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Relevance of the colloidal stability of chitosan/PLGA nanoparticles on their cytotoxicity profile

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ABSTRACT

The application of nanoparticles on a sub-cellular level necessitates an in depth study of their biocompatibility. However, complete characterization of the particles under the physiological conditions relevant for biological evaluation is still lacking. Our goal is therefore to evaluate the possible toxicity aspects of chitosan-modified PLGA nanoparticles on different cell lines and relate them to the parameters affecting the colloidal stability of the nanoparticles. The impacts of different factors such as nanoparticle concentration, exposure time, chitosan content in the particles and pH fluctuations on the cell viability were investigated. Meanwhile, the colloidal stability of the particles in cell culture media was checked by measuring their size and charge as well as visualizing the particles in media by scanning force microscopy (SFM). A slight shift in the pH of the culture medium to the acidic side allows the protonation of chitosan; thus the increased positive surface charge induced membrane damage (~50% increase in LDH released). Besides, cell viability is reduced by 15% in the absence of serum; serum in the culture medium forms a protective shell around the particles; such interaction influences the surface charge of the particles and was found to be a function of chitosan content in the particles. In conclusion, there is an undeniable impact of cell type, medium, presence/absence of serum on the colloidal state of the particles that consequently influence their interaction with the cells.

1. Introduction

Nanomedicine, a new attractive term frequently applied nowadays that implies for the medical application of nanotechnology as an alternative to the classical drug formulations. In the last decade, an increasing number of investigations concerning the use of nanoscale structures for drug and gene delivery purposes have been developed (Jin and Ye, 2007; Azarmi et al., 2008). Despite the significant scientific interests and promising potential in numerous applications, the safety aspects of nanoparticulate systems remain a growing concern as the processing of nanoparticles in biological systems could lead to unpredictable effects. In addition, due to the greater surface area-to-volume ratio for nanoscale material, the toxicity could differ from a similar bulk material (Xia et al., 2006). Indeed dealing with metal-based nanoparticles for drug delivery is much more crucial; therefore, a new sub-discipline of nanotechnology called nanotoxicology has emerged (Fischer and Chan, 2007).

One of the main goals in nanomedicine is the use of body-friendly and biodegradable materials and polymeric excipients. Poly(D,L-lactide-*co*-glycolide) (PLGA) is a biodegradable, synthetic polymer frequently used in drug/gene delivery (Panyam and Labhasetwar, 2003). The slight negative surface charge of PLGA nanoparticles (PLGA NP) tends to limit their interaction with the negatively charged plasmids and their intracellular uptake. Therefore, attempts have been made to modify the surface of PLGA NP using cationic polymers such as chitosan (Nafee et al., 2007; Ravi Kumar et al., 2004) retrieved from biological sources. Chitosan has been shown to be relatively safe (Corsi et al., 2003; Lee et al., 2001). Moreover, chitosan is approved as a food additive in Japan, Italy and Finland and as a wound dressing in the USA (Illum, 1998) and is widely used in drug delivery owing to its biocompatibility, mucoadhesive and permeability enhancing properties (Dodane et al., 1999). Nowadays, chitosan and its derivatives, e.g., trimethyl chitosan and thiolated chitosan gained a great interest as non-viral transfection reagents (Issa et al., 2005; Amidi et al., 2007; Martien et al., 2007; Hohne et al., 2007; Hwang et al., 2008). However, the derivatization and degree of deacetylation was sometimes found to influence the safety of the polymer (Kean et al., 2005; Guggi et al., 2004). Other

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studies also revealed a size-dependent toxicity (Qi et al., 2005; Yin et al., 2005).

The degree of toxicity of polymeric nanomedicines is strongly influenced by the biological conditions of the local environment, which influence the rate of degradation or release of polymeric nanomedicines. Many cationic polymers have been found to be toxic and it has been suggested that this toxicity is due to charge interactions with the plasma membrane and/or with negatively charged cell components and proteins (Fischer et al., 2003; Lv et al., 2006). On this basis, the physicochemical properties such as size distribution, surface charge and the presence of functional groups on the particle surface are considered key factors in judging the cytotoxicity. However, a complete understanding of the size, shape, composition and aggregation-dependent interactions of nanostructures with biological systems is currently still lacking.

Therefore, the aim of our study was not only the regular *in vitro* testing of the toxicity of chitosan-modified PLGA nanoparticles but also a deeper understanding of the factors responsible for the observed cytotoxicity assays results. In this context we investigated the influence of the surface modification of PLGA NP with chitosan, with emphasis on the importance of the colloidal stability of the particles along the study. Three different cell lines were used; African green monkey kidney cells COS-1 cells, human alveolar cancer cells A549 cells, and human bronchial epithelial cells Calu-3 cells. The safety of the particles was checked at different biological endpoints, including membrane integrity, mitochondrial activity, ATP release and integrity of the cell monolayer. The impact of pH changes, which are expected in the body, on the surface charge and subsequently on cytotoxicity was investigated. In addition, surface interaction of serum proteins and the multiple components in the cell culture medium with nanoparticle surface and their influence on toxicity and colloidal stability of the particles was verified. Scanning force microscopy was applied to visualize and evidence these surface interactions.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-*co*-glycolide) 70:30 (Polysciences Europe GmbH, Eppelheim, Germany), polyvinyl alcohol Mowiol® 4-88 (Kuraray Specialities Europe GmbH, Frankfurt, Germany), ultrapure chitosan chloride: Protasan® UP CL113 (molecular weight of 50–150 kDa and a degree of deacetylation between 75 and 90%) from NovaMatrix (FMC BioPolymer AS, Oslo, Norway), ethyl acetate (Fluka Chemie GmbH, Buchs, Switzerland) were used as obtained.

2.2. Methods

2.2.1. Preparation of nanoparticles

Chitosan-modified PLGA nanoparticles were prepared by an emulsion–diffusion–evaporation technique as previously described (Nafee et al., 2007; Ravi Kumar et al., 2004). In brief, 5 ml of PLGA dissolved in ethyl acetate (20 mg/ml) was added dropwise to 5 ml of an aqueous solution of the stabilizer PVA (2.5%, w/v) and the cationic polymer chitosan under magnetic stirring. The emulsion was stirred at 1000 rpm for 1 h. Afterwards, it was homogenized using an UltraTurrax T25 (Janke & Kunkel GmbH & Co-KG, Staufen, Germany) at 13,500 rpm for 10 min. The homogenized emulsion was diluted to a volume of 50 ml under constant stirring with MilliQ-water to form the nanoparticles. Remaining ethyl acetate was evaporated by continuous stirring overnight at room temperature. The concentration of chitosan in the aqueous phase was varied to obtain nanoparticles with different surface charges, Table 1. The abbreviations stated in the table will be used throughout the document as reference to the different particle preparations.

Table 1
Summary of the different chitosan concentrations and the respective nanoparticles obtained with their hydrodynamic size, their polydispersity index and their ζ-potential.

	NP-0	NP-3	NP-6	NP-9
Chitosan (%, w/v)	0	0.3	0.6	0.9
Particle size (nm)	148.2 (2.3)[a]	163.6 (2.9)	186.2 (5.5)	247.4 (5.64)
PI	0.03 (0.01)	0.14 (0.01)	0.17 (0.01)	0.19 (0.01)
ζ-Potential (mV)	−8.62 (0.2)	32.3 (1.96)	46.4 (1.65)	58.0 (1.01)

[a] Values in brackets denote the standard deviations ($n = 3$).

2.2.2. Measurement of colloidal characteristics

Nanoparticles were characterized with respect to mean diameter, polydispersity index (PI) and ζ-potential using the ZetaSizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). In general, the colloidal properties were determined in MilliQ-water. To check the colloidal stability nanoparticle suspensions were also diluted (0.9 mg/ml) and investigated in the biological media applied in the study. All measurements were performed in triplicates.

2.2.3. Cell cultures and treatments

COS-1 cells (CRL-1650, ATCC, Manassas, VA, USA), passage no. 10-20, were cultivated in DMEM supplemented with 10% fetal calf serum (FCS), 4500 mg/l glucose, Glutamax™ and 1 mM sodium pyruvate (all from Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Cells were seeded at a density of 100,000 cells/ml and allowed to attach for 24 h, for longer term experiments a lower cell density was applied.

A549 cells (CCL-185; ATCC, Manassas, VA, USA) were cultivated in RPMI with L-glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FCS. One day prior to experiments, A549 cells were detached using trypsin–EDTA and seeded in multiwell plates at a density of 100,000 cells/ml.

Calu-3 cells (HTB-55; ATCC, Manassas, VA, USA) were cultivated in Minimum Essential Medium (MEM) with Earl's Salts and L-glutamine (PAA Laboratories GmbH, Pasching Austria) supplemented with 10% FCS, 1% MEM non-essential amino acid (NEAA) solution and 1 mM sodium pyruvate (all from Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Calu-3 cells were seeded at a density of 200,000 cells/ml 3 days prior to the experiment.

All cells were kept in an incubator set to 37 °C, 5% CO_2 and 95% humidity. On the day of experiment, cells were washed with PBS and medium was changed.

2.2.4. Biological endpoints

2.2.4.1. MTT assay. Cells were incubated with nanoparticle samples in different concentrations for different time periods as will be described later. In addition, cells grown in culture medium only were considered as high control (100% cell viability) and others incubated with Triton X-100 (2%, w/v) were used as low control (0% cell viability). Afterwards, cells were washed with PBS and allowed to grow in the culture medium. On the next day, MTT solutions (5 mg/ml in PBS pH 7.4) were added for 3 h. The precipitated formazan was dissolved using acidified isopropanol for 0.5–1 h and quantified by measuring the absorbance at 550 nm in a multiwell plate reader (Tecan Deutschland GmbH, Crailsheim, Germany). Samples were applied in quadruplicates. Cell viability (%) was calculated by the following equation:

$$\%\,\text{cell viability} = \frac{\text{Abs}^{550}_{\text{exp}} - \text{Abs}^{550}_{\text{low conrol}}}{\text{Abs}^{550}_{\text{high conrol}} - \text{Abs}^{550}_{\text{low conrol}}} \times 100$$

Means and relative standard deviations (RSD) were calculated.

2.2.4.2. LDH assay. LDH assay is based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of dead or plasma membrane-damaged cells into the culture supernatant. LDH assay was performed on the same plates applied for MTT assay. After incubation of cells with the samples, the supernatants (100 μl) were transferred to 96-well plate for LDH assay whereas the original plates were used for MTT assay. Equal volume of the reaction mixture was added per well. Absorbance was measured at 492 nm and the cytotoxicity (%) was calculated relative to Triton X-100 as high control (100% cytotoxicity), and cells in culture medium as low control (0% cytotoxicity) as follows:

$$\%\,\text{cytotoxicity} = \frac{\text{Abs}^{492}_{\text{exp}} - \text{Abs}^{492}_{\text{low conrol}}}{\text{Abs}^{492}_{\text{high conrol}} - \text{Abs}^{492}_{\text{low conrol}}} \times 100$$

Interaction of the samples with the assay procedure (substance control) was also checked and not detectable.

2.2.4.3. ATP (Vialight® Plus) assay. In this assay, ATP (adenosine triphosphate) was used to assess the functional integrity of living cells, since all cells require ATP to remain alive. Any form of cell injury results in a rapid decrease in cytoplasmic ATP levels which can be detected utilizing the luciferase enzyme to catalyze the formation of light from ATP and luciferin. The emitted light intensity is linearly related to the ATP concentration. After incubation of A549 cells with the nanoparticle samples, 50 μl/well cell lysis reagent® was added for 10 min. Equal volumes of cell lysate and ATP monitoring reagent Plus® (100 μl) were incubated in white walled luminometer plate for 2 min at room temperature then the bioluminescence was measured. Substance control was done in parallel.

$$\%\,\text{cell viability} = \frac{\text{Lum}_{\text{exp}} - \text{Lum}_{\text{low control}}}{\text{Lum}_{\text{high control}} - \text{Lum}_{\text{low control}}} \times 100$$

2.2.4.4. Measurement of transepithelial electrical resistance (TEER). Calu-3 cells were seeded on tissue culture treated polyester membrane inserts for 12-well plate at a seeding density of 200,000 cells/ml. TEER values were measured using EVOM voltohmmeter (World Precision Instruments Inc., Sarasota, FL, USA) and corrected with respect to the background of the Transwell® insert with medium. When the resistance readings were between 1000 and 1500 Ω, nanoparticle samples, NP-3, were applied in different concentrations to the apical chamber and TEER measured at 2 and 4 h. Thereafter, nanoparticles were removed and replaced with culture medium. The reversibility of the effect was checked by measuring TEER after 24 h.

2.2.5. Parameters investigated

In order to understand the possible effects of the nanoparticles on the biological activity of the cells, several variables were investigated including:

- *Concentration of nanoparticles*: Different concentrations of the nanoparticle suspension (NP-3), Table 1, ranging from 0.1 to 2.5 mg/ml were incubated with two different cell types, COS-1 and A549 for 6 h, then MTT and LDH assays were performed.
- *Surface charge of the nanoparticles*: Different nanoparticle suspensions containing increasing concentrations of chitosan and accordingly carrying higher surface charges were prepared (Table 1). The effect of the surface charge on the cytotoxicity of the particles was studied on COS-1 and A549. Furthermore, Calu-3 cell lines were studied because of the anticipated effect of the charge especially on the barrier integrity.
- *Contact time*: Immediate and long-term toxicity of the particles (NP-3), Table 1, was studied by incubating A549 and COS-1 cells with the particles for 2, 4, 6, 8, 24 and 48 h after which the cell viability was determined.
- *pH of the culture medium*: RPMI mixed with HEPES buffer 100 mM of two different pH values 4.7 and 7.4 were applied during the incubation of the particles with A549 cells. The experimental pH of these mixtures was 6.5 and 7.4, respectively. The possible effects on cell proliferation (MTT assay) and/or membrane integrity (LDH assay) were investigated.
- *Presence of FCS in the culture medium*: FCS was suggested to affect the colloidal stability of the nanoparticles, therefore, nanoparticle samples in serum-free media as well as media supplemented with serum were applied, the viability of A549 and COS-1 cells was determined.
- *Assay procedure*: Cytotoxicity of nanoparticles with increasing chitosan content was assessed by three different assays (MTT, LDH and ATP assays).

2.2.6. Scanning force microscopy (SFM)

In order to investigate the surface morphology of the nanoparticles in the culture media, different nanoparticles containing increasing amounts of chitosan were examined by scanning force microscopy with a Bioscope™ equipped with a Nanoscope IV™ controller (Digital Instruments, Veeco, Santa Barbara, CA, USA). Dried samples (nanoparticles/culture media) were investigated under ambient conditions in tapping mode using a scanning probe with a force constant of 40 N/m at resonant frequency of ~170 kHz (Anfatec, Oelsnitz, Germany).

2.2.7. Statistical analysis

Data are expressed as mean ± standard deviation and analyzed by two-way ANOVA with the Holm–Sidak method for paired comparisons of means (SigmaStat 3.0, SPSS Inc. Chicago, IL, USA). Values of $p \leq 0.05$ were indicative of significant differences.

3. Results

Chitosan-modified PLGA nanoparticles prepared by the emulsion–diffusion–evaporation technique were characterized by a homogeneous size distribution and positive surface charge in MilliQ-water. Increasing chitosan content gradually increases the surface charge from 21 to 58 mV, NP-3–NP-9, as well as the width of the size distribution as can be seen from the increasing P.I. values, Table 1.

3.1. Nanoparticle concentration

Our previous studies demonstrated the efficacy of chitosan-modified PLGA nanoparticles, NP-3, to be taken up by A549 cells within 6 h incubation (Nafee et al., 2007; Taetz et al., 2009). The effects of these particles on different cell lines were investigated by testing membrane integrity via the LDH release and metabolic activity via mitochondrial enzymes. The viability of the cells, estimated by the MTT assay, after incubation with nanoparticles, NP-3, of increasing concentrations (0.1–2.5 mg/ml) for 6 h was found to be clearly dependent on the cell type; the viability of COS-1 cells was remarkably decreased with increasing NP concentration to reach ~35% with the highest NP concentration, Fig. 1A. On the other hand, 80–90% of A549 cells remained metabolically active after incubation with NP in the whole concentration range investigated. Cell morphology observed by optical microscopy also showed that the cytotoxicity of the nanoparticles was quite low. The effect of NP on the membrane integrity (LDH assay) was negligible and independent of the NP concentration with both cell lines (within the experimental error), Fig. 1B.

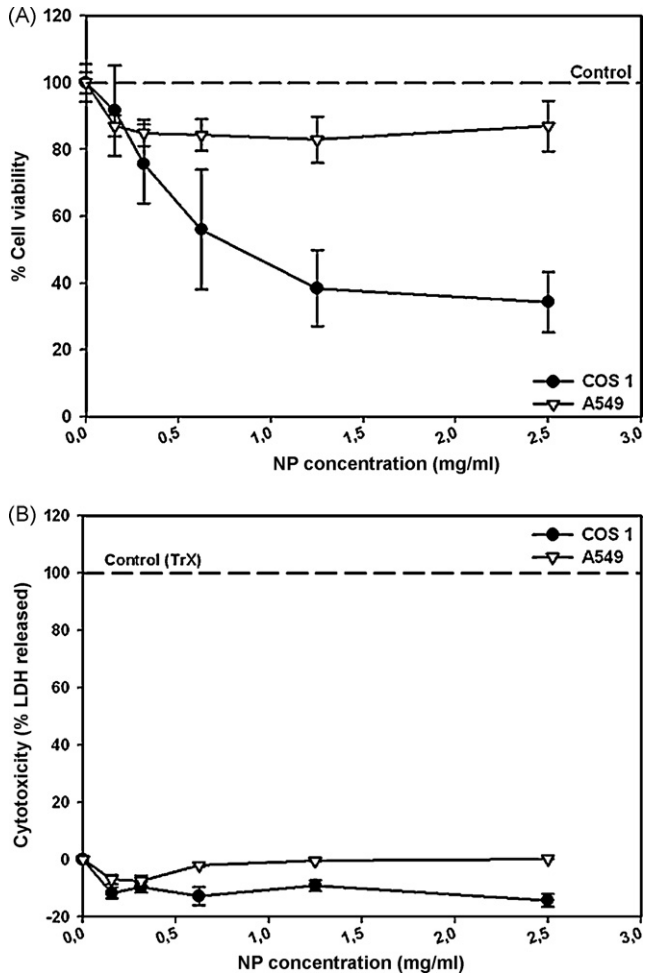

Fig. 1. (A) MTT assay and (B) LDH assay of chitosan-modified PLGA nanoparticles at different concentrations in COS-1 and A549 cell lines.

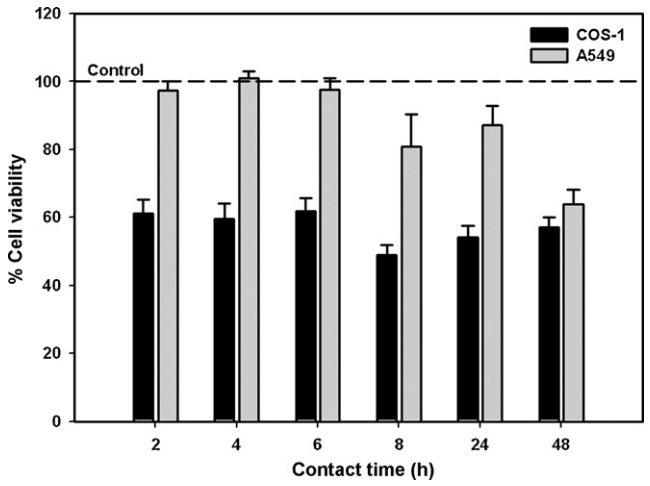

Fig. 2. MTT assay for chitosan-modified PLGA nanoparticles (0.9 mg/ml) at different contact times with A549 and COS-1 cell lines.

3.2. Contact time

PLGA nanoparticles are known to be slowly biodegraded; therefore, testing prolonged toxicity is as interesting as short-term toxicity. Accordingly, A549 cells were incubated with the nanoparticles NP-3 (0.9 mg/ml) for different time periods ranging from 2 to 48 h. As shown in Fig. 2, the survival rate of A549 cells was comparable to the control during the first 6 h (statistically insignificant, two-way ANOVA, $p < 0.05$), and then started to decrease to ~85% after 24 h and further to 64% after 48 h. In comparison, there has been a significant difference between the cell types (two-way ANOVA, $p < 0.05$); incubation of nanoparticles with COS-1 cells showed a remarkable toxicity directly after 2 h and along the study period as observed from the lower viability rates (Fig. 2).

3.3. Chitosan content on the nanoparticles

Cationic polymers are known to exhibit cytotoxic effects by inducing cell membrane damage (Fischer et al., 2003). For this reason, it was interesting to study the effect of different nanoparticles (NP-3–NP-9) prepared with increasing chitosan concentrations (0.3–0.9 mg/ml) – thus characterized by higher surface charge – on the viability of different cell lines. NPs were used in a concentration of 0.9 mg/ml. The survival rate, estimated by the MTT-test, was significantly dependent on the cell type (two-way ANOVA, $p > 0.05$) and was in the ranking order: Calu-3 > A549 > COS-1 cells, Fig. 3A. Around 90% of A549 and Calu-3 cells were metabolically active after incubation with NP-9 compared to only 40% in case of COS-1 cells.

Particle size measurement of the nanoparticles in different culture media indicated uniform distribution of PLGA nanoparticles, NP-0, in water, RPMI and MEM, Fig. 3B. In contrast, agglomerates were observed when chitosan-modified particles, NP-3–NP-9, were measured in culture media, Fig. 3C–E. Agglomeration was more pronounced in MEM than in RPMI. The higher the chitosan content (ζ-potential) the keener are the particles to agglomeration in medium and the less toxic they appear on A549 cells.

3.4. Incubation medium

The impact of the medium applied during the incubation of the particles with the cells and especially its pH is expected to play an essential role. Chitosan is known to acquire a positive charge in acidic media (Agnihotri et al., 2004), while chitosan-modified nanoparticles in neutral pH (as in cell culture medium) are found to have low or negligible surface charge. It is therefore essential to investigate the behavior of the particles when subjected to pH fluctuation as would be the case in the human body. In this context, non-modified PLGA nanoparticles, NP-0, and chitosan-modified PLGA nanoparticles, NP-3 (0.9 mg/ml) were diluted in three different media: RPMI, RPMI + (HEPES pH 7.4) in 1:1 mixture and RPMI + (HEPES pH 4.7), the experimental pH of the latter was found to be 6.5, and incubated with A549 cells for 6 h. Values were always normalized to the corresponding control treated with the same conditions (pH, medium composition). LDH assay revealed no destructive effect of the nanoparticles, NP-0 and NP-3, on the cell membrane in neutral media as seen before; RPMI and RPMI + (HEPES pH 7.4), Fig. 4, where chitosan is expected to be uncharged. On the other hand, a significant increase in LDH release was noticed and even more pronounced in NP-3 when (HEPES pH 4.7) was mixed with the culture medium (two-way ANOVA, $p < 0.05$). Despite the influence of the particles on the membrane integrity, negligible effect on the cell proliferation was observed as recorded by the MTT assay (data not shown).

It was hence necessary to check the colloidal stability of the nanoparticles in the different media used in this test. Particle size measurements revealed a monomodal distribution of PLGA nanoparticles, NP-0, in all media, while a bimodal distribution was observed in case of chitosan-modified particles, NP-3, indicating higher affinity of the latter to interact with the culture media independent of the presence of HEPES buffer, Fig. 4B and C. Similarly, PLGA nanoparticles retained their negative ζ-potential whereas a

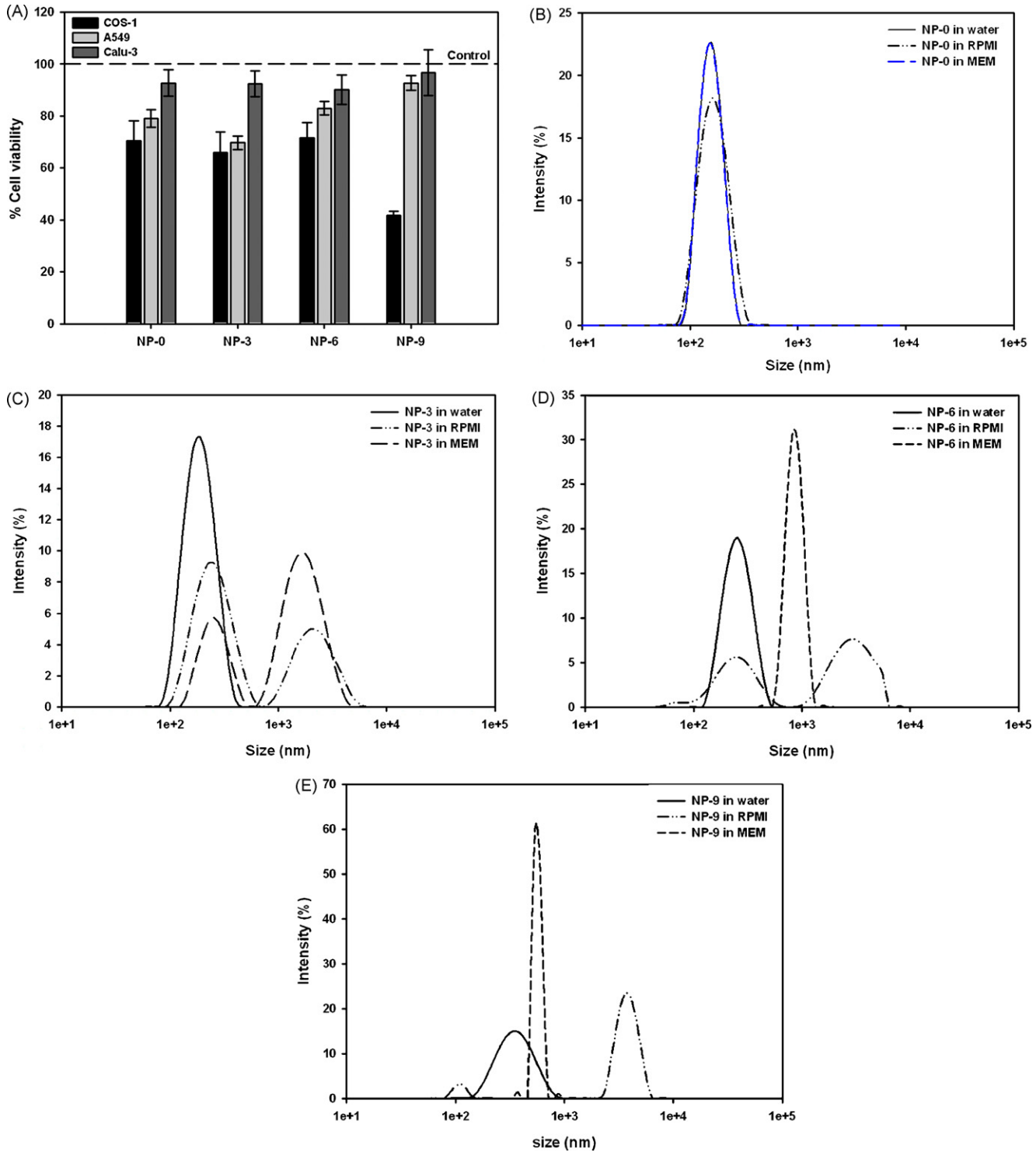

Fig. 3. (A) MTT assay for nanoparticles with increasing concentrations of chitosan on different cell types, (B–E) size distribution curves of nanoparticles (B) NP-0, (C) cNP-3, (D) cNP-6 and (E) cNP-9 in MilliQ-water and different culture media.

broad distribution of charges was observed for chitosan in culture media giving a mathematical mean of nil ζ-potential.

3.5. FCS in culture medium

Another important aspect regarding the influence of the culture medium on the toxicity of the particles is the presence of serum. COS-1 and A549 cells were incubated with nanoparticles, NP-3 (0.9 mg/ml) suspended in RPMI both in the presence and absence of 10% FCS; the cell viability was then determined. From Fig. 5A, one can notice a significant reduction in viability in the absence of FCS (∼15%) (two-way ANOVA, $p < 0.05$), which might indicate the protective role of serum. Several authors reported the adsorption of negatively charged serum proteins on the positively charged nanoparticles surfaces, therefore shielding or masking their original (probably harmful) effect on the cells (Schulze et al., 2008).

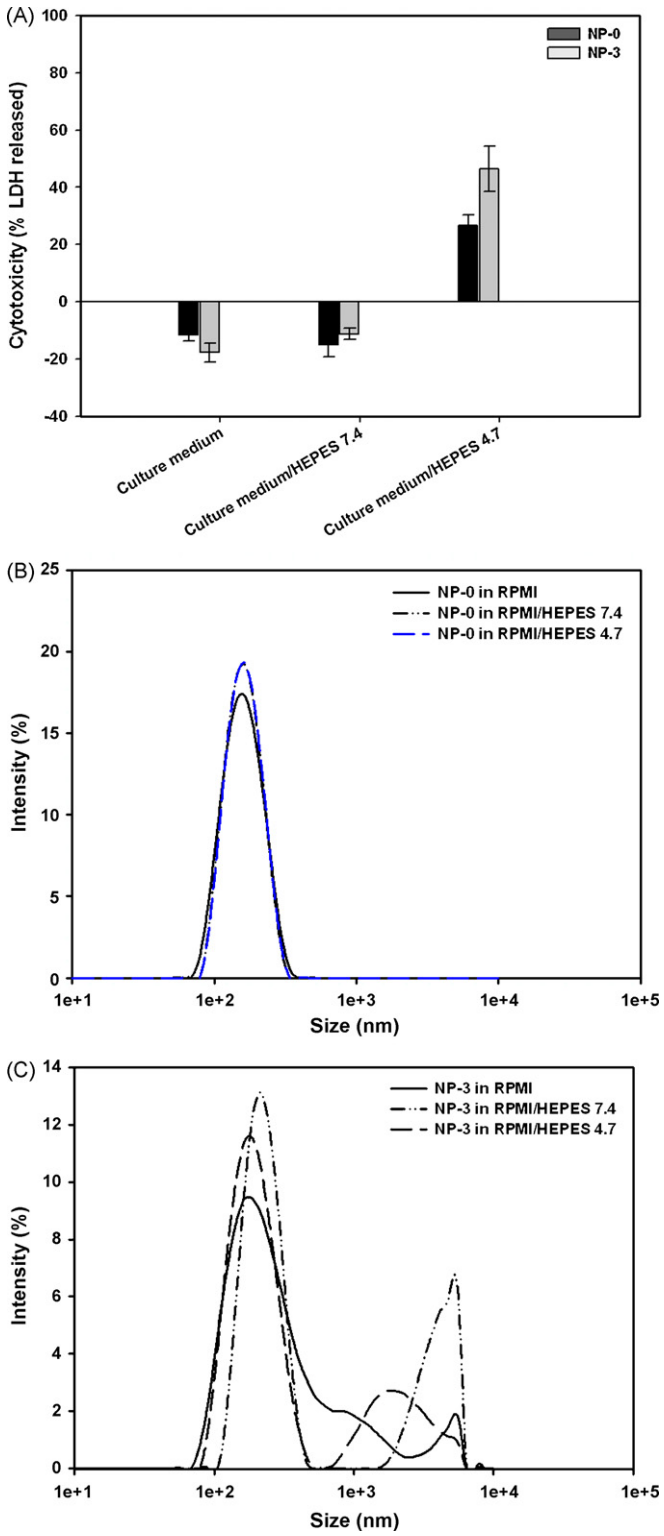

Fig. 4. (A) LDH assay for PLGA and chitosan-modified PLGA nanoparticles (0.9 mg/ml) incubated with A549 cells in different media, (B and C) size distribution curves of (B) PLGA NPs and (C) chitosan/PLGA NPs in these media.

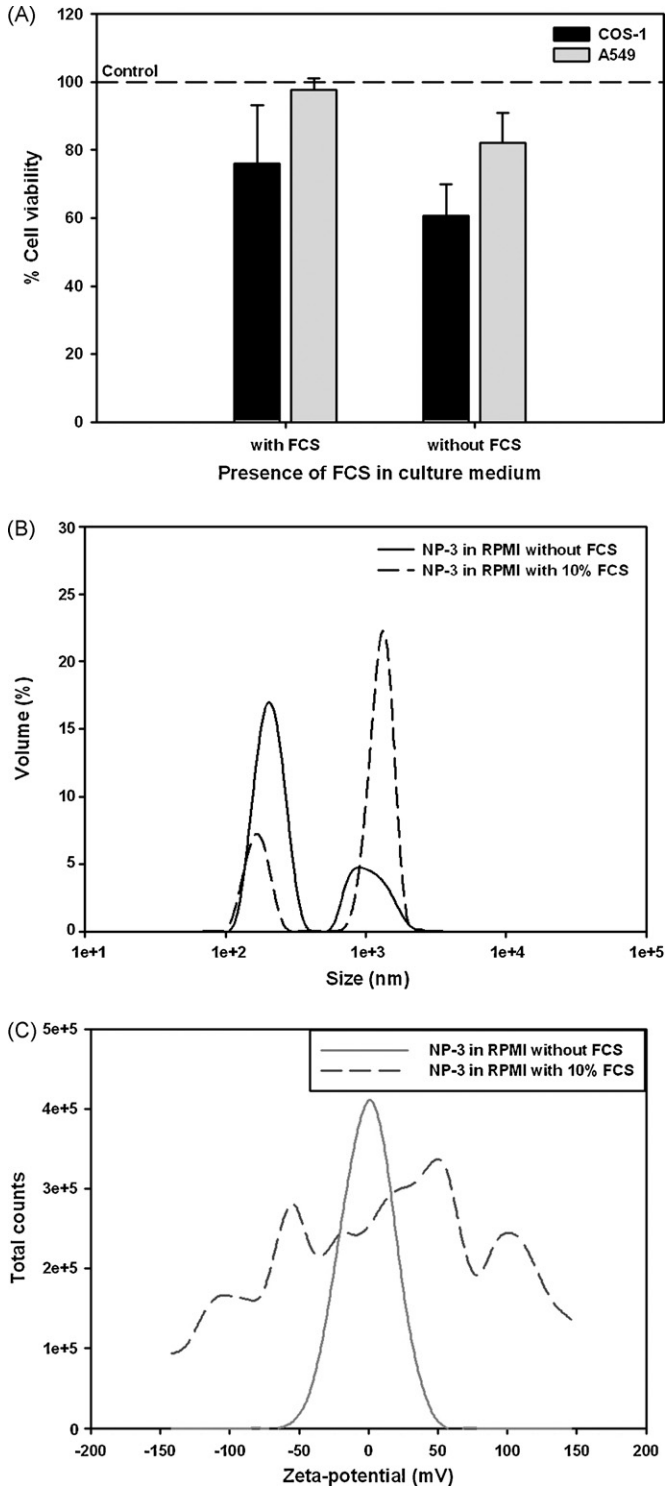

Fig. 5. Effect of the presence of FCS in the culture medium on (A) the viability of A549/COS-1 cells, (B) size distribution and (C) ζ-potential of chitosan/PLGA nanoparticles.

Accordingly, it was necessary to investigate the colloidal stability of the nanoparticles in the culture medium both in the presence and absence of FCS. Measurement of the particle size indicated the presence of some agglomerates (~1 μm) whereas a significant amount of the particles retained their original state in the nano-size range independent of the presence of serum, Fig. 5B. On the other hand, in the absence of serum the ζ-potential was found to have a mean value of zero indicating a risk of colloidal instability, while in serum-supplemented medium a broad undetermined range of surface charges was detected, Fig. 5C, which clearly reveals the strong uncontrolled interaction of the serum proteins with the nanoparticles surfaces.

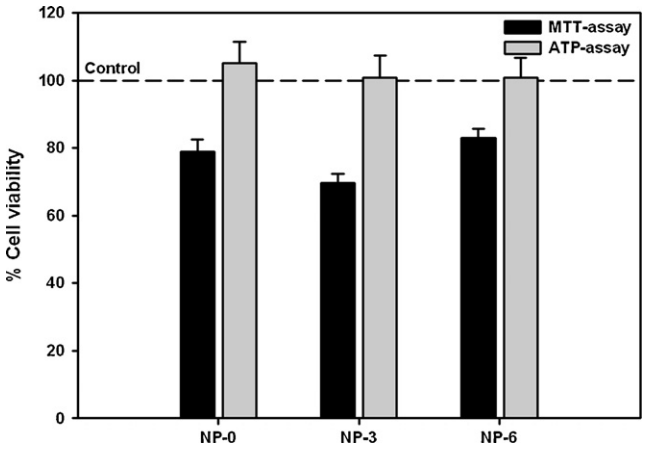

Fig. 6. Comparison between the viability of A549 cells after incubation with different nanoparticles (0.9 mg/ml) as determined by MTT and ATP assays.

3.6. Viability assays (MTT vs. ATP assay)

MTT assay is a widespread method to assess cell viability; a more recent method is the ATP assay. In order to check their validity to investigate the toxicity of our nanoparticles, a comparative experiment was done using nanoparticles (0.9 mg/ml) with different chitosan content; NP-0, NP-3, and NP-6. As shown in Fig. 6, viability of A549 cells incubated with nanoparticles was similar to the control as tested with the ATP assay, whereas a slight reduction in viability by ~15–25% was determined with the MTT assay. Despite the significant difference in magnitude (which is always expected due to the difference in assay principle and protocol), both assays showed the same tendency (two-way ANOVA, $p < 0.05$).

3.7. Colloidal stability of nanoparticles in culture media by SFM

In order to get a deeper insight on the nanoparticle behavior in different culture media as well as nanoparticles–serum surface interaction, the morphology of nanoparticles in different culture media, RPMI and MEM, was examined by SFM. Fig. 7 represents the arrangement of the particles, containing increasing amounts of chitosan, in RPMI and MEM. Generally speaking, one can see that the culture media tend to form complex patterns when dried on mica surfaces applied during the investigation (diffusion limited aggregation). It can be also noticed that PLGA nanoparticles, NP-0, remain evenly dispersed and can be clearly distinguished from these drying induced arrangements of the culture media, Fig. 7A. As seen from the height, amplitude and phase images PLGA NPs retain their smooth surface, their spherical well-defined shape either in RPMI or MEM. On the other hand, chitosan-modified particles, NP-3 and NP-6, were observed to be imbedded in the medium structures and usually surrounded by various small structures, which are most probably representing smaller protein units, Fig. 7B and C. Phase images reveal a distinct change in phase around each nanoparticle, indicating the absorption of other molecules from the medium to the nanoparticle surface.

3.8. TEER measurements

Chitosan-modified nanoparticles, NP-3, were incubated in different concentrations with Calu-3 cells, the TEER values were measured after 2 and 4 h. The reduction in TEER values is calculated as % of the initial values measured prior to the addition of particles. As shown in Fig. 8, diluted nanoparticle concentrations (0.02–0.3 mg/ml) resulted in slight decrease in the TEER values to ~80% of the baseline values, which is similar to the reduction caused in case of the control. Higher nanoparticle concentrations (1.3 mg/ml) induced a distinct temporary reduction in TEER values to ~45% after 2 h which started to recover again even in presence of the particles. Measurement of TEER values 24 h after starting the test reveals complete recovery of the monolayer integrity at all concentrations tested.

4. Discussion

Insights into the cytotoxic effects of nanoparticulate carriers are essential especially when they are intended to be applied on a sub-cellular level. PLGA is known to be benign to the cells. On the contrary, cationic polymers are reported to induce certain cell damage through their interaction with anionic components (sialic acid) of the glycoproteins on the surface of epithelial cells (Fischer et al., 2003). Nevertheless, chitosan was found to be less toxic than other cationic polymers such as poly-L-lysine and polyethyleneimine *in vivo* and *in vitro* (Carreno-Gomez and Duncan, 1997; Richardson et al., 1999). Therefore, chitosan was chosen to modify the surface of PLGA nanoparticles aiming to improve their binding potential with negatively charged plasmids and enhance their cellular uptake. The toxicity of these particles was measured by assessing cellular damage indicated by reduction in metabolic activity (MTT and ATP assays), or leakage of plasma membrane (LDH release).

Cytotoxicity is known to be a function of the cell type (Kean et al., 2005; Mueller et al., 2004), therefore, three cell lines were used in this study; COS-1 and A549 as fast growing cancerous cell types and Calu-3 cells to investigate the effect of the particles on the integrity of a cell monolayer. In case of COS-1 cells, cytotoxicity of chitosan-modified particles was found to be dose-dependent, this feature did not hold for A549 cells in the investigated dose range. Similarly, the survival of COS-1 cells was negatively affected after incubation with particles containing higher amounts of chitosan, while A549 and Calu-3 cells were found to be more robust. It is important to note that chitosan/PVA solutions of the same concentration used for the nanoparticles showed the same tendency (MTT, LDH assay) as chitosan/PLGA nanoparticles (data not shown).

Since the toxicity of cationic polymers is considered an interesting issue, several authors discussed the influence of polymer properties such as molecular weight, charge density, type of cationic functionalities, structure and sequence (block, random, linear, branched) and conformational flexibility (Choksakulnimitr et al., 1995; Ferruti et al., 1997; Singh et al., 1992).

Toxicity was checked over 48 h; the short time points give an indication of the toxicity of the particles over the time of an *in vitro* transfection experiment, while longer incubation time points have been tested to mimic the tissue-therapeutic contact time, which is expected in an *in vivo* experiment where clearance would take longer. Furthermore, the 24-h exposure is important as the cells would be within an exponential growth phase in this period meaning that any toxicity, due to inhibition of proliferation and/or cell death, would be clearly visible in the assay. As seen, different cell types react differently on the incubation with time. The COS-1 cells exhibit a reduced viability already for short incubation times while the A549 only responded after 8 h.

One of the interesting points to be discussed is the relation of particle toxicity with the composition and pH of the medium, yet this factor was rarely investigated. It is generally expected that diverse *in vivo* routes of administration can present different toxicological outcomes that vary with the surrounding pH. In our case, this can be considered a key factor, where the toxicity is thought to be due to the positive charge of the particles and the surface charge of the NP is pH-dependent. Normally, optimum conditions for cell cultures maintain pH 7.4, at which chitosan is mostly non-ionized

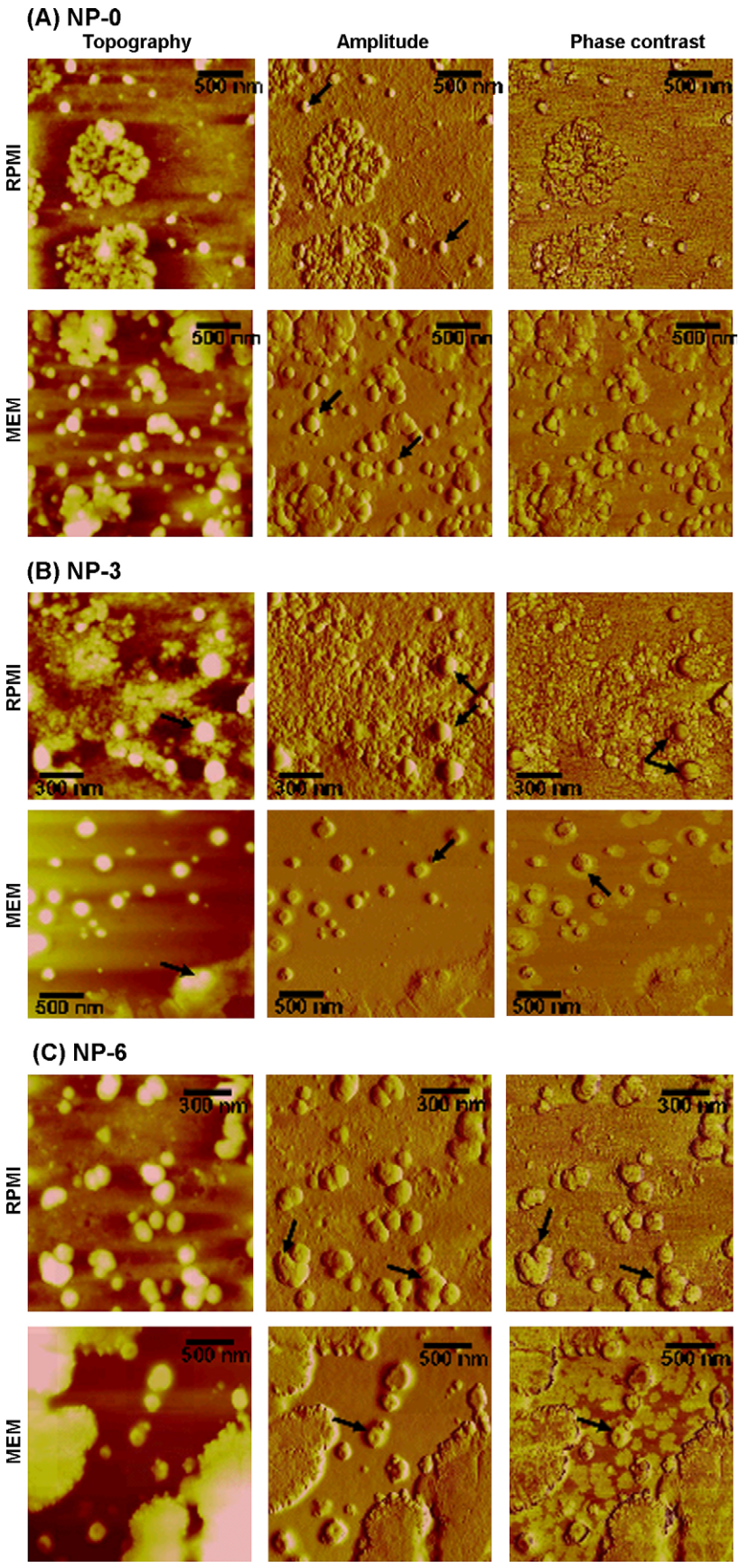

Fig. 7. Surface morphology of (A) NP-0, (B) NP-3 and (C) NP-6 in RPMI and MEM as examined by SFM. Arrows demonstrate nanoparticles either dispersed (NP-0) or surrounded by medium components (NP-3, NP-6).

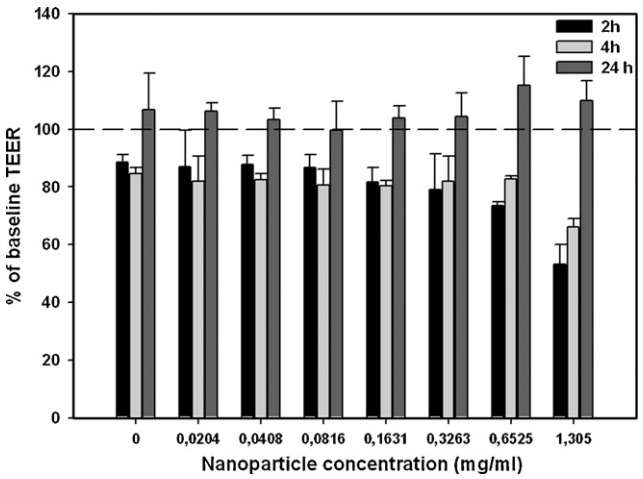

Fig. 8. Change in the TEER values of Calu-3 cells after incubation with chitosan-modified PLGA nanoparticles in different concentrations for different time periods.

and hence apparently non-toxic. The decrease of pH by adding HEPES buffer of pH 4.7 reduced the overall experimental pH of the medium to ~6.5, the survival rate of the cells under these conditions was high enough to perform the study but was relatively lower than cells grown in culture medium only. The relative reduction in pH allows a considerable amount of chitosan molecules to be ionized; which enhances their interaction with the plasma membrane, which was clearly demonstrated by the increase in LDH release indicating membrane damage. This suggests that the toxicity of chitosan is mediated by electrostatic interaction with the negatively charged membrane. The presence of the particles impacts on the toxicity in addition to the unfavorable conditions with the medium at pH 6.5. These conditions may further facilitate the toxic impact of the particles on the cells what is reflected also in the increased toxicity of the PLGA particles.

The colloidal behavior of nanoparticles in different buffers and culture media with respect to acute toxicity is still missing. Generally speaking, the main goal of using nanoparticles is their improved cellular uptake compared to larger size carriers. This fact still holds for our PLGA NP stabilized by PVA on their surface, which can be explained by the low absorption of serum proteins on PVA surface (Barrett et al., 2001). Nevertheless, a considerable fraction of the chitosan-modified PLGA particles are forming agglomerates in culture media as revealed by particle size measurements and morphological examination by SFM. This agglomeration is thought to reduce the effective concentration of the particles in the nanosize range, which in turns correlates with the 'apparently' reduced toxic effect of NP with higher chitosan content on A549 and Calu-3 cells. Therefore, it is not possible to refer our results exclusively to their nanoscale properties. Besides, many authors referred the cytotoxic effects of polycations to their charge interaction with cell membrane. However, ζ-potential measurements demonstrated that this positive surface charge does not exist any longer due to high ionic strength, the presence of divalent ions and the possible surface association of serum proteins to the nanoparticle surface, even when higher concentrations of chitosan were applied to the nanoparticles. This finding supports the hypothesis of the essential role of serum by forming a protective shell around nanoparticulate carriers, which is much of interest in case of metal or inorganic nanoparticles. The presence of a variety of nanoscale components in the culture media often complicates the measurement and interpretation of size and ζ-potential measurements. Many researchers tried to avoid the problem of the colloidal instability of the nanoparticle in culture media by using other buffers, which is far away from the *in vivo* conditions. Therefore, there is still a need to establish more physiologically relevant *in vitro* testing models that can efficiently substitute *in vivo* nanotoxicology studies (Fischer and Chan, 2007).

We have demonstrated that SFM can be a promising approach to visualize the distribution of the nanoparticles within the air dried culture medium. The distribution of the particles gives a hint to the presence of interactions within the medium. PLGA particles were evenly distributed whereas the chitosan particles were always found within the solid residues of the dried media indicating the favored interaction with the included materials. It is reasonable to assume that the interactions of nanoparticles with serum proteins and the diverse components of the culture media mask not only the charge of the particles but also their recognition by the cells as foreign bodies, which might be responsible for their enhanced cellular uptake. However, the overall arrangement in dendritic structures is not representative for the general interactions because the arrangement of the molecules will be influenced by the drying process of the sample, the changing concentrations and the hindered diffusional processes. Therefore, a next step should be the SFM measurement of the nanoparticles in culture media under liquid to get a better insight in the situation in the medium and to avoid possible artifacts. Furthermore, studying nanoparticle–cell surface interactions is an ongoing topic.

The presence of tight junctions between neighboring epithelial cells prevents the free diffusion of hydrophilic molecules across the epithelium by the paracellular route. A sensitive indicator for sub-lethal toxicity is the loss of integrity of tight junctions revealed by TEER measurements. Our results indicate a temporary, concentration-dependent opening of the tight junctions of Calu-3 cells when exposed to chitosan-modified nanoparticles. Similarly, Smith et al. (2004) found that chitosan cause a dose-dependent reduction in TEER of Caco-2 monolayers of up to 83% that is caused by a translocation of tight junction proteins from the membrane to the cytoskeleton.

Our findings can be summarized in the following; the toxicity of chitosan-modified PLGA NP is dependent on the cell type and was found to be in the order COS-1 > A549 > Calu-3 cells. The colloidal stability of the nanoparticles is remarkably reduced when suspended in the culture media. Due to the formation of agglomerates, a significant reduction in the amount of nanosized fraction decreases the actual effective concentration of the particles along the study. This is supported by the data of the chitosan/PLGA particles with different chitosan amounts. The higher the amount (ζ-potential) the keener are the particles to agglomeration in medium and the less toxic they are on A549 cells. For COS-1 cells there seems to be no impact of lower charges (or uncharged); only the highest chitosan concentration showed a different toxicity level. Besides, the adsorption of multicomponents of the culture medium on the NP surface (as visualized by SFM) limits their recognition by the cells as foreign bodies, and hinders the 'real' surface interaction between the nanoparticle and the cell. Both factors support the idea of underestimated nanotoxicity in general. On the other hand, stimulation of chitosan ionization by reducing the pH of the incubation medium relatively increases the positive surface charge of the particles and in turns destabilizes the cell membrane. This clearly demonstrates the role of charge in NP-cell surface interaction.

Although our chitosan/PLGA NPs did not show evident acute harmful effects to the investigated cell lines, the real interactions between the nanoparticles and the target cells on sub-cellular and molecular levels are still poorly understood. Therefore, the identification of fundamental cellular responses to nanoparticles (such as generation of reactive oxygen and activation of redox-sensitive signaling cascades) is still necessary to complement the toxicological testing with a mechanistic approach.

5. Conclusions

From this study, it can be concluded that the cytotoxicity of chitosan-modified PLGA nanoparticles is a function of the cell line. Nevertheless, the toxicity is underestimated due to the colloidal instability in culture media, reduction in effective nanoparticle concentration on the nanosize range, in addition to adsorption of medium components to the nanoparticle surface. Slight shift of the surrounding pH allows ionization of chitosan and increase in surface charge of the nanoparticles, which as a consequence lead to more pronounced loss of membrane integrity of the cells.

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