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# Cytotoxicity assessment of heparin nanoparticles in NR8383 macrophages

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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.

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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.