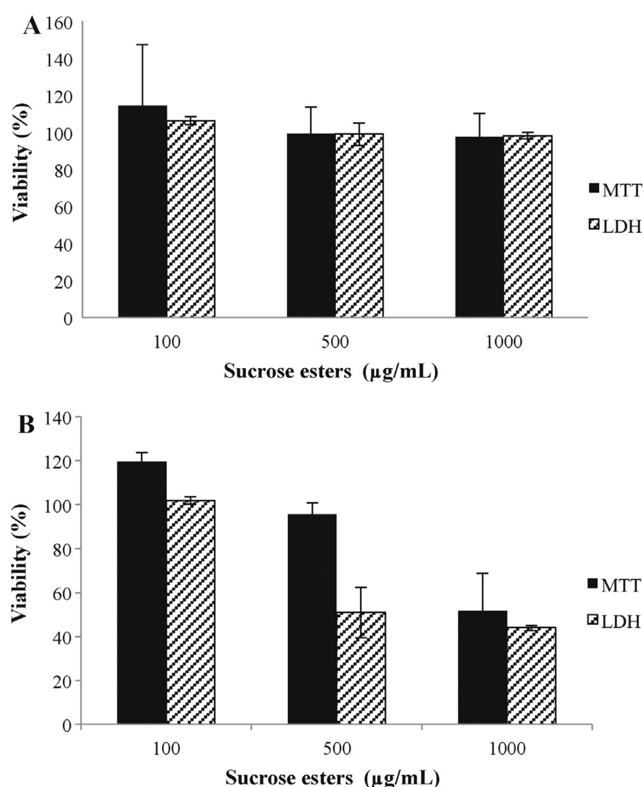

Fig. 5. Cytotoxicity of NSVs on Caco-2 cells expressed as percentage cell viability. Toxicity was measured by MTT and LDH leakage in response to different concentrations of vesicles prepared with S-570 (A) and S-770 (B) (mean ± SD; $n = 5$).

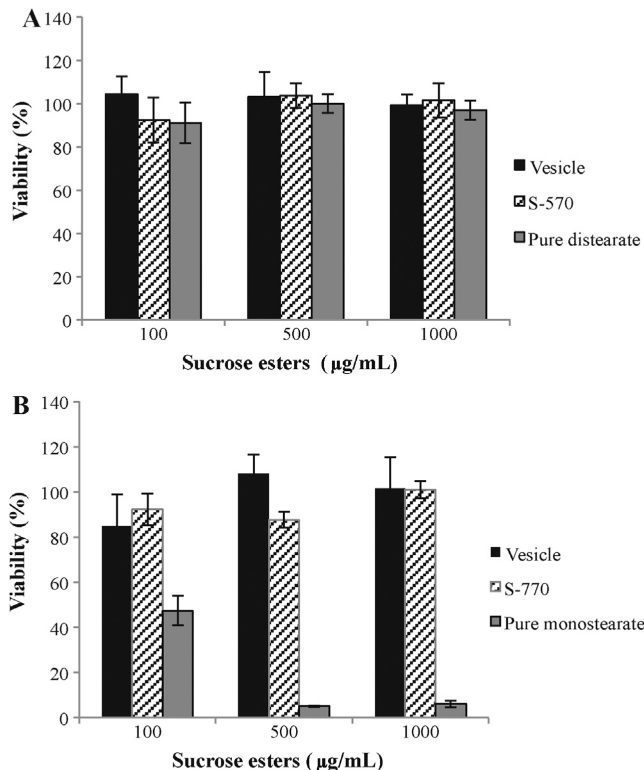

Fig. 6. Cytotoxicity of NSVs and commercial and pure sucrose esters on Caco-2 cells. Toxicity was measured by the MTT assay after 24 h of incubation with different concentrations of vesicles and clean surfactants (A) vesicles of S-570, S-570 and pure sucrose distearate and (B) vesicles of S-770, S-770, and sucrose monostearate (mean ± SD; $n = 5$).

human lung fibroblasts (HLF). Authors report that cytotoxic effect was associated only with surfactant concentration and not with time exposure.

One of the advantages claimed for sucrose esters is safety; they do not irritate the skin or mucous membranes [28]. However, our results showed a toxic effect associated with monoester proportion in sample formulation. Cell culture was exposed to free sucrose esters and prepared vesicles in independent tests, to establish the origin of the observed cytotoxic effect. In order to remove non-encapsulated material from vesicles, after sonication samples were centrifuged, and re-suspended to perform cytotoxicity tests. Fig. 6(A) and (B) shows viability results of Caco-2 cells exposed to various concentrations of 'clean' vesicles of the two grades of commercial sucrose stearate, S-570 and S-770. Additionally, to check cytotoxicity of free sucrose esters, samples of S-570, S-770, pure sucrose monoester and pure sucrose distearate were prepared. Results showed that cell viability is not affected after contact with commercial S-570 or pure sucrose distearate. On the other hand, while commercial S-770 slightly reduces cell viability, pure sucrose monostearate diminishes it to a 10%. Cytotoxicity observed for pure sucrose monostearate free (compared with monostearate integrated to the vesicles) can be explained with a high partition coefficient to membrane observed for long chain sucrose esters [48,49], sucrose monostearate incorporation into the cell membrane, alters permeability and homeostasis, as observed with assay measuring LDH released into the medium when the cell membranes are damaged.

4. Conclusion

Nonionic surfactant vesicles of sucrose ester mixtures as a vehicle for oral antigen delivery proved adequate in size and stability to a surfactant, but were unable to overcome the barrier of acid pH, agglomerating and thereby losing one of the most important features when developing a system that targets the Peyer's patches. A possible alternative to solve this problem is to manage the vesicles using enteric capsules that protect the stomach and then release into the intestine. Easiest alternative is to use this vehicle by other mucosal routes, such as nasal. Similar results are achieved where the stimulation of the immune system is without making contact with an acidic pH. In order to obtain a safer carrier, with lowered or absent cytotoxic effects, a clean-up step to remove any free sucrose monostearate present (not incorporated into the structure) is necessary.

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