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Cytotoxicity assessment of heparin nanoparticles in NR8383 macrophages

H. Eidi[a], O. Joubert[a,1], G. Attik[a,1], R.E. Duval[b], M.C. Bottin[a], A. Hamouia[c], P. Maincent[a], B.H. Rihn[a,*]

[a] Nancy-Université, Faculty of Pharmacy, EA 3452, 5 rue Albert Lebrun, 54001 Nancy Cedex, France
[b] Nancy-Université, Faculty of Pharmacy, Equipe GEVSM, SRSMC, CNRS, 5 rue Albert, Lebrun, 54001 Nancy Cedex, France
[c] University of Al-Baath, Pharmacology and Toxicology Department, Faculty of Veterinary Medicine, Hama, Syria

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ABSTRACT

The bioavailability of low molecular weight heparin (LMWH) has been increased by encapsulation in nanoparticles. As a complement to these results, the cytotoxicity and apoptosis induced by LMWH nanoparticles prepared by two methods [nanoprecipitation (NP) and double emulsion (DE)] using Eudragit® RS (RS) and poly-ε-caprolactone (PCL) have been analysed. Particle sizes varied from 54 to 400 nm with zeta potential values between −65 and +63 mV. Our results showed that the method of nanoparticle preparation affects their properties, especially in terms of drug incorporation and cell tolerance. Cell viability ranged from 6% to 100% depending on the preparation method and physicochemical properties of the particles and the type of toxicity assay. Particle diameter and zeta potential seemed to be the most valuable cytotoxicity markers when cell viability was measured by Trypan blue exclusion and MTT respectively. Nanoparticles prepared by DE were better tolerated than those of NP. LMWH encapsulation into the cationic nanoparticles reduces remarkably their toxicity. Apoptosis evaluation showed activated caspases in exposed cells. However, no nuclear fragmentation was detected in NR8383 cells whatever the tested nanoparticles. DE nanoparticles of RS and PCL can be proposed as a good LMWH delivery system due to their low toxicity (IC_{50} ~2.33 and 0.96 mg/mL, respectively).

1. Introduction

Nanotechnology has a large number of potential applications in many different areas and as a result concern about the safety of nanomaterials has become a major concern. Novel engineered nanomaterials have unique physicochemical properties including small size, shape, high surface area, surface activity and chemical composition. Therefore, these nanomaterials might exhibit new toxic effects and thereby may represent a considerable health hazard (Kabanov, 2006). Therefore, the risk/benefit ratio for the use of nanoparticles needs to be evaluated for any technological or medical development (Donaldson et al., 2004; Medina et al., 2007). Although numerous *in vitro* "nanotoxicology" studies have already been published, most of the experiments carried out so far, have not used particles that have been very well characterized for their composition and physicochemical properties (Kroll et al., 2009). This characterization is necessary because nanoparticles might interact differently with assay components, especially culture medium proteins, or interfere with detection systems resulting in unreliable data (Lison et al., 2008; Schulze et al., 2008).

Nanosystems with different compositions and biological properties have been extensively investigated for drug and gene delivery applications (Brannon-Peppas and Blanchette, 2004; Yokoyama, 2005; Pison et al., 2006; Schatzlein, 2006). Nanoparticles used as drug delivery vehicles are generally <500 nm in diameter and consist of different biodegradable or non-biodegradable materials such as natural or synthetic polymers, lipids or metals (Suri et al., 2007; Kroll et al., 2009). There are many publications describing the advantages of drug delivery systems (DDS) nanoparticles (Ferrari, 2005; Couvreur and Vauthier, 2006; Mohanraj and Chen, 2006; Zhang et al., 2008), but data on their toxicity are scarce. The majority of studies have focused on cytotoxicity of nanoparticles present in the environment rather than nanoparticles designed for drug delivery. Additionally, most of *in vitro* and *in vivo* studies investigated the toxicity of whole nanosized DDS but scarcely the nanoparticle itself. Beside their size, the toxicity of these DDS can be different according to their source: material-biological-metal based or polymeric,

Abbreviations: Aspec., specific surface area; DDS, drug delivery system; DE, double emulsion; DMSO, dimethyl sulfoxide; DQ12, quartz microparticles; FITC, fluorescein isothiocyanate; FMK, fluoro methyl ketone; IC_{50}, half-maximal inhibitory concentration; LMWH, low molecular weight heparin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP, nanoprecipitation; PCL, poly-ε-caprolactone; PI, polydispersity index; PLGA, poly(lactic-*co*-glycolic acid); PVA, polyvinyl alcohol; QESD, quasi-emulsion solvent diffusion; RS, Eudragit® RS; UFH, unfractionated heparin; VAD, valine-alanine-aspartate; + and −, nanoparticle with and without LMWH respectively.

* Corresponding author at: Faculty of Pharmacy, 5 rue Albert Lebrun, BP 80403, 54001 Nancy Cedex, France. Tel.: +33 0383 682 355; fax: +33 0383 682 301.
E-mail address: bertrand.rihn@pharma.uhp-nancy.fr (B.H. Rihn).
[1] Both these authors contributed equally to this work.

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their preparation methods and their degradation products or biopersistence (Committee on Toxicity of Chemicals in Food Consumer Products and the Environment, 2006).

Poly-ε-caprolactone (PCL) and Eudragit® RS (RS) are widely used for manufacturing medical device and dosage forms, respectively. Indeed, PCL, a biocompatible and biodegradable polyester, is used for resorbable sutures whereas RS is often used for tablet coating: consequently, both are accepted by health authorities in major countries (Europe, Japan, USA) (Hoffart et al., 2006). Actually, RS was widely used because of its well-established mucoadhesive characteristics. This polycation is insoluble at physiological pH but swells in water (Pignatello et al., 2002). Moreover, this non-biodegradable positively charged polymer has been used for developing nanoparticles for the ophthalmic and oral administration of ibuprofen (Pignatello et al., 2002) and cyclosporins (Ubrich et al., 2005), as well as nano- and micro-fibers used as scaffolds in tissue engineering (Vaquettea et al., 2008). Few *in vitro* studies are dedicated to the toxicity of oral nanoparticulate DDS based on RS or PCL. Furthermore, the majority of these studies have been used only one assay (often MTT) to evaluate the toxicity of nanosized DDS. Although many studies have evaluated the nanosized-DDS toxicity depending on cell type, preparation methods, nanoparticle concentration and experimental conditions, they are estimated as limited data (Pignatello et al., 2002; Gargouri et al., 2009; Lopedota et al., 2009).

The injection of low molecular weight heparin (LMWH) does not necessarily require patient hospitalization, and for this reason it is now replacing unfractionated heparin (UFH) in many countries, thereby decreasing the cost in health care (Hull et al., 1998; Hoffart et al., 2006). However, both UFH and LMWH are still administered by injection only. So, it would be an important breakthrough in the care of such patients to be able to administer LMWH orally. In early eighties, Maincent et al. (1984, 1986) demonstrated that orally nanosized DDS were an improvement in bioavailability of drugs previously administered by parenteral route. Recently, an oral LMWH formulation based on nanoparticles formed from mixtures of PCL and RS has been developed. Although good oral bioavailability has already been demonstrated with these LMWH nanoparticles namely 20–40% of administered dose in rabbits (Jiao et al., 2002), pre-clinical and clinical studies will be necessary before they can be considered for pharmaceutical use. The effects of LMWH nanoparticles made of RS on human epithelial cell line have been investigated by Lamprecht et al. (2006) and found to have low toxicity. Thus, our work aim is to assess the toxic effects of these nanoparticles using another related cell model namely the macrophages. Actually, the largest database of the nanoparticles toxicity is provided by inhalation studies; then numerous *in vitro* assays are performed using macrophage cell lines (Nguea et al., 2008). Through their capacity to cross different biological barriers and their phagocytic activity, macrophages play a crucial role in determining the biopersistence of foreign particles and initiating inflammatory of non-specific or specific immune responses. Used in a convenient assay, macrophages allow to qualifying and comparing the cytotoxicity profiles of a range of nanoparticles according to size, surface activity, dissolution rate. Thus, macrophages can be considered as a valuable cell model to evaluate nanoparticle toxicity (Oberdorster et al., 2005b; AFSSAPS, 2009; Lanone et al., 2009).

The goal of this study was to evaluate and compare the cytotoxicity of a set of nanosized DDS obtained with two different LMWH encapsulation methods using well known pharmaceutical polymers. The cytotoxicity was explored through assessment of cell growth and apoptosis markers in a rat alveolar macrophage cell line NR8383 regarding to the physicochemical characteristics, composition and preparation method of the nanoparticles.

2. Materials and methods

2.1. Materials

LMWH, *i.e.*, bemiparin (mean MW = 3600 Da [91449-79-5]) was generously provided by Rovi (Madrid, Spain). Two polymers were used to prepare nanoparticles: Eudragit® RS PO (MW = 150,000 Da [33434-1]), an acrylic polycationic copolymer of acrylic and methacrylic acid esters with a proportion of quaternary ammonium groups (0.5–0.8%), was a gift of Evonik polymers (Darmstadt, Germany). Poly(-ε-caprolactone) (PCL) (MW = 42,000 Da [24980-41-4]) was purchased from Sigma (l'Isle d'Abeau Chesnes, France). Pluronic F 68 [11104-97-5] and poly(vinyl alcohol) (99% hydrolyzed, [9002-89-5]) (PVA) were used as surfactants, and obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). Quartz, type DQ12 microparticles (87% α-quartz + 13% amorphous, 2.99 μm) were provided by the Institute of Toxicology and Experimental Medicine (Hanover, Germany).

For the culture of NR8383 cell line and cytotoxicity assay: Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Invitrogen, Cergy Pontoise, France), foetal calf serum was furnished by Eurobio (Eurobio, Les Ullis, France). Penicillin [113-98-4], streptomycin [128-46-1], amphotericin B [1397-89-3], L-glutamine [78354-52-6], 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [57360-69-7] and paraformaldehyde [30525-89-4] were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). Hoechst dye 33258 and CaspACE™ FITC-VAD-FMK (Fluorescein Iso-Thio Cyanate conjugate of the caspase inhibitor: Valine-Alanine-Aspartate Fluoro Methyl Ketone) were obtained from Promega (Promega, France).

2.2. Preparation of nanoparticles

2.2.1. Nanoparticles by nanoprecipitation method (NP)

Eudragit® RS (RS) and polycaprolactone (PCL) nanoparticles were prepared by the nanoprecipitation method as already described (Fessi and Puisieux, 1989; Bodmeier et al., 1991b). Briefly, 300 mg of polymer was dissolved in 15 mL of acetone (organic phase). In the case of LMWH-loaded particles, 1 mL of an aqueous LMWH solution (5000 IU) was added to the organic phase. The organic solution was poured in the body of a syringe, and flowed slowly, under stirring, in 40 mL of a Pluronic® F68 (0.5%, w/v) aqueous phase. The solvent was removed by rotary evaporation under vacuum at 40 °C (Heidolph, Schwabach, Germany) until 5 mL of nanoparticles suspension were obtained. RS or PCL nanoparticles prepared according to this method with and without LMWH (+ and −) were named NP/RS− and NP/RS+ or NP/PCL− and NP/PCL+, respectively.

2.2.2. Nanoparticles by double emulsion (DE)

Nanoparticles were prepared by using the double emulsion/solvent evaporation technique (w/o/w) (Bodmeier et al., 1991a,b). Briefly, 125 mg of PCL or RS was dissolved in 5 mL of the organic solution (ethylacetate or methylene chloride). Either water (1 mL) or an aqueous solution of LMWH (1 mL, 5000 IU) was emulsified into this organic phase by sonication (80 W for 30 s) using an ultrasonic homogenizer (Vibracell 75022, Bioblock, Illkirch, France) for the preparation of empty or LMWH-loaded nanoparticles, respectively. This primary w/o emulsion was then dispersed by sonication (80 W for 1 min) into 40 mL of an aqueous solution of PVA (0.1%, w/v), thus producing a secondary w/o/w emulsion. The resulting nanoparticles were obtained by the evaporation of the organic phase down to 5 mL RS or PCL nanoparticles prepared by this technique with and without LMWH (+ and −) were named DE/RS− and DE/RS+ or DE/PCL− and DE/PCL+, respectively.

2.3. Colloidal stability study

The stability of nanoparticles was determined by monitoring the changes in size, polydispersity and zeta potential as a function of storage time at 4 °C. To check the colloidal stability, nanoparticle suspensions were also diluted and investigated in the biological media used in the study namely DMEM and in double distilled water. All measurements were performed in triplicate.

2.3.1. Particle size measurement

Particle sizes were investigated by photon correlation spectroscopy (PCS) using a Zetasizer™ (Malvern Instruments, UK). Each sample was diluted with double distilled water until the appropriate concentration of particles to avoid multiscattering events was achieved. The concentration and dilution were kept constant for all samples. The particle size (*z*-average) and size distribution (polydispersity index, PI) of equivalent hydrodynamic spheres were calculated using the Malvern software associating analysis and an exponential sampling method. Each measurement was performed, at least, in triplicate. The particle *z*-average diameter was used to calculate the specific surface area ($A_{spec.}$ given in m^2/g) of empty nanoparticles assuming a spherical particle shape (Eq. (1)). RS and PCL densities (ρ) were 0.82 and 1.05 g/cm^3, respectively (Schubert and Muller-Goymann, 2005).

$$A_{spec.} = \frac{\text{Surface area}}{\text{density} \times \text{volume}} = \frac{4\pi r^2}{\rho 4/3\pi r^3} = \frac{3}{\rho \times r} \quad (1)$$

Eq. (1) is calculation of particle specific surface area ($A_{spec.}$).

2.3.2. Zeta potential measurement

Nanoparticle zeta potential is calculated by electrophoretic mobility which is determined by laser Doppler anemometry in a microelectrophoresis cell (Zetasizer™, Malvern Instruments, UK). Each sample was measured at least in triplicate.

2.4. LMWH encapsulation efficiency

The amount of LMWH entrapped within nanoparticles was determined by nephelometry (Ardry, 1967; Hoffart et al., 2006) measuring the amount of free LMWH in the aqueous PVA or Pluronic® solution recovered after centrifugation of the nanoparticle suspension (42,000 × *g*; Biofuge Stratos, Heraeus Instrument GmbH). Typically, triplicate aliquots (500 μL) of aqueous samples were incubated at 37 °C for 1 h with sodium acetate [127-09-3] buffer (pH 5.25) and 1 M aqueous solution cetylpyridinium chloride [27841-61-8]. The mix absorbance is measured by UV spectroscopy at λ = 500 nm (Uvikon 922, Kontron, Eching, Germany). Drug entrapment efficiency was expressed as the percentage of LMWH entrapped with respect to the amount added to the preparation.

2.5. Cells and cell culture

The NR8383 rat alveolar macrophage cell line was purchased from the American Type Culture Collection (CRL-2192, ATCC, Manassas, VA) as a homogenous and expandable source of alveolar monocyte/macrophage-like cells not chemically or virally transformed. Cultures exhibited 50% adherent and 50% floating cell phenotypes. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplied with 200 mM L-glutamine, 15% of heat-inactivated foetal calf serum and a mixture of antibiotic/antimycotic compounds (100 U/mL of penicillin, 100 μg/mL of streptomycin and 0.25 μg/mL of amphotericin B). Cultures were incubated at 37 °C under 5% CO_2 and split every 3 or 4 days.

2.6. Cell viability

Cytotoxicity was evaluated based on cell viability relative to controls as proposed by Kong et al. (2009):

- Non-cytotoxic > 90% cell viability.
- Slightly cytotoxic = 60–90% cell viability.
- Moderately cytotoxic = 30–59% cell viability.
- Severely cytotoxic ≤ 30% cell viability.

Particle toxicity ranking was determined using the half-maximal inhibitory concentrations (IC_{50}) for both of viability assays used in this study.

2.6.1. Cell viability evaluation by Trypan blue exclusion

Trypan blue is one of the many dye recommended techniques of exclusion staining, counting and evaluation of cellular population and acute cellular toxicity (Wong et al., 2006). This method is based on the principle that the living cells do not incorporate the dye, whereas dead cells incorporate it owing to their damaged membrane. Trypan blue exclusion assays were carried out as previously described (Liu et al., 1999). Cells were dispensed into 24-well microplates (at 10^5 cells/mL), in 1 mL of DMEM medium/well and incubated overnight at 37 °C, 5% CO_2 and 95% air. Cells are then treated with different concentrations of particles for 24 h. Unexposed control cultures were maintained under the same conditions. The enumeration is carried out by immediate microscopic observation using a Malassez counting cell. Six numerations per sample were performed to limit the risk of error. The percentage of viability corresponds to the percentage of living cells resistant to any adverse effect of the nanoparticles.

2.6.2. Cell viability evaluation by MTT assay

Colorimetric MTT assays were performed to assess the activity of living cells by their mitochondrial dehydrogenase activity. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple formazan crystals that are insoluble in aqueous media. NR8383 cells were seeded in 96-well plates at 1×10^5 cells/mL. Two hundreds microlitres of suspended cells were added in DMEM medium/well and incubated overnight. Cells were exposed to different concentrations of nanoparticles for 24 h. Unexposed control cultures were maintained in the same conditions. After incubation, the percentage of cell viability was determined by using a procedure previously used in our laboratory (Attik et al., 2008), adapted from Mosmann (1983). Briefly, cells were washed once with PBS and a solution (2.5 mg/mL) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well. After a 3-h incubation at 37 °C under 5% CO_2, the MTT solution was discarded carefully and the blue formazan crystals, formed by reduction of MTT, were dissolved in dimethyl sulfoxide (DMSO [67-68-5], Sigma–Aldrich). The amount of formazan was determined spectrophotometrically by measuring the absorbance at λ = 540 nm using a microplate reader (Model 3550-UV, Bio-Rad, Marnes-la-Coquette, France). Each concentration was tested in quadruplicate and three independent experiments were done. Since absorbance is proportional to the number of living cells, cell viability was represented by the absorbance ratio of exposed to control cells expressed as a percentage.

2.7. Evaluation of apoptosis

One of the hallmarks in apoptosis is the cleavage of the genomic DNA in nucleosome units. Furthermore, the caspases cascade activation plays a central role in this process.

Table 1
Physicochemical properties of nanoparticles: diameter (by photon correlation spectroscopy), polydispersity index (PI), specific surface area ($A_{spec.}$), Zeta potential (by laser Doppler anemometry) and LMWH encapsulation efficiency (by nephelometry).

Particles	Diameter [nm] ($n = 8 \pm$ SD)	PI	$A_{spec.}$ [m^2/g]	Zeta potential [mV] ($n = 8 \pm$ SD)	LMWH encapsulation efficiency [%] ($n = 4 \pm$ SD)
NP/RS−	54.2 ± 5	0.5 ± 0.04	68	40.63 ± 5	–
NP/RS+	195.6 ± 3	0.2 ± 0.01	NA[a]	−56.10 ± 2	56.0 ± 0.8
NP/PCL−	291.5 ± 63	0.4 ± 0.06	10	−13.06 ± 7	–
NP/PCL+	248.7 ± 17	0.1 ± 0.07	NA	−10.20 ± 12	31.0 ± 1.3
DE/RS−	364.0 ± 138	0.4 ± 0.20	13	62.93 ± 8	–
DE/RS+	241.6 ± 15	0.1 ± 0.03	NA	−65.30 ± 12	32.0 ± 0.9
DE/PCL−	269.8 ± 44	0.2 ± 0.06	11	−5.40 ± 5	–
DE/PCL+	401.1 ± 45	0.2 ± 0.04	NA	−3.60 ± 3	61.5 ± 0.6

[a] NA: non-analysed.

2.7.1. DNA fragmentation

DNA fragmentation was quantified using the *Cell Death Detection ELISA*^{PLUS} (Biohit Diagnostics, Meylan, France). This assay based on the ELISA immunoassay principle uses anti-histone and anti-DNA mouse monoclonal antibodies to quantify specifically mono- and oligonucleosomes released during apoptosis.

NR8383 cells were seeded in 96-well plates at the density of 1×10^5 cells in 100 μL of medium/well. Following overnight incubation (37 °C, 5% CO_2, 95% air), nanoparticle suspensions were added to the culture wells at 400 μg/mL for 6, 24, 48 and 72 h. Quartz microparticles (DQ12) was used as a positive control because they were known to induce apoptosis (Gao et al., 2001; Attik et al., 2008). Unexposed control cultures were maintained under the same conditions. Cells were then treated with lysis buffer for 30 min at 15–25 °C. After centrifugation (200 × *g*), supernatant was transferred carefully into streptavidin-coated microplates; a mixture containing anti-DNA-peroxidase [EC 1.11.1.7] and anti-histone–biotin labelled antibodies was added to each well. After shaking, the solution was removed thoroughly by tapping or aspiration. After three washes with the incubation buffer, 100 μL of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS solution, [28752-68-3]) was added to each well. The plates were shaked until the colour development. The absorbance was measured at λ = 405 nm against an ABTS blank solution using a microplate reader (Model 3550-UV reader, Bio Rad, Marnes-la-Coquette, France). Three independent experiments were performed, each in triplicate. The specific enrichment factor of mono- and oligonucleosomes released into the cytoplasm was expressed as the absorbance ratio of exposed to unexposed cells.

2.7.2. In situ FITC-VAD-FMK labelling

The FITC-VAD-FMK *In situ* Marker Assay System is a cell-permeable FITC conjugate of the caspase inhibitor VAD-FMK (Duval et al., 2002). Briefly, according to manufacturer instructions, 2–3 mL of a NR8383 suspension (10^5 cells) was added to a slide-flask (Nunc) and exposed 24 h to nanoparticles (400 μg/mL). After the incubation period, medium from samples was removed and the slide was rinsed with PBS. FITC-VAD-FMK marker was added at a final concentration of 1×10^{-4} M. The samples were returned to the humidified chamber at 37 °C until the appearance of fluorescence. Cells were fixed with 4% para-formaldehyde [30525-89-4] in PBS, examined by fluorescent microscopy (Zeiss), fixed and photographed at 400× magnification.

2.7.3. FITC-VAD-FMK/Hoechst double staining

The Hoechst dye 33258 is an intercalating DNA agent binding preferentially to A:T and it is widely used to observe the cell nucleus. Double staining experiments were done as follows: after FITC labelling, cells were washed in PBS and stained 1 min with Hoechst dye (1:2000). Cells were simultaneously examined under fluorescence microscopy with appropriate filters.

2.8. Statistical analysis

One-way ANOVA with Fisher's Least Significant Difference (LSD) *post hoc* comparisons at 95% confidence interval was used for statistical comparisons in both the MTT and Trypan blue assays.

3. Results and discussion

3.1. Physiochemical characteristics of nanoparticles

Since many unique properties of nanomaterials stem from their size, nanoparticle sizing is a prerequisite of cytotoxicity assessment. Additionally, because of intrinsic high dispersion and elevated surface energy, nanoparticle aggregation is thought to be common in complex experimental conditions such as biological media. Surprisingly, only few studies report such aggregation data. At the nanoscale, aggregation is extremely difficult to detect, especially in biological milieu, but could have a strong influence on the specific properties of nanomaterials when they interact with cells and tissues. These properties include colloidal stability and homogeneity, electronic and optical behaviour and their uptake and targeting to cells and bacteria, especially in the presence of proteins (Jones and Grainger, 2009).

In this report, two techniques of nanoparticle preparation: double emulsion (DE) and nanoprecipitation (NP) were compared to investigate whether the preparation method could affect LMWH incorporation, particle properties and cytotoxicity. The main properties of the prepared nanoparticles are given in Table 1. The nanoparticles have nominal mean diameters ranging from 54 to 401 nm and entrapment efficiencies for LMWH ranging from 31% to 61%. The mean specific surface area ($A_{spec.}$) was calculated as being between 10 and 68 m^2/g, depending on the diameter. Therefore, particles with a shorter diameter have the highest $A_{spec.}$ (Table 1). Our data showed that nanoparticles prepared by DE were bigger than those formed by NP using the same polymer (401 nm *vs* 54 nm). Indeed, NP/RS− (54 nm) and DE/PCL+ (401 nm) were the biggest and smallest particles, respectively (Table 1). The relatively low encapsulation efficiency is probably due to the loss of LMWH into the external aqueous phase as a result of its hydrophilic nature favouring its diffusion before the precipitation of the polymers (Hoffart et al., 2006). RS nanoparticles obtained by NP have a higher entrapment efficiency than those produced by the DE method (56% *vs* 32%). On the reverse, PCL nanoparticles prepared according to DE have a higher encapsulation efficiency than those of NP (61% *vs* 31%).

The zeta potential of the particles under study varied from −65 to +63 mV depending on the preparation method, the polymer used and whether LMWH was loaded or not (Table 1). Although unloaded RS nanoparticles were positively charged owing to the quaternary ammonium groups of the polycationic RS polymer: +40 and +63 mV for NP/RS− and DE/RS−, respectively, a dramatic

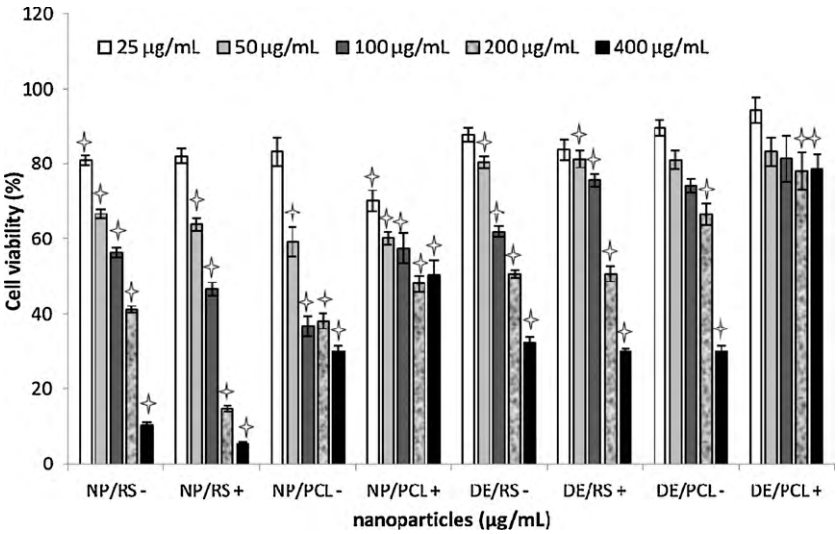

Fig. 1. Cell viability assayed by cellular membrane damage assessment using the Trypan blue exclusion test. Cells were treated with different concentrations of nanoparticles for 24 h. Results are expressed as mean ± standard deviation ($n = 9$). ✢ Significant difference (LSD test) at $p < 0.05$.

change in zeta potential occurred when LMWH was encapsulated within these nanoparticles namely −56 and −65.4 mV for NP/RS+ and DE/RS+, respectively. The shift in the zeta potential is due to LMWH loading on the particle surface rather than incorporation into the particle matrix. Indeed, Positively charged quaternary ammonium groups interact with the negatively charged sulphate and carboxylate groups of LMWH, anchoring the polysaccharide on the surface and conferring a strong negative charge to the LMWH-loaded nanoparticles (Hoffart et al., 2006).

Nanoparticle stability was assessed by monitoring changes in size, polydispersity and zeta potential. Our data demonstrated that most nanoparticle formulations were stable for 4 weeks after dilution in double distilled water, whereas DE/RS+ nanoparticles were only stable for 2 weeks (13 ± 5 days). However, tested nanoparticles were much less stable in the culture medium namely DMEM: RS nanoparticles kept their colloidal stability for at most 24 h, whereas PCL nanoparticles were more stable: 3 and 28 days for NP/PCL− and DE/PCL+, respectively. Furthermore, PCL nanoparticles prepared by DE were more stable (26 ± 3 and 28 ± 1 days for DE/PCL− and DE/PCL+, respectively) than those prepared by NP (3 ± 2 and 4 ± 2 days for NP/PCL− and NP/PCL+, respectively). In all cases, the colloidal stability of nanoparticles was dramatically reduced (∼97% in RS nanoparticles) when they were suspended in DMEM. This decrease in stability may be due to the adsorption of culture medium components onto the polycationic nanoparticle surface. The comparison of different data of nanoparticle zeta potential, size and stability shows that nanoparticles have a broad tendency to form micro-sized agglomerates (5–10 µm) in culture medium (DMEM). These agglomerates can induce a significant reduction in the effective concentration of the nanosized fraction along the current study. We can also hypothesise the opsonisation of nanoparticles by adsorption of medium serum proteins which facilitate their uptake by the macrophage (Schulze et al., 2008). These key elements can affect the biodistribution and biocompatibility of nanoparticles throughout the body (Aggarwal et al., 2009). Indeed, the present results could reflect the underestimation of nanoparticle toxicity. Therefore, the results obtained cannot be attributed solely to the nanoscale properties of the particles. The nanoparticles produced for industrial applications are not designed to be compatible with *in vitro* cell culture tests which are carried out in isotonic solutions at physiological pH and often in presence of proteins (Schulze et al., 2008). Thus, many researchers tried to avoid the colloidal instability of nanoparticles in culture media by using other buffers that are unfortunately far away from the *in vivo* conditions. Therefore, it remains a very important need to achieve more physiologically relevant *in vitro* testing models that can substitute for *in vivo* "nanotoxicology" studies (Fischer and Chan, 2007).

3.2. Cell viability

To evaluate interaction of nanoparticles and the immune system, the NR8383 macrophage cell line was used as a model to study *in vitro* cytotoxicity, since macrophages are the first line of defence in the body. As a mediator, the mononuclear system can modify advantageously the cytotoxicity and the bioavailability of the nanosized-DDS (Soma et al., 2000), or, in contrast as a target of nanoparticles toxicity, it can induce adverse effects such as oxidative stress and be less effective in the clearance of these potentially damaging particles. Human or rodent macrophages cell line are widely used and recommended to evaluate the cytotoxicity of environmental and engineered nanoparticles at first as historical "standard" in *in vitro* studies (Oberdorster et al., 2005b; Lanone et al., 2009). In fact, macrophages are able to migrate into many different organs where they have the important roles of engulfing and clearing of pathogens as well as small particles (Ahsan et al., 2002). Besides, nanomaterials introduced into the blood stream (*e.g.*, via systemic injection, oral absorption, or inhalation) would immediately encounter a high number of platelets and red blood cells and would also have frequent contact with the blood-filtering cells of the liver and the mononuclear phagocytic system (*i.e.*, reticuloendothelial system) including macrophages (Jones and Grainger, 2009). Thus, as NR8383 macrophages have been reported to represent the best currently available cell line for studying the macrophage function (Lane et al., 1998), they were considered as a satisfactory cell model for cytotoxicity assessment. Moreover, the survival of macrophages is considered as one of key prerequisites for an effective elimination of potentially harmful particles (Albrecht et al., 2009). Our preliminary data from electron microscopy showed that RS particles were taken up by NR8383 macrophages (data not shown). Therefore, it was interesting to test the influence of nanoparticle on this macrophage cell line. Additionally, the unwanted exposure route to DDS nanoparticles (mainly respiratory) during their manufacture process should be taken into account.

Nanoparticle suspended in culture medium collide with cells mainly as a result of convection forces and exert their biological

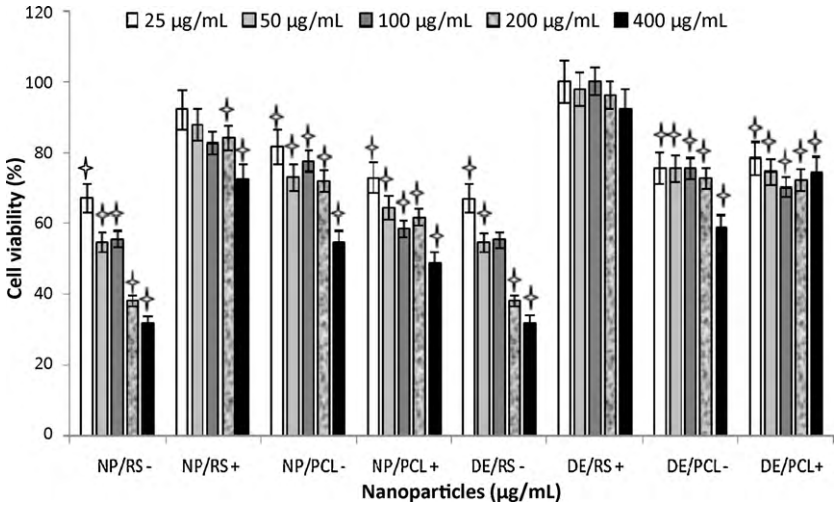

Fig. 2. Cell viability assayed by mitochondrial metabolism assessment (MTT test). Cells were incubated with different concentrations of nanoparticles for 24 h. Viability was normalized to the value determined in untreated cells. Results are expressed as mean ± standard deviation ($n = 12$). ✢ Significant difference (LSD test) at $p < 0.05$.

effects after these contacts (Lison et al., 2008). It was important to assess nanoparticle cytotoxicity after a 24-h exposure period since the cells would be in an exponential growth phase during this period and any toxicity due to inhibition of proliferation and/or cell death would be clearly visible (Nafee et al., 2009).

Figs. 1 and 2 show that cell viability ranged from 6% to 100% depending on the particle concentration, preparation method, size and zeta potential of the tested particles. Most of nanoparticles showed a dose-dependent cytotoxicity, whereas DE/PCL+ nanoparticles have a low dose-independent toxicity (Fig. 1). However, our results of MTT assay showed a dose-dependent cytotoxicity of empty RS nanoparticles (Fig. 2). Data displayed in Fig. 2 and Table 2 showed that empty nanoparticles exhibit more toxicity than LMWH-loaded ones. According to the MTT results, DE/RS+ nanoparticles were rated as non-toxic whatever the dose, whereas the other nanoparticles varied from slightly to severely cytotoxic depending on the nanoparticle type and the concentration in the culture medium. Indeed, cell viability was 74% ± 0.03 and 32% ± 0.03 for DE/PCL+ and NP/RS−, respectively at a 400 µg/mL concentration. When the viability was measured by Trypan blue exclusion, cytotoxicity was well inversely correlated with particle diameter, as the particles with smaller diameters were the most toxic (Fig. 3).

When possible, IC_{50} was calculated in each viability assay in order to compare the nanoparticles cytotoxicity (Pennati et al., 2006). Table 2 shows a strong discrepancy in IC_{50} results between the two assays depending perhaps on the cell death mechanism. The nature of the nanoparticles such as their surface charge, their adsorption capacity and their uptake may interfere with the detection system (Kroll et al., 2009).

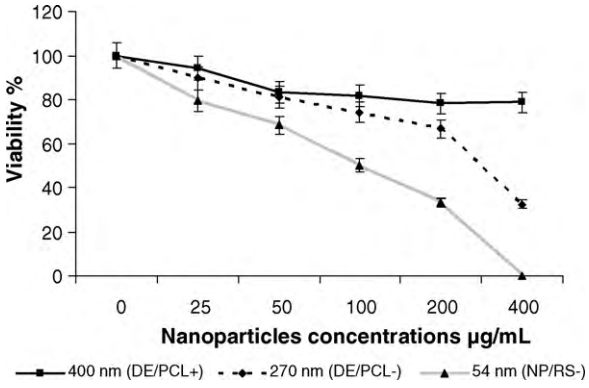

Fig. 3. Influence of nanoparticle diameter on cell viability assayed by the Trypan blue assay. Cells were incubated with nanoparticles for 24 h ($n = 9 \pm$ mean SD).

$A_{spec.}$ is often proposed as an important physical determinant of cytotoxicity (Lanone et al., 2009), therefore we propose an "$A_{spec.} \times IC_{50}$-index" to illustrate $A_{spec.}/IC_{50}$ relationships. The index values reported in Table 2 clearly show that there is a positive relationship between toxicity and $A_{spec.}$ since the particles with the largest $A_{spec.}$ are the most toxic. In case of Trypan blue, this index was $11{,}968 \times 10^{-2}$ cm^{-1} for NP/RS−, and was found to be much lower when the particle $A_{spec.}$ decreased: 3198, 1790 and 2760×10^{-2} cm^{-1} for DE/RS−, NP/PCL− and DE/PCL−, respectively. Since the Trypan blue test assesses damage to the cell membrane (Liu et al., 1999), it is seams reasonable that nanoparticle $A_{spec.}$ would be one of main factors affecting the cytotoxicity since it

Table 2
Half-maximal inhibitory concentration (IC_{50}) and "$A_{spec.} \times IC_{50}$ index" of nanoparticles for both viability tests: Trypan blue and MTT.

Particles	IC_{50} ($\times 10^{-6}$ g/cm^3)		$A_{spec.}$ ($\times 10^4$ cm^2/g)	[$A_{spec.} \times IC_{50}$] index ($\times 10^{-2}$ cm^{-1})	
	Trypan blue	MTT		Trypan blue	MTT
NP/RS−	176	188	68	11,968	12,784
NP/RS+	142	>400 (781)	NA[a]	NA	NA
NP/PCL−	179	>400 (445)	10	1790	4450
NP/PCL+	317	339	NA	NA	NA
DE/RS−	246	187	13	3198	2431
DE/RS+	259	>400 (2329)	NA	NA	NA
DE/PCL−	276	>400 (506)	11	2760	5060
DE/PCL+	>400 (1037)	>400 (959)	NA	NA	NA

[a] NA: non-analysed.

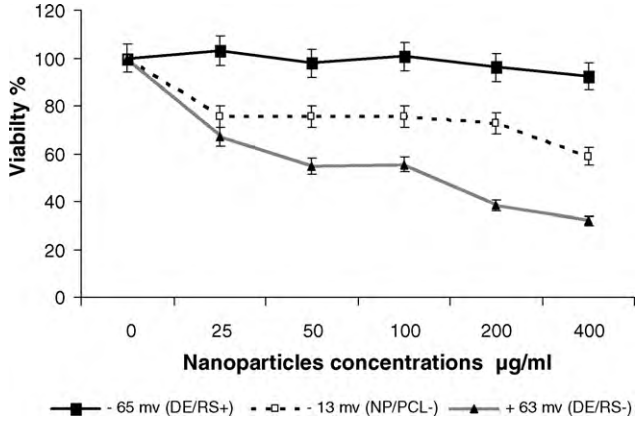

Fig. 4. Influence of nanoparticle zeta potential on cell viability assayed by the MTT assay. Cells were incubated with nanoparticles for 24 h ($n = 12 \pm$ mean SD).

would determine the extent of interactions between particles and cellular membranes.

According to IC_{50} results obtained with Trypan blue, the tested particles showed the following order of cytotoxic potency:

$$NP/RS+ > NP/RS- \approx NP/PCL- > DE/RS- \approx DE/RS + > DE/PCL- > NP/PCL+ > DE/PCL+.$$

When the endpoint was MTT reduction, the zeta potential of the nanoparticles seemed to be a valuable marker of cytotoxicity, since this parameter showed a positive relationship with their cytotoxic effects (Fig. 4). The IC_{50} values calculated from the MTT measurements showed that cytotoxic effect of the tested nanoparticles could be ranked as:

$$NP/RS- \approx DE/RS- > NP/PCL+ > NP/PCL- > DE/PCL- > NP/RS+ > DE/PCL+ > DE/RS+.$$

Heparin encapsulation dramatically changed the nanoparticle toxicity. The MTT data showed that, in contrast to PCL nanoparticles, RS nanoparticles without LMWH were more toxic than those with LMWH because of the lower zeta potential of LMWH-loaded nanoparticles, namely −56 and −65 mV for NP/RS+ and DE/RS+, respectively. Indeed, while the IC_{50} values were 188 and 781 μg/mL for NP/RS− and NP/RS+, they were 445 and 339 μg/mL for NP/PCL− and NP/PCL+, respectively. Furthermore, RS nanoparticles without LMWH were more toxic than PCL ones, whereas LMWH-loaded RS nanoparticles were less toxic than LMWH-loaded PCL particles obtained by the same preparation method. For example, the IC_{50} values were 188 and 445 μg/mL for NP/RS− and NP/PCL−, respectively as compared to 781 and 339 μg/mL for NP/RS+ and NP/PCL+, respectively. The cytotoxicity of nanoparticles prepared by DE varied in the same manner (Table 2).

The results obtained from the MTT and Trypan blue assays reveal that nanoparticles with the smallest diameter and the highest zeta potential, namely NP/RS− nanoparticles, were the most toxic (Figs. 3 and 4). Thus, DE/RS+ and DE/PCL+ can be proposed as a good delivery system for LMWH due to their relative high encapsulation efficiency (32% and 61%, respectively) and low toxicity ($IC_{50} \sim 1.0$ and 2.3 mg/ml, respectively).

The main factors affecting cytotoxicity of polycationic agents have been reported to be molecular weight, charge density and type of cationic functionality, structure and sequence (block, random, linear, and branched) and conformational flexibility (Unger et al., 2007). Polymers with a linear (PCL) or branched (RS) and flexible structure showed higher cell damaging effects as compared to other structures (Fischer et al., 2003). According to some authors, cationic agents such as RS could affect the cell viability through their positive charge interacting with the negatively charged cell membrane (Morgan et al., 1988). Furthermore, cationic agents have been reported to induce some cell damage through their interaction with anionic components (sialic acid) of glycoproteins on the epithelial cell surface (Fischer et al., 2003). Verma and Stellacci (2010) reported that regardless of the type of particle, cationic particles penetrate cell membranes which may contribute to the cytotoxicity observed with such particles. Our study confirms these observations for RS nanoparticles (Fig. 4).

It is also noteworthy that our results are in accordance with those of Delie et al. (2001) and Gargouri et al. (2009) since nanoparticles of the same polymer, prepared by the DE technique were less cytotoxic than those obtained by NP. This result could be explained by the small size of NP nanoparticles compared with those of DE. Indeed, whereas the IC_{50} values obtained by Trypan blue were 142 and 176 μg/mL for NP/RS+ and NP/RS− respectively, they were very different for the same particles prepared by DE (259 and 246 μg/mL for DE/RS+ and DE/RS−, respectively) (Table 2). In contrast, Sun et al. (2008) reported that cationic biodegradable micellar nanoparticles prepared by nanoprecipitation displayed good cytocompatibility against HEK293 cells even at high concentration. Such differences in cytotoxicity may be due to the active principle, physicochemical modifications, polymer biodegradability and cell type (Mueller et al., 2004; Kean et al., 2005). Lanone et al. (2009) suggested that the different sensitivity of cell lines has to be carefully taken into account when assessing nanoparticles toxicity. For example, Lamprecht et al reported that RS nanoparticles produced by oil/water emulsification-solvent evaporation had a low toxicity on Caco-2 cells (Lamprecht et al., 2006). This cancerous cell line used as model for the gastrointestinal tract epithelium is characterized by its high robustness compared to other cell lines (Pertuit et al., 2007). Additionally, Lamprecht et al. used a different nanoparticle preparation method, namely oil/water emulsification-solvent evaporation as well as a different cell density in the MTT assay. The latter parameter has been reported to be an important factor that affects nanoparticle cytotoxicity data (Geys et al., 2009). Another study reported that

Table 3
Apoptosis assessment in NR8383 by quantification the mono- and oligonucleosomes release (DNA fragmentation assay). DQ12 microparticles were used as a positive control of apoptosis. Cells were treated with 400 and 200 μg/mL of tested nanoparticles and DQ12 microparticles, respectively.

Nanoparticles	Enrichment factor			
	6 h	24 h	48 h	72 h
Unexposed cells	1.00 ± 0.01	1.00 ± 0.03	1.00 ± 0.01	1.00 ± 0.01
NP/RS−	0.76 ± 0.01	0.86 ± 0.01	0.73 ± 0.01	0.70 ± 0.05
NP/PCL−	0.74 ± 0.01	0.61 ± 0.03	1.10 ± 0.01	1.62 ± 0.08
DE/RS−	0.61 ± 0.01	0.61 ± 0.03	0.85 ± 0.01	0.77 ± 0.01
DE/PCL−	0.87 ± 0.01	0.78 ± 0.01	0.14 ± 0.03	1.32 ± 0.01
DQ12	23.20* + 0.30	3.95* + 0.08	ND	ND

ND: not determined. Results expressed as mean ± standard deviation ($n = 9$). *Significant difference at $p < 0.05$.

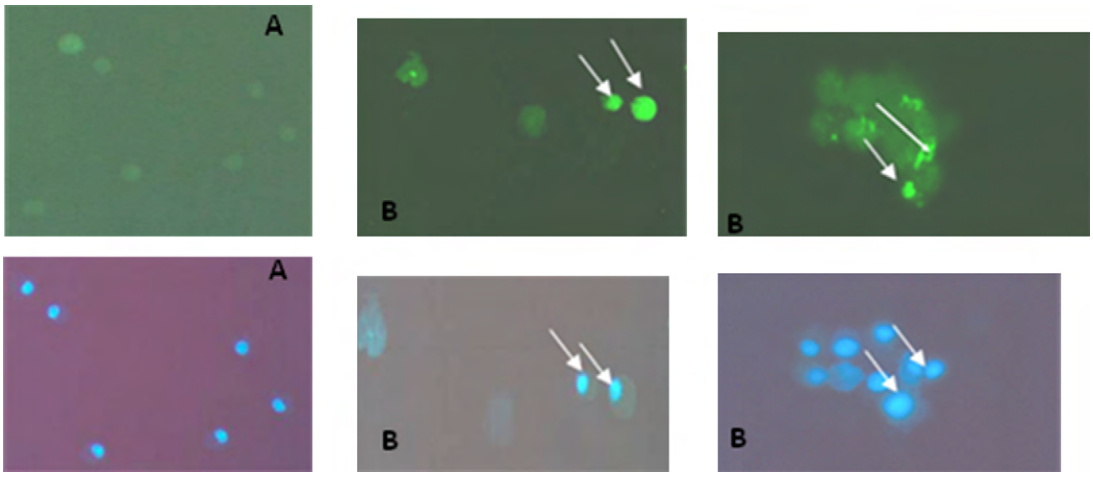

Fig. 5. Double staining FITC-VAD-FMK (green)/Hoechst dye 33258 (blue) of NR8383. Arrows show caspase activation and chromatin condensation using FITC-VAD-FMK and Hoechst staining, respectively. (A) Unexposed cells, B: cells incubated with 400 μg/mL of NP/RS− for 24 h. Each field was viewed at a magnification of ×400. This figure is representative of three experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

hydroxylapatite particles showed low toxicity towards NR8383 cells (Albrecht et al., 2009). Indeed, these differences in toxicity data were due to the use of other nanoparticles-hydroxylapatite-at higher cell density (1×10^6 cells/mL) compared with our work, namely 1×10^5 cells/mL. Furthermore, Gargouri et al. (2009) showed that unloaded RS nanoparticles prepared by NP and DE showed cytotoxic effects depending on both cell type and nanoparticle concentration. These nanoparticles were found to be less toxic towards MDA-MB 231 and MCF-7 cells than towards FaDu cells using the MTT assay without serum, which also may affect nanoparticle cytotoxicity (Geys et al., 2009). Glutathione-loaded RS/cyclodextrin nanoparticles prepared using the quasi-emulsion solvent diffusion technique did not show any cytotoxicity towards the RAW264.7 macrophage cell line using the MTT assay (Lopedota et al., 2009).

Few studies are realized to evaluate the toxicity of nanosized-DDS based on the biodegradable PCL polymer. No toxic effect of PCL itself was noticed in cell culture using neutral red and MTT assays (Dang et al., 1997). PCL nanoparticles prepared by NP and a simple oil/water emulsification technique showed a higher cytotoxic effect in the HEK293 cell line as compared to Caco-2 (Pertuit et al., 2007). However, PCL microparticles prepared by DE were reported to be slightly toxic towards the J774.2 cell line (Murillo et al., 2002a,b). Furthermore, Luong-Van et al. (2006) demonstrated that heparin-containing PCL electrospun fibers did not induce any inflammatory response in the mouse RAW264.7 macrophage cell line.

Our findings confirm the hypothesis that nanoparticle toxicity is not only due to the preparation method and composition of nanoparticles. In fact, the overall physicochemical properties and assay conditions have to be taken in account.

3.3. Apoptosis

Apoptosis is a genetically regulated of cell death characterized by cell shrinkage, plasma membrane blebbing, nuclear chromatin condensation and DNA fragmentation (Matute-Bello and Martin, 2003; Shih et al., 2003). The results exposed in Table 3 show that no nuclear fragmentation occurs when the cells were treated with a cytotoxic dose of unloaded nanoparticles (400 μg/mL) whatever the nanoparticles and the time of exposure. In addition, nuclear fragmentation featured by the enrichment factor value is significantly enhanced when cells are exposed to 200 μg/mL of DQ12 microparticles (23.20) as previously demonstrated by Attik et al. (2008) and Gao et al. (2001). Caspases activation plays a central role in the apoptosis process. *In situ* double labelling of NR8383 with FITC-VAD-FMK/Hoechst dye after a 24 h exposure to 400 μg/mL of the same nanoparticles shows activated caspases in the cytoplasm and chromatin condensation without DNA fragmentation (Fig. 5). The discrepancy between the two detection methods could be explained by the apoptosis asynchronous process. A limited number of cells will be progressing through programmed cell death as indicated by the immunohistological method. So, the level of DNA laddering obtained from apoptotic cells is below the detection limit of our biochemical method. However, Dursun et al. (2006) have shown that caspases, the major mediators of programmed cell death, can also cause necrosis. Our findings showed that NP/RS− nanoparticles induced cell death by either necrosis or apoptosis distinguished by caspase activation without DNA fragmentation.

4. Conclusion

In summary, our results show that the preparation method for nanoparticles and the assay conditions influence their properties, especially in terms of LMWH encapsulation and cytotoxic effect profiles. DE/RS+ and DE/PCL+ can be considered as a satisfactory nanosized delivery system for LMWH because of their high encapsulation efficiency and low toxicity. LMWH encapsulation dramatically reduces the cationic nanoparticles toxicity owing to the serious modifications in particle zeta potential.

Our work also showed that nanoparticles can induce cell death. Which kind of cell death (necrosis or apoptosis) involved in this cytotoxicity should be investigated further. Whether the cell death induced by tested nanoparticles is due to their internalisation by the cells or their interaction with cell membranes should also be further investigated. Although many researchers have earlier reported cytotoxicity evaluations of DDS nanoparticles based on physicochemical properties, more relevant biological and molecular assays such as apoptosis, *in situ* caspase marker assay and transcriptomic microarray require attention. IC_{50} varies as a function of the cytotoxicity endpoint since each endpoint screens a specific mechanism of toxicity. Thus, in order to obtain comparable results of "nanotoxicology", it is necessary to standardize the *in vitro* toxicity experiments in terms of cell line type, cell density, cytotoxicity assay type and the different *in vitro* conditions. Since nanoparticle cytotoxicity data differ for different cell types, complimentary *in vivo* studies will be needed to confirm our findings *in vitro*.

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