1 **Nanoparticles incorporating pH-responsive surfactants as a viable approach**

2 **to improve the intracellular drug delivery**

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1 **Abstract**

2 The pH-responsive delivery systems have brought new advances in the field of functional

3 nanodevices and might allow more accurate and controllable delivery of specific cargoes, which

4 is expected to result in promising applications in different clinical therapies. Here we describe a

5 family of chitosan–TPP (tripolyphosphate) nanoparticles (NPs) for intracellular drug delivery,

6 which were designed using two pH-sensitive amino acid-based surfactants from the family

7 $N^{\alpha},N^{\varepsilon}$-dioctanoyl lysine as bioactive compounds. Low and medium molecular weight chitosan

8 (LMW-CS and MMW-CS, respectively) were used for NP preparation, and it was observed that

9 the size distribution for NPs with LMW-CS were smaller (~168 nm) than that for NPs prepared

10 with MMW-CS (~310 nm). Hemolysis assay demonstrated the pH-dependent biomembrane

11 disruptional capability of the constructed NPs. The nanostructures incorporating the surfactants

12 cause negligible membrane permeabilization at pH 7.4. However, at acidic pH, prevailing in

13 endosomes, membrane-destabilizing activity in an erythrocyte lysis assay became evident. When

14 pH decreased to 6.6 and 5.4, hemolytic capability of chitosan NPs increased along with the raise

15 of concentration. Furthermore, studies with cell culture showed that these pH-responsive NPs

16 displayed low cytotoxic effects against 3T3 fibroblasts. The influence of chitosan molecular

17 weight, chitosan to TPP weight ratio, nanoparticle size and nature of the surfactant counterion on

18 the membrane-disruptive properties of nanoparticles was discussed in detail. Altogether, the

19 results achieved here showed that by inserting the lysine-based amphiphiles into chitosan NPs,

20 pH-sensitive membranolytic and potentially endosomolytic nanocarriers were developed, which,

21 therefore, demonstrated ideal feasibility for intracellular drug delivery.

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23 **Keywords:** chitosan nanoparticles; cytotoxicity; hemolysis assay; lysine-based surfactants;

24 membrane disruption; pH-sensitivity

25

1. Introduction

Crescent studies on nanomaterials that respond to physical, chemical or biological stimuli have been reported in the literature with a wide range of therapeutic applications [1-3]. Stimulus-responsive nanomaterials have greater potential than traditional delivery systems [4-6] and, among the different types of stimuli, the chemical stimuli, especially the pH, have been the most widely used to design smart nanocarriers for drug delivery [7-9]. pH-responsive nanocarriers have remarkable properties that allow them to circumvent biological barriers and achieve targeted intracellular drug delivery [10]. Unfortunately, nanocarriers without this special function lack the ability to escape from endosome–lysosome timely, resulting in poor therapeutic index of the loaded active agents [11-12].

One approach to achieve a nanocarrier responsive to pH is to introduce ionizable chemical groups, such as amines or carboxylic acids, into its structure. Such groups can accept or donate protons and undergo pH-dependent changes in physical or chemical properties, i.e swelling ratio or solubility. In turn, these changes can be exploited to trigger the release of a drug from a delivery system in a pH-dependent fashion [10]. In this context, surfactants derived from $N^{\alpha},N^{\varepsilon}$-dioctanoyl lysine, which contain two hydrophobic chains and a hydrophilic head group with protonatable carboxylic acid, are expected to be protonated at acidic pH. Our previous study has demonstrated the capability of these compounds to destabilize cell membranes in mildly acidic conditions found in the endocytic pathway [13]. Furthermore, earlier studies by the authors' group demonstrated the biocompatibility and low *in vitro* toxicity of this class of amino acid-based surfactants [14-16]. Therefore, the type of chemical structure, together with the biocompatible properties, attracted us to confirm whether they have pH-dependent membranolytic activity when incorporated in polymeric nanocarriers.

The majority of studies reported in the literature deals with pH-sensitive polymers, peptides or polymerizable surfactants to assemble nanoparticles (NPs) responsive to pH

[9,17,18]. Here, we use the natural polymer chitosan (CS) and incorporated biocompatible lysine-based surfactants to prepare pH-responsive NPs. CS has been attracting the interest of many researchers as a polymeric drug carrier material in dosage form design, due to its attractive properties such as biodegradability, biocompatibility, low toxicity and relatively low production cost from abundant natural sources [19]. CS has a pKa of around 6.0-6.5 and is, therefore, positively charged at low pH values (below its pKa value). This positive charge allows its spontaneously gelling on contact with anionic counterparts in solution [20], which relies on the formation of inter and intramolecular cross-linkages mediated by the polyanions (i.e. tripolyphosphate, TPP). Important factors that have been shown to affect the characteristics of these NPs are the CS concentration, the CS-to-TPP ratio, the chitosan's molecular weight, the pH, and the ionic strength of the dissolution medium used in the particle preparation [21].

Due to their sub-cellular and sub-micron size, CS–TPP NPs can penetrate deep into tissues and promote an efficient delivery of therapeutic agents to target sites in the body. Moreover, when these nanocarriers have pH-responsive behavior, they may have the capability to release the drug content into the intracellular compartments after endosomal rupture. Therefore, the focus of this study was to develop NPs with the ability to release bioactive molecules into the cytoplasm by sensing low pH in endosomes. To achieve this aim, we incorporated into NP matrix two pH-sensitive lysine-based surfactants differing in the type of counterion. It is worth pointing out that we have previously demonstrated that this class of bioactive compounds is biocompatible, low cytotoxic and membranolytic at mildly acidic conditions [13-16]. Here, we also evaluated the potential toxicity of NPs using an *in vitro* cell model. Moreover, the influence of CS molecular weight, CS to TPP weight ratio, NP size and nature of the surfactant counterion on the membrane-disruptive properties of NPs was also discussed in detail.

2. Materials and Methods

2.1. Materials

Chitosan (CS) of low (LMW-CS) and medium molecular weight (MMW-CS) (deacetylation degree, 75-85%; and viscosity of 20-300 cP or 200-800 cP, respectively, according to the manufacturer's data sheet), pentasodium tripolyphosphate (TPP), 2,5-diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS) and trypsin-EDTA solution (170,000 U l-1 trypsin and 0.2 g l-1 EDTA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM), supplemented with L-glutamine (584 mg/l) and antibiotic/antimicotic (50 mg/ml gentamicin sulphate and 2 mg/l amphotericin B), were purchased from Vitrocell (Campinas, SP, Brazil). All other reagents were of analytical grade. NaCl, Na_2HPO_4 and KH_2PO_4 were from Merck (Darmstadt, Germany).

2.2. pH-responsive surfactants

Two anionic amino acid-based surfactant derived from $N^{\alpha},N^{\varepsilon}$-dioctanoyl lysine and with distinct inorganic counterions were included in the NP formulations: a salt with sodium counterion (77KS) and a salt with lithium counterion (77KL). They have molecular weight of 421.5 g/mol and 405.6 g/mol, and critical micellar concentration (CMC) of 3 x 10^3 µg/ml and 2.9 x 10^3 µg/ml, respectively. Two alkyl chains form their chemical structure, each one with eight carbon atoms [15,16]. The synthesis procedure of both surfactants was previously described [22].

2.3. Nanoparticle preparation

1   CS-NPs were spontaneously obtained by ionotropic gelation technique, according to the

2   methodology previously developed by Calvo et al. [23], but with some modifications. The NPs

3   were prepared varying the molecular weight of CS, the type of surfactant and the amount of TPP.

4   Six different formulations were obtained, as described in Table 1. All CS-NPs were prepared by

5   dropwise addition of a solution containing the cross-linker TPP (0.1%, w/v) or a premixed

6   solution containing TPP and the surfactant 77KS or 77KL (both at 0.1%, w/v) over the CS

7   solution under magnetic stirring. Agitation was maintained for 20 min under room temperature

8   to allow the complete formation of NPs. Both the LMW-CS and MMW-CS solutions were

9   prepared at a concentration of 0.1% (w/v) and were dissolved in acetic acid solution (1%, v/v).

10 The pH of the CS final solution was adjusted to 5.5 with 1 M NaOH.

11

12 **Table 1.** Composition of the chitosan-based nanoparticles

Label	Formulation (Ratio, w/w or w/w/w)	Chitosan	Surfactant
CS-NP01	CS:TPP (5:1)	MMW-CS	-
CS-NP02	CS:TPP:77KL (5:1:0.5)	MMW-CS	77KL
CS-NP03	CS:TPP (5:1)	LMW-CS	-
CS-NP04	CS:TPP:77KS (5:1:0.5)	LMW-CS	77KS
CS-NP05	CS:TPP (5:2)	LMW-CS	-
CS-NP06	CS:TPP:77KS (5:2:0.5)	LMW-CS	77KS

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15 *2.4. Nanoparticle characterization*

16 The mean hydrodynamic diameter and the polydispersity index (PDI) of the NPs were

17 determined by dynamic light scattering (DLS) using a Malvern Zetasizer ZS (Malvern

18 Instruments, Malvern, UK), without any dilution of the samples. The zeta potential (ZP) values

6

1   of NPs were assessed by determining electrophoretic mobility using also the Malvern Zetasizer

2   ZS equipment. Transmission electron microscopy (TEM) was used to assess the NP morphology.

3   The formulations CS-NP01 and CS-NP02 were set as models for this assay. First, a 5 µl-droplet

4   of each NP suspension was placed on a carbon-coated cooper grid to form a thin liquid film.

5   After that, a 2% (w/v) uranyl acetate solution was used to perform the negative staining of

6   samples. Finally, the images were obtained with a Jeol JEM-1010 electron microscope (Jeol Ltd.,

7   Tokyo, Japan) operating at an acceleration voltage of 80 kV.

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9   *2.5. Preparation of red blood cells suspensions*

10   Erythrocytes were isolated from human blood, which was obtained from discarding

11   samples of the Clinical Analysis Laboratory of the University Hospital of Santa Maria. Tubes

12   containing EDTA as anticoagulant were used for blood collection. Red blood cells were isolated

13   by centrifugation at 3,000 rpm for 10 min, and washed three times in an isotonic phosphate buffer

14   solution (PBS) containing 123.3 mM NaCl, 22.2 mM Na_2HPO_4 and 5.6 mM KH_2PO_4 in distilled

15   water (pH 7.4; 300 mOsmol/l). The cell pellets were then suspended in PBS at a cell density of

16   8×10^9 cell/ml.

17

18   *2.6. Hemolysis assay*

19   The pH-dependent membrane-lytic activity of the NPs was assessed using the hemolysis

20   assay, with the erythrocytes as model of the endosomal membrane [18,24]. PBS buffers in the

21   pH range of 5.4 – 7.4 were prepared to be isosmotic to the inside of the erythrocyte and cause

22   negligible hemolysis. The 25-µl aliquots of erythrocyte suspension were exposed to NPs at

23   concentrations up to 500 µg/ml (referred to total content in the NP dispersion) dissolved in PBS

24   solution in a total volume of 1 ml. Two controls were prepared by resuspending erythrocyte

25   suspension either in buffer alone (negative control) or in distilled water (positive control). The

1   samples were incubated at room temperature under constant shaking for 10 or 60 min, and then

2   centrifuged at 10,000 rpm for 5 min. Supernatants were taken, the absorbance of the hemoglobin

3   release was measured at 540 nm using a double-beam Shimadzu UV–1800 UV-VIS

4   spectrophotometer (Shimadzu, Kyoto, Japan), and the percentages of hemolysis were determined

5   by comparison with the positive control samples totally hemolyzed with distilled water.

6

7   *2.7. Cell culture*

8   The murine Swiss albino 3T3 fibroblast cell line was grown in DMEM medium

9   supplemented with 10% (v/v) FBS, L-glutamine (584 mg/l) and antibiotic/antimicotic (50 mg/ml

10 gentamicin sulfate and 2 mg/l amphotericin B), at 37 °C, 5% CO_2. The 3T3 cells were routinely

11 cultured in 75 cm^2 culture flasks and were trypsinized using trypsin-EDTA when the cells

12 reached approximately 80% confluence.

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14 *2.8. Cytotoxicity assays*

15 The cytotoxic effect of the CS-based NPs containing the surfactants was measured by

16 tetrazolium salt MTT assay [25] and neutral red uptake (NRU) assay [26]. 3T3 cells were seeded

17 into the central 60 wells of a 96-well plate at a density of 1 x 10^5 cells/ml. After incubation for

18 24 h under 5% CO_2 at 37 ºC, the spent medium was replaced with 100 μl of fresh medium

19 supplemented with 5% FBS containing NP dispersions at the required concentration range (25-

20 300 μg/ml, referred to total content in the NP dispersion). After 24 h, the NP-containing medium

21 was removed, and 100 μl of MTT in PBS (5 mg/ml) diluted 1:10 in medium without FBS was

22 then added to the cells. Similarly, 100 μl of 50 μg/ml NR solution in DMEM without FBS was

23 added in each well for the NRU assay. The plates were further incubated for 3 h, after which the

24 medium was removed, and the wells of the NRU assay were washed once in PBS. Thereafter,

25 100 μl of DMSO was then added to each well to dissolve the purple formazan product (MTT

1 assay). Likewise, for the NRU assay 100 µl of a solution containing 50% ethanol absolute and

2 1% acetic acid in distilled water was added to extract the dye. After 10 min shaking at room

3 temperature, the absorbance of the resulting solutions was measured at 550 nm using a

4 SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA) microplate reader. The effect of each

5 treatment was calculated as a percentage of cell viability inhibition against the respective

6 controls.

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8 *2.9. Statistical analysis*

9 Each hemolysis and cytotoxicity experiment was performed at least three times using

10 three replicate samples for each NP concentration tested. Results are expressed as mean ±

11 standard error of the mean (SE). Statistical analyses were performed using one-way analysis of

12 variance (ANOVA) to determine the difference between the sets of data, followed by Tukey's

13 *posthoc* test for multiple comparisons, using the SPSS® software (SPSS Inc., Chicago, IL, USA).

14 $p < 0.05$ was considered statistically significant, and $p < 0.005$ were considered highly

15 statistically significant.

16

17 **3. Results and discussion**

18

19 *3.1. Preparation and characterization of the NPs*

20 CS-NPs have been investigated as a promising drug carrier for targeted delivery to

21 specific sites. The ionic cross-linking between CS, as the polycation, and TPP, as the polyanionic

22 partner, is a simple production method that avoids the use of organic solvent, while is also an

23 effective way to obtain stable and size-controlled NPs [27]. Moreover, CS-NPs prepared via

24 ionotropic gelation avoid the use of chemical cross-linking or emulsifying agents that can be

25 toxic to cells and affect the biological properties [28,29].

1   CS undergoes ionotropic gelation and precipitates to form spherical particles that are

2   distinguishable by opalescence of solution. The size of NPs could be controlled especially by

3   modifying the molecular weight of CS and, in a lesser extent, by changing the CS-to-TPP ratios

4   (Table 2). Noteworthy that besides its influence on the NP size, the polymer weight ratio (w/w)

5   can affect the biological performance of CS-NPs, as demonstrated in the next sections of this

6   paper. Our results demonstrated that the size distribution for CS-NPs with LMW-CS were

7   smaller (~168 nm) than that for NPs prepared with MMW-CS (~310 nm). Likewise, we observed

8   that with increasing amount of the counterion TPP, scattering intensity increases with the

9   accompanying decrease in particle size. The formulations CS-NP03 and CS-NP04, with CS:TPP

10   ratio of 5:1, have a slight opalescence and a larger mean diameter (162.9 and 183 nm,

11   respectively). On the other hand, CS-NP05 and CS-NP06 showed a medium opalescence and a

12   smaller size (156.9 and 170.4 nm, respectively), which might be attributed to the higher quantity

13   of the polyanion TPP (ratio CS:TPP of 5:2). These results are in agreement with the work

14   reported by Gan et al. [21], in which it was demonstrated a linear decrease of size with decreasing

15   CS to TPP weight ratio. However, it is worth pointing out that the addition of excess polyanion

16   neutralized all the positive charges of CS, which in turn resulted in the loss of potential cross-

17   linking sites and in NP flocculation. Low to moderate CS to TPP weight ratio is necessary to

18   achieve stable and size-controlled NPs. Finally, it is worth pointing out that the NPs incorporating

19   77KL or 77KS exhibited increased mean diameter. Additional dimensions of 36.9, 20.1 and 13.5

20   nm were observed for CS-NP02, CS-NP04 and CS-NP06, respectively, in relation to those

21   formulations without surfactants.

22   All CS-NPs exhibited a positive surface charge around + 25 to + 30 mV (Table 2),

23   indicating good dispersion stability [30]. The comparatively low molecular weight anionic

24   molecule TPP become interspersed between the large CS polymer chains [31], which resulted in

25   the positive net charge of the formed NPs. Noteworthy that the net positive charge of CS-NPs

decreased with increasing polyanion quantity. The formulations CS-NP05 and CS-NP06 presented lower ZP values (~ 25 mV), which appear to be attributed to the lower CS to TPP mass ratio (5:2 w/w, in comparison to the ratio 5:1 w/w of the other formulations). With increased amount of TPP, the ionotropic complexation between CS and TPP will increase and, in turn, the number of free positive charges of CS will be diminished, leading to a decrease in the ZP. However, it can be inferred that as long as the particles have sufficient repulsive force (ZP +/- 25 ± 5 mV), NPs remain colloidally stable [29]. Finally, in contrast to the results observed for NP size, the presence of 77KS and 77KL did not affect significantly the ZP values of all designed formulations.

Table 2. Characterization parameters of the different chitosan-based nanoparticles

| Formulation	Hydrodynamic size (nm) ± SD*	PDI ± SD*	Zeta potential (mV) ± SD*	pH
CS-NP01	291.47 ± 11.25	0.308 ± 0.044	28.21 ± 1,65	5.58
CS-NP02	328.37 ± 9.54	0.271 ± 0.009	30.07 ± 1.18	5.49
CS-NP03	162.9 ± 1.405	0.221 ± 0.02	29.4 ± 2.08	5.52
CS-NP04	183.0 ± 1.234	0.206 ± 0.015	30.7 ± 1.21	5.55
CS-NP05	156.9 ± 0.231	0.187 ± 0.015	25.2 ± 1.87	5.46
CS-NP06	170.4 ± 1.137	0.201 ± 0.019	24.0 ± 1.82	5.47

* SD = standard deviation

In order to gain insight into the morphology of NPs, TEM analysis of the formulations CS-NP01 and CS-NP02 were performed (Fig. 1). As the composition of the NPs is rather similar, we chose only two formulations in order to compare whether the presence of the surfactant affects or not the NP morphology. The results showed that both NP suspensions dispersed in water

1 showed clearly mono-dispersed particles, and had a regular and roughly spherical shape,

2 independently on the presence of surfactant. It is noteworthy that TEM images revealed NPs

3 much smaller $(40-80 \text{ nm})$ than the hydrodynamic diameters measured by DLS. This discrepancy

4 in the NP size between DLS and TEM can be attributed to the fact that NPs swell in aqueous

5 medium and DLS gives the size of the particle surrounded by the solvation layers, while TEM

6 gives the diameter of particles alone in the dry state [29,32]. Additionally, the divergence

7 between nanosizes may also be related to surface used for TEM analysis, which does not allow

8 the particles to agglomerate, but let them separated.

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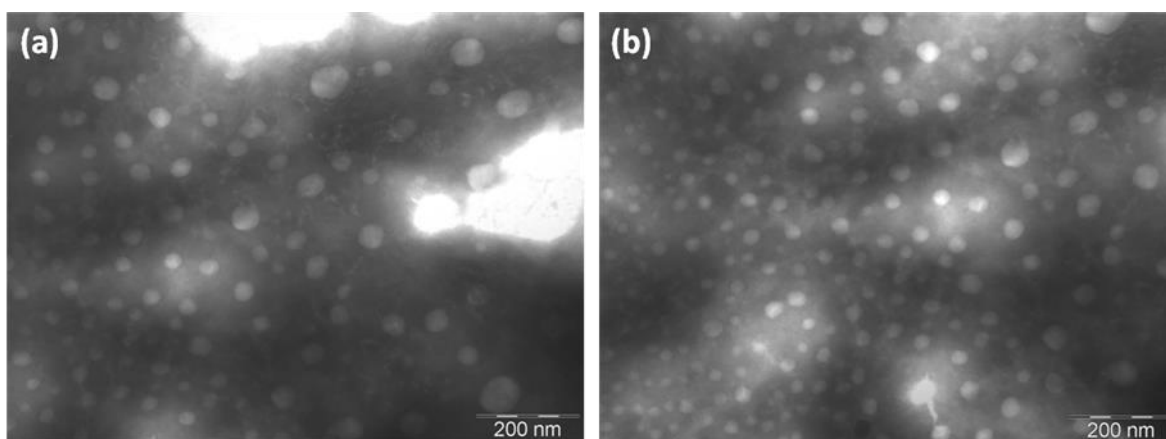
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11 **Fig. 1.** TEM images of the NPs containing MMW-CS and the surfactant 77KL: (a) CS-NP01

12 and (b) CS-NP02. Scale bars correspond to 200 nm.

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14 *3.2. pH-dependent membrane-lytic activity*

15   Hemolysis assay was performed to evaluate the pH-sensitive membranolytic activity and

16 predict the endosomolytic capability of CS-NPs. Erythrocytes are considered a simple model of

17 the endosomal membrane [18,33] and, therefore, have been used as a convenient cell membrane

18 system to study NP-membrane interactions as a function of pH [7,34,35]. Moreover, they have

19 enough functions in common with more specialized cells [36], and the given results may be good

20 indicators of the biological activity of the NPs at physiological conditions. Early endosomal

1   compartments have a pH from 6.5 to 6.8, while the lumen of late endosomes has a lower pH, of

2   about 5.5 [37,38]. Therefore, we explored the membrane-lytic activity of the NPs at the pH range

3   of 5.4 to 7.4, mimicking the environment that these nanocarriers are expected to encounter when

4   translocating through the endocytic pathway.

5   Fig. 2 shows the membrane-lytic activity of the NPs incorporating or not a surfactant as

6   a function of pH, with varying concentration and incubation time. At low concentrations, the NPs

7   incorporating either of the surfactants showed low or negligible hemolytic activity at pH 7.4.

8   When pH decreased to 6.6, CS-NP02 and CS-NP04 had increased membrane-lytic capability and

9   so did CS-NP06, but to a lesser extent, resulting in a maximum hemolysis of 61.23, 77.46 and

10 24.73%, respectively. At pH 5.4, all NPs with 77KS or 77KL showed stronger membrane-lytic

11 activity ($p < 0.005$), reaching maximum of about 87% hemolysis. On the other hand, all

12 formulations without the surfactants showed no pH-sensitive activity throughout the endosomal

13 pH range and, thus, do not seen to have any ability to facilitate endosomal destabilization.

14 Altogether, these results confirmed that the improved membrane-lytic behavior of NPs at acidic

15 environment is due to the presence of surfactants.

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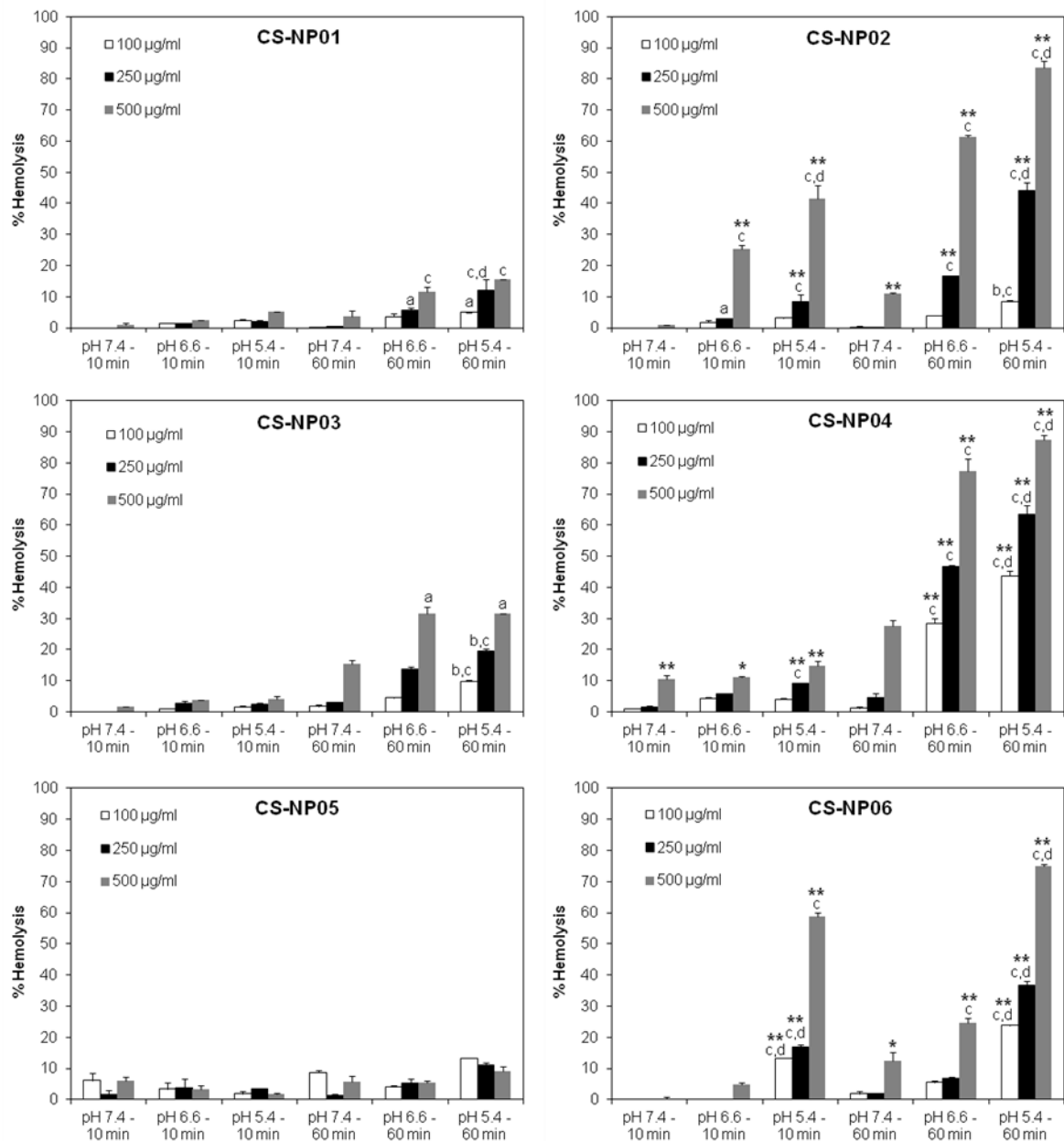
Fig. 2. pH-responsive membrane-lytic behavior of CS-NPs with and without 77KS or 77KL. NP-induced membrane-lysis was expressed as a function of pH, concentration and incubation time. Results are expressed as the mean ± SE of three independent experiments. Statistical analyses were performed using ANOVA followed by Tukey's multiple comparison test. [a] Statistically different from pH 7.4 ($p < 0.05$), [b] statistically different from pH 6.6 ($p < 0.05$), [c] highly statistically different from pH 7.4 ($p < 0.005$) and [d] highly statistically different from pH 6.6 (p

14

< 0.005). * Statistically different from CS-NPs without surfactant (p < 0.05) and ** highly statistically different from CS-NPs without surfactant (p < 0.005).

It is worth mentioning that the pH-responsive membrane-lytic activity of NPs is not dependent on their size. CS-NP02, with mean diameter of about 328 nm, displayed similar hemolysis with decreasing pH values as did CS-NP04 and CS-NP06, with smaller mean size of about 183 and 170 nm, respectively.

To better visualize the pH-dependent membranolytic activity of the NPs, graphics were constructed expressing the hemolysis rate of each formulation as a function of pH (Fig. 3). The pK50, which means the pH at which 50% hemolysis is obtained, was calculated for the formulations containing the surfactant 77KS or 77KL. This value was estimated from the pH-response curves showed in Fig. 3b [39]. The pK50 values found for CS-NP02, CS-NP04 and CS-NP06 were 6.78, 7.04 and 5.84, respectively. From these results we can observe that the formulation with higher amount of TPP (CS-NP06) displayed a hemolysis response curve shifted toward lower pH, while the other two NPs, with lower concentration ratio of TPP, showed a higher sensitivity to a small variation of pH. This might be attributed to a lower incorporation of the surfactant 77KS into the NP structure when greater amount of TPP is present. It seems that there is a bigger competition between 77KS and TPP for the positive sites of CS in CS-NP06. Therefore, these results support the early lytic activity of CS-NP02 and CS-NP04 at the pH range of early endosomal compartments (pH 6.6), whereas CS-NP06 induced considerable lysis only at the pH characteristic of late endosomes (pH 5.4).
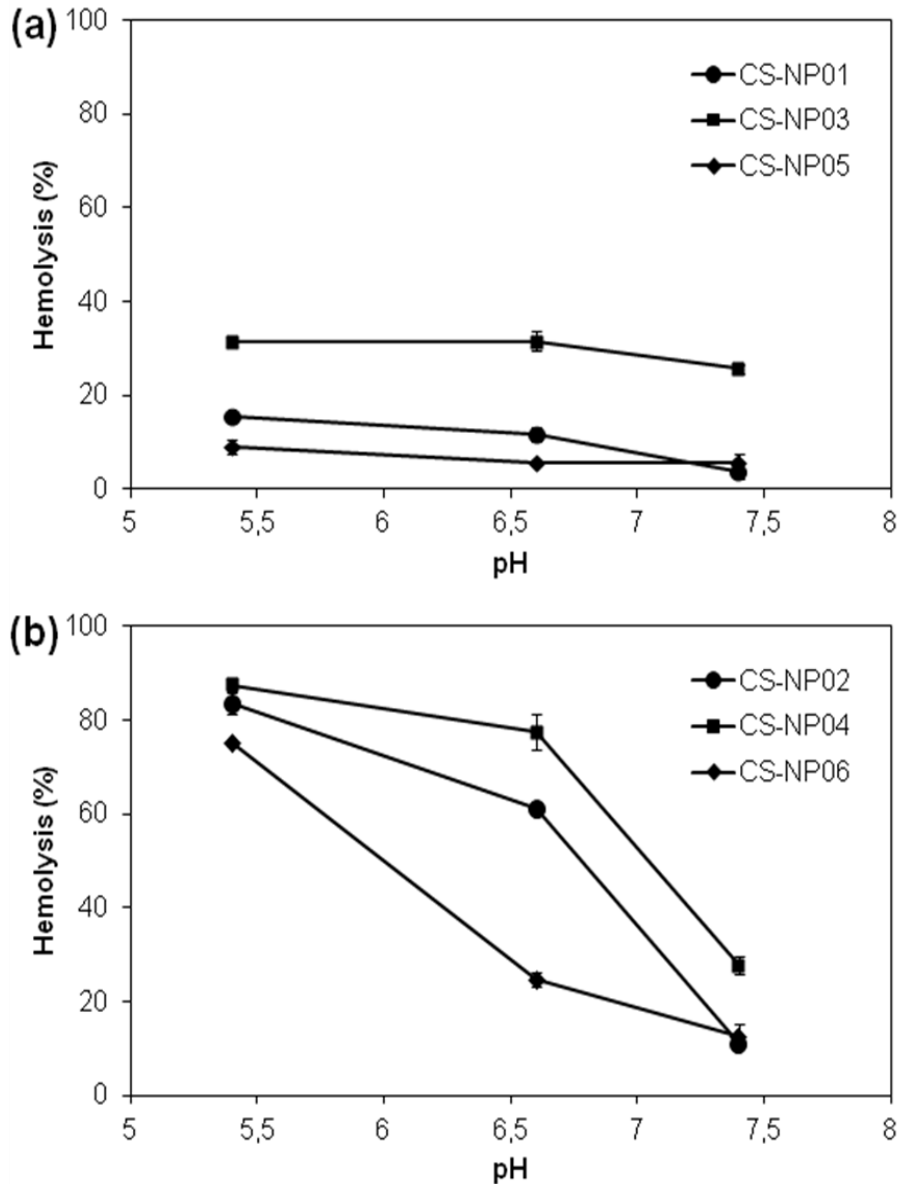
Fig. 3. Nanoparticle-induced membrane lysis as a function of pH. (a) CS-NPs without surfactants and (b) CS-NPs incorporating the pH-sensitive surfactants. CS-NPs were added to give final concentration of 500 μg/ml and were incubated for 60 minutes with human erythrocytes. Each point represents the mean of three independent experiments ± SE (error bars).

Despite the individual differences within the pH range tested, all NPs displayed hemolytic activity dependent on the concentration. Here, this enhanced membrane-lytic activity with increasing NP concentration could be due to the stronger affinity of positively charged NPs

towards the negatively charged cell membrane [40]. Furthermore, the positive charge of the NPs is expected to facilitate their cellular transport and adhesion [41].

As the pH-responsive activity of NPs was clearly demonstrated to be dependent on the presence of 77KS or 77KL, it can be inferred that the most significant hemolysis at acidic environments must be due to a modification in the hydrophobic/hydrophilic balance of these compounds at the acidic pH range. Noteworthy is the enhanced membrane lysis at pH 6.6 and 5.4 independently on the surfactant counterion. Therefore, it was assumed that the carboxylic group of the amino acid lysine undergoes an increase in its pKa value (reported to be originally of the order of 2.2) when it is included in the surfactant molecule. In order to confirm this hypothesis, we determined the pKa of 77KL and found that it is 6.2. Therefore, the enhanced hemolysis induced by NPs incorporating the pH-responsive surfactants might be due to the increasing protonation state of their carboxylic group at the acidic endosomal pH range. The protonation of the surfactant molecule makes it non-ionic and enhanced its hydrophobicity, which would increase binding to the membrane and, therefore, its lysis. This mechanism described for the behavior of the surfactants at acidic conditions, together with the fact that the positively charged NPs appear to be easily endocytosed [41], substantiate the potential of CS-NP02, CS-NP04 and CS-NP06 to facilitate the intracellular release of their cargo in response to a chemical stimuli as is the acidic pH of the endosomal compartments.

3.3. Kinetics of pH-dependent membrane-lytic activity

Considering that the formulations without surfactant did not displayed a pH-sensitive activity throughout the endosomal pH range, the kinetics of pH-dependent membrane-lytic activity was evaluated especially for NPs incorporating a surfactant. The results are also shown in Fig. 2.

1   Negligible membrane lysis was induced by all NPs with 77KS or 77KL after 10 min

2   incubation at physiological pH, while 60 min incubation prompted a slight increased hemolysis

3   rate (between 10 and 27% hemolysis). It is noteworthy that when the same NPs were formulated

4   without surfactants, a lesser extent of hemolysis was observed (between 3 and 15% hemolysis).

5   These results demonstrated that the higher hemolytic activity of the first formulations is due to

6   the presence of surfactant. It is well known that amphiphilic substances usually have some

7   unspecific interactions with lipid bilayer, leading to membrane lysis [42]. Between 77KS and

8   77KL, it is possible to notice that CS-NP04, incorporating 77KS, were more hemolytic than CS-

9   NP02, incorporating 77KL. Both NPs have the same CS:TPP ratio and the given results may be

10   related to the higher membrane-lytic activity of 77KS itself, as demonstrated in our previous

11   report [13]. At pH 7.4, 77KS has a HC_{50} (concentration inducing 50% hemolysis) of 298.90

12   µg/ml, while for 77KL the value is 489.22 µg/ml. Likewise, the greater hemolysis could be

13   attributed to the smaller size of the NPs incorporating 77KS, which might facilitate their insertion

14   into the lipid bilayer of the plasma membrane and, thus, it lysis.

15   As the pH decrease to 6.6 or 5.4, the membrane-lytic activity of the NPs incorporating

16   77KS or 77KL (CS-NP02, CS-NP04 and CS-NP06) increased significantly ($p < 0.005$) in a dose-

17   and, especially, time-dependent manner. This demonstrated that the presence of surfactant into

18   the formulations improved kinetics of hemolytic activity at both stages of endosomal

19   acidification. After 60 min incubation at pH 6.6, the membrane disruptive activity of CS-NP02,

20   CS-NP04 and CS-NP06 was approximately 2.4, 7.03 and 5.25-fold higher than that observed

21   after 10 min of incubation. At pH 5.4, the formulations CS-NP02 and CS-NP06 prompted high

22   hemolysis levels (41.59 and 58.72% hemolysis, respectively) after only 10 min incubation, while

23   CS-NP04 caused relatively weak hemolysis (14.90%). After 60 min, a sharp increased of

24   membrane lysis was observed to a maximum of about 83.45% (CS-NP02), 87.51% (CS-NP04)

25   and 75.06% (CS-NP06). The formulations CS-NP01, CS-NP03 and CS-NP05, all without any

surfactant, did not show any significant dependence on incubation time at the two mildly acidic conditions assessed, confirming their inability to disrupt endosomal membranes. Noteworthy is the rather unexpected kinetics of hemolytic activity presented by CS-NP03. However, this activity is significantly lower than that observed for the same formulation incorporating 77KS (CS-NP04).

The significant hemolytic kinetics at acidic conditions shown by NPs incorporating 77KS or 77KL indicates that a previous step seem to be required before the erythrocyte membrane becomes permeable to hemoglobin, i.e. the formation of pores or channels that lead to the efflux of low molecular weight solutes. It is worth mentioning that the molecules taken up by endocytosis are trafficked from early endosomes to lysosomes within several hours [43]. Thus, timely permeabilization of the endosomal membrane is a prerequisite for cytosolic translocation of drugs in order to exert their pharmacological effect [44]. Therefore, given the improved hemolytic kinetics and pH-responsive membrane activity of CS-NP02, CS-NP04 and CS-NP06, these formulations might have the ability to disrupt endosomal membranes before fusion of the endocytic vesicles with lysosomes, thus avoiding non-productive intracellular trafficking, a critical feature for potential intracellular drug delivery applications.

3.4. Cytotoxicity studies

The cytotoxicity of the NPs was assessed using *in vitro* cell-based assays with 3T3 fibroblasts as cell model, and MTT and NRU as endpoints to determine the cell viability. We evaluated the cytotoxicity of the NPs containing 77KS or 77KL, as the occurrence of any damaging effects is expected to be due the presence of surfactants. Noteworthy that our previous study on the cytotoxicity of these surfactants revealed their low cytotoxic effects against different cell lines in comparison to the typical commercial surfactant sodium dodecyl sulfate (SDS) [14].

1   As shown in Fig. 4, all tested CS-NPs either with 77KS or 77KL, exhibit low or negligible

2   cellular toxicity as determined by NRU and MTT assays. By the NRU assay, no cytotoxicity was

3   detected with any concentration tested. Likewise, the viability remained above 95% for all

4   formulations at the lowest concentrations by the MTT assay. In contrast, NPs showed some

5   cytotoxicity towards 3T3 cells by the MTT assay at the highest concentration tested. CS-NP04

6   and CS-NP06 were slightly cytotoxic (90.93% and 86.70% cell survival, respectively), while CS-

7   NP02 showed a small increase in the cytotoxicity level (81.22% cell viability) at the same

8   concentration (250 μg/ml). The higher cytotoxic effects of the formulation CS-NP02 could be

9   attributed to the surfactant 77KL or to the MMW-CS. These are the two variables that differ from

10   the other formulations. In fact, these results contrast with our previous study [14], in which the

11   cytotoxicity of the surfactant 77KL was lower than that of 77KS. However, from these results, it

12   can be inferred that the complexation of the surfactants into the polymeric NPs changed their

13   cytotoxic profiles.

14   Despite the presence of a surfactant as a bioactive compound into the formulations, it was

15   showed that the proposed nanocarriers displayed general low or negligible cellular toxicity and,

16   thus, can be suitable intracellular drug delivery systems without nonspecific cytotoxicity. The

17   biocompatible properties and natural source of these surfactants clear collaborate for the

18   biocompatibility of the designed NPs.

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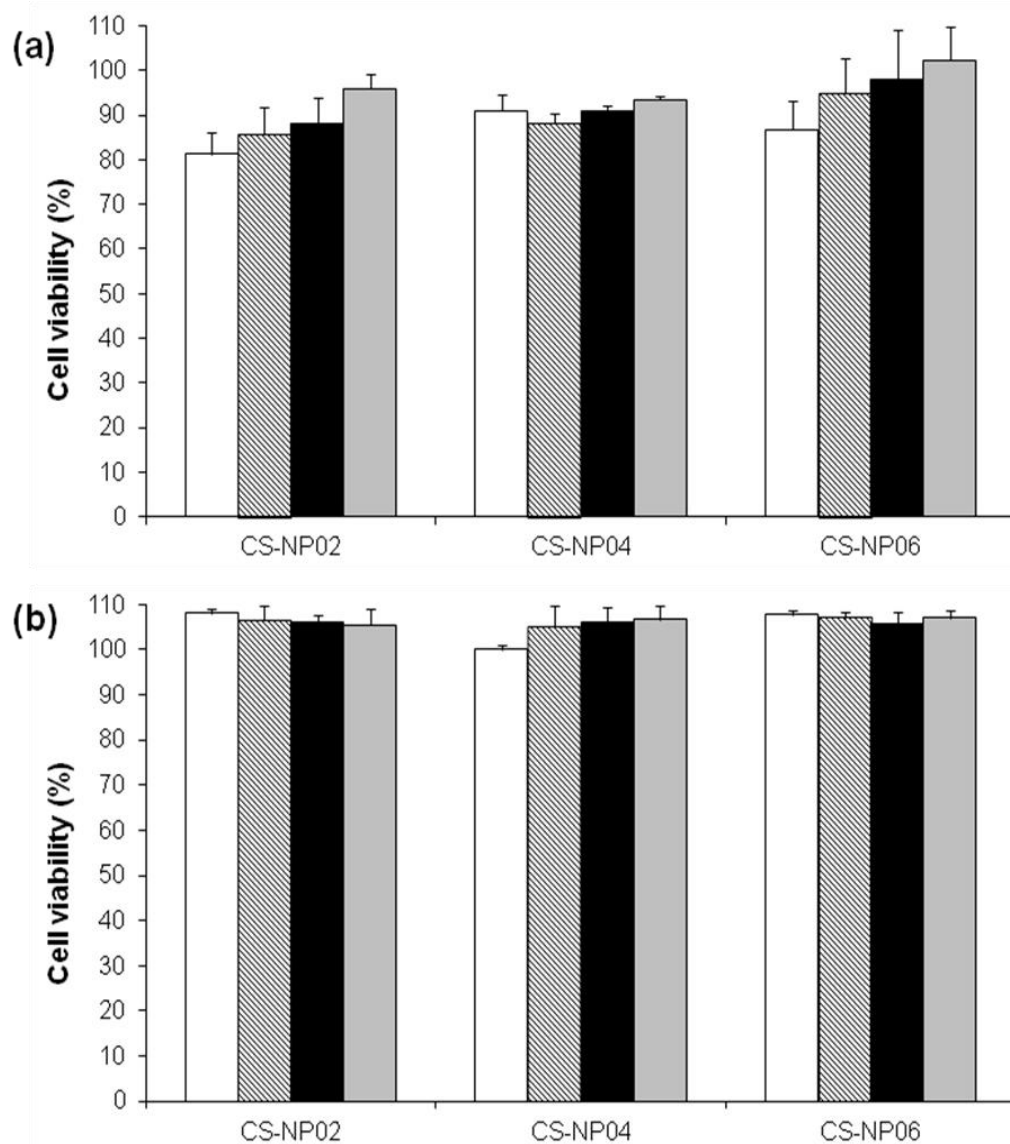
Fig. 4. Cytotoxicity of CS-NPs incorporating 77KL or 77KS on 3T3 cells as a function of concentration, as determined by (a) MTT and (b) NRU viability assays. Concentrations tested (from left to right) of 250 μg/ml (blank), 125 μg/ml (striped), 50 μg/ml (black) and 25 μg/ml (gray). The data represent the mean of three independent experiments ± SE (error bars).

4. Conclusions

Chitosan-based NPs with pH-responsive behavior were prepared by a mild process without using organic solvents. The designed NPs exhibited changes in their hydrodynamic size and membrane-disruptive activity as a function of the chitosan molecular weight, chitosan to TPP

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1   weight ratio and type of the surfactant counterion. It was clearly demonstrated that the NPs

2   containing the lysine-based surfactants have membranolytic activity specifically in mildly acidic

3   conditions found in the endosomal compartments. Moreover, the improved kinetics of the

4   hemolytic activity supports the ability of these functional nanodevices to disrupt endosomal

5   membranes before vesicular evolution from endosomes to lysosomes, where many drugs may

6   suffer degradation. Altogether, the results suggested the potential capability of these pH-

7   responsive nanocarriers to promote an improved delivery of bioactive compounds to the

8   intracellular compartments. This hypothesis is partially proven by a membrane lysis study on

9   human erythrocyte. However, further *in vitro* and *in vivo* studies should be performed to improve

10   the knowledge underlying this process.

11

12   **Conflict of interest statement**

13   The authors state that they have no conflict of interest.

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References

[1] F. Yan, L. Li, Z. Deng, Q. Jin, J. Chen, W. Yang, C.K. Yeh, J. Wu, R. Shandas, X. Liu, H. Zheng, Paclitaxel–liposome-microbubble complexes as ultrasound-triggered therapeutic drug delivery carriers, J. Control. Release 166 (2013) 246–255.

[2] A.E. Felber, M.H. Dufresne, J.C. Leroux, pH-sensitive vesicles, polymeric micelles, and nanospheres prepared with polycarboxylates, Adv. Drug Deliver. Rev. 64 (2012) 979–992.

[3] R.V. Ulijn, Enzyme-responsive materials: a new class of smart biomaterials, J. Mater. Chem. 16 (2006) 2217–2225.

[4] G.H. Gao, Y. Li, D.S. Lee, Environmental pH-sensitive polymeric micelles for cancer diagnosis and targeted therapy, J. Control. Release 169 (2013) 180–184.

[5] T. Liu, X. Li, Y. Qian, X. Hu, S. Liu, Multifunctional pH-disintegrable micellar nanoparticles of asymmetrically functionalized beta-cyclodextrin-based star copolymer covalently conjugated with doxorubicin and DOTA–Gd moieties, Biomaterials 33 (2012) 2521–2531.

[6] S.P. Victor, C.P. Sharma, Poly methacrylic acid modified CDHA nanocomposites as potential pH responsive drug delivery vehicles, Colloids Surf. B Biointerfaces 108 (2013) 219-228.

[7] J.T. Wilson, A. Postma, S. Keller, A.J. Convertine, G. Moad, E. Rizzardo, L. Meagher, J. Chiefari, P.S. Stayton, Enhancement of MHC-I antigen presentation via architectural control of pH-responsive, endosomolytic polymer Nanoparticles, AAPS J. (2014). Doi: 10.1208/s12248-014-9697-1

[8] M. Amoli-Diva, K. Pourghazi, M.H. Mashhadizadeh, Magnetic pH-responsive poly(methacrylic acid-co-acrylic acid)-co-polyvinylpyrrolidone magnetic nano-carrier for controlled delivery of fluvastatin, Mater. Sci. Eng. C Mater. Biol. Appl. 47 (2015) 281-289.

[9] A. Car, P. Baumann, J.T. Duskey, M. Chami, N. Bruns, W. Meier, pH-responsive PDMS-b-PDMAEMA micelles for intracellular anticancer drug delivery, Biomacromolecules 15 (2014) 3235-3245.

[10] J. Liu, Y. Huang, A. Kumar, A. Tan, S. Jin, A. Mozhi, X.J. Liang, pH-sensitive nano-systems for drug delivery in cancer therapy, Biotechnol. Adv. 32 (2014) 693-710.

[11] J. Van den Bossche, W.T. Al-Jamal, A. Yilmazer, E. Bizzarri, B. Tian, K. Kostarelos, Intracellular trafficking and gene expression of pH-sensitive, artificially enveloped adenoviruses in vitro and in vivo, Biomaterials 32 (2011) 3085-3093.

[12] R. Singh, K.T. Al-Jamal, L. Lacerda, K. Kostarelos, Nanoengineering artificial lipid envelopes around adenovirus by self-assembly, ACS Nano 2 (2008) 1040-1050.

[13] D.R. Nogueira, M. Mitjans, M.R. Infante, M.P. Vinardell, The role of counterions in the membrane-disruptive properties of pH-sensitive lysine-based surfactants, Acta Biomater. 7 (2011) 2846-2856.

[14] D.R. Nogueira, M. Mitjans, M.R. Infante, M.P. Vinardell, Comparative sensitivity of tumor and non-tumor cell lines as a reliable approach for in vitro cytotoxicity screening of lysine-based surfactants with potential pharmaceutical applications, Int. J. Pharm. 420 (2011) 51-58.

[15] L. Sanchez, M. Mitjans, M.R. Infante, M.P.Vinardell, Potential irritation of lysine derivative surfactants by hemolysis and HaCaT cell viability, Toxicol. Lett. 161 (2006) 53-60.

[16] L. Sánchez, M. Mitjans, M.R. Infante, M.T. García, M.A. Manresa, M.P. Vinardell, The biological properties of lysine-derived surfactants, Amino Acids 32 (2007) 133-136.

[17] A. Ahmad, S. Ranjan, W. Zhang, J. Zou, I. Pyykkö, P.K. Kinnunen, Novel endosomolytic peptides for enhancing gene delivery in nanoparticles, Biochim. Biophys. Acta 1848 (2015) 544-553.

[18] X.-L. Wang, S. Ramusovic, T. Nguyen, Z.-R. Lu, Novel polymerizable surfactants with pH-sensitive amphiphilicity and cell membrane disruption for efficient siRNA delivery, Bioconjug. Chem. 18 (2007) 2169-2177.

[19] J.H. Hamman, Chitosan based polyelectrolyte complexes as potential carrier materials in drug delivery systems, Mar. Drugs 8 (2010) 1305-1322.

[20] H. Jonassen, A.L. Kjøniksen, M. Hiorth, Stability of chitosan nanoparticles cross-linked with tripolyphosphate, Biomacromolecules 13 (2012) 3747-3756.

[21] Q. Gan, T. Wang, C. Cochrane, P. McCarron, Modulation of surface charge, particle size and morphological properties of chitosan-TPP nanoparticles intended for gene delivery, Colloids Surf. B Biointerfaces 44 (2005) 65-73.

[22] M.A. Vives, M.R. Infante, E. Garcia, C. Selve, M. Maugras, M.P. Vinardell, Erythrocyte hemolysis and shape changes induce by new lysine-derivate surfactants, Chem. Biol. Interact. 118 (1999) 1-18.

[23] P. Calvo, C. Remuñan-López, J.L. Vila-Jato, M.J. Alonso, Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines, Pharm. Res. 14 (1997) 1431-1436.

[24] T. Akagi, H. Kim, M. Akashi, pH-dependent disruption of erythrocyte membrane by amphiphilic poly(amino acid) Nanoparticles, J. Biomater. Sci. 21 (2010) 315-328.

[25] T. Mosmann, Rapid colorimetric assay to cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55-63.

[26] E. Borenfreund, J.A. Puerner, Toxicity determined in vitro by morphological alterations and neutral red absorption, Toxicol. Lett. 24 (1985) 119-124.

[27] E.N. Koukaras, S.A. Papadimitriou, D.N. Bikiaris, G.E. Froudakis, Insight on the formation of chitosan nanoparticles through ionotropic gelation with tripolyphosphate, Mol. Pharm. 9 (2012) 2856-2862.

[28] T. Ramasamy, T.H. Tran, H.J. Cho, J.H. Kim, Y.I. Kim, J.Y. Jeon, H.G. Choi, C.S. Yong, J.O. Kim, Chitosan-based polyelectrolyte complexes as potential nanoparticulate carriers: physicochemical and biological characterization, Pharm. Res. 31 (2014) 1302-1314.

[29] W. Fan, W. Yan, Z. Xu, H. Ni, Formation mechanism of monodisperse, low molecular weight chitosan nanoparticles by ionic gelation technique, Colloids Surf. B Biointerfaces 90 (2012) 21-27.

[30] Z. Wang, Y. Yu, W. Dai, J. Lu, J. Cui, H. Wu, L. Yuan, H. Zhang, X. Wang, J. Wang, X. Zhang, Q. Zhang, The use of a tumor metastasis targeting peptide to deliver doxorubicin-containing liposomes to highly metastatic cancer, Biomaterials 33 (2012) 8451−8460.

[31] A. Malhotra, X. Zhang, J. Turkson, S. Santra, Buffer-stable chitosan-polyglutamic acid hybrid nanoparticles for biomedical applications, Macromol. Biosci. 13 (2013) 603-613.

[32] F. Gao, Y. Cai, J. Zhou, X. Xie, W. Ouyanf, Y. Zhang, X. Wang, X. Zhang, X. Wang, L. Zhao, J. Tang, Pullulan acetate coated magnetic nanoparticles for hyperthermia: preparation, characterization and in vitro experiments, Nano Res. 3 (2010) 23-31.

[33] R. Chen, S. Khormaee, M.E. Eccleston, N.K.H. Slater, The role of hydrophobic amino acid grafts in the enhancement of membrane-disruptive activity of pH-responsive pseudo-peptides, Biomaterials 30 (2009) 1954-1961.

[34] M. Meyer, A. Zintchenko, M. Ogris, E. Wagner, A dimethylmaleic acid–melittin-polylysine conjugate with reduced toxicity, pH-triggered endosomolytic activity and enhanced gene transfer potential, J. Gene Med. 9 (2007) 797–805.

[35] S. Li, Z. Su, M. Sun, Y. Xiao, F. Cao, A. Huang, H. Li, Q. Ping, C. Zhang, An arginine derivative contained nanostructure lipid carriers with pH-sensitive membranolytic capability for lysosomolytic anti-cancer drug delivery, Int. J. Pharm. 436 (2012) 248-257.

[36] H. Kleszczynska, D. Bonarska, J. Luczynski, S. Witek, J. Sarapuk, Hemolysis of erythrocytes and erythrocyte membrane fluidity changes by new lysosomotropic compounds. J. Fluoresc. 15 (2005) 137-141.

[37] N.M. Moore, C.L. Sheppard, T.R. Barbour, S.E. Sakiyama-Elbert, The effect of endosomal escape peptides on in vitro gene delivery of polyethylene glycol-based vehicles, J. Gene Med. 10 (2008) 1134-1149.

[38] P.S. Stayton, A.S. Hoffman, N. Murthy, C. Lackey, C. Cheung, P. Tan, L.A. Klumb, A. Chilkoti, F.S. Wilbur, O.W. Press, Molecular engineering of proteins and polymers for targeting and intracellular delivery of therapeutics, J. Control. Release 65 (2000) 203-220.

[39] Y.-J. Lee, G. Johnson, J.-P. Pellois, Modeling of the endosomolytic activity of HA2-TAT peptides with red blood cells and ghosts, Biochemistry 49 (2010) 7854-7866.

[40] K. Seo, D. Kim, pH-dependent hemolysis of biocompatible imidazole-grafted polyaspartamide derivatives, Acta Biomater. 6 (2010) 2157-2164.

[41] Y. Huang, Y. Lapitsky, Salt assisted mechanistic analysis of chitosan/tripolyphosphate micro- and nanogel formation, Biomacromolecules 13 (2012) 3868–3876.

[42] V. Martínes, L. Sánchez, M.A. Busquets, M.R. Infante, M.P. Vinardell, M. Mitjans, Disturbance of erythrocyte lipid bilayer by amino acid-based surfactants, Amino Acids 33 (2007) 459-462.

[43] D.W. Pack, A.S. Hoffman, S. Pun, P.S. Stayton, Design and development of polymers for gene delivery, Nat, Rev, Drug Discov, 4 (2005) 581-593.

[44] A. Asokan, M.J. Cho, Cytosolic delivery of macromolecules 4. Head group-dependent membrane permeabilization by pH-sensitive detergents, J. Control. Release 106 (2005) 146-153.