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Biodegradable nanoparticles designed for drug delivery: The number of nanoparticles impacts on cytotoxicity

Lívia Palmerston Mendes [a], Jorge Miguel Ferreira Delgado [b], Angela Daniela A. Costa [b], Marcelo Sousa Vieira [c], Poliana Lopes Benfica [c], Eliana Martins Lima [a,*], Marize Campos Valadares [c,*]

[a] Laboratory of Pharmaceutical Nanotechnology and Drug Delivery Systems, School of Pharmacy, Federal University of Goiás, Goiânia, Goiás, Brazil
[b] Escola Superior de Tecnologia e Gestão, Instituto Politécnico de Bragança, Bragança, Portugal
[c] Laboratory of Cellular Pharmacology and Toxicology, School of Pharmacy, Federal University of Goiás, Goiânia, Goiás, Brazil

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ABSTRACT

Nanostructured drug delivery systems are based on biocompatible and biodegradable components. Composition, size and membrane surface properties are characteristics that may influence cell viability in cytotoxicity assays. In this work, four nanostructured systems commonly used for drug delivery were prepared and cytotoxicity was evaluated on human lymphocytes and Balb/c 3T3 fibroblasts. The hemolytic potential was also investigated. Polymeric nanocapsules (NC) and nanospheres (NS), nanostructured lipid carriers (NLC) and liposomes were prepared and characterized for size, distribution, zeta potential and number per volume of the colloidal dispersion. Cell viability was evaluated, 24 and 48 h, by MTT and neutral red assays (NR). Cells were incubated with each particle in eight different dilutions varying from 2.1×10^4 to 2.1×10^{11} particles/mL. Diameter of nanoparticles was between 130 and 200 nm, all samples exhibited narrow size distribution (polydispersity index below 0.1) and zeta potential varied from −6.8 to −19.5 mV. NC, NS and NLC reduced cell viability in a dilution dependent manner. For these nanoparticles, the higher number of particles induced cell death for both cell types. Liposomes did not cause loss of cell viability even at the highest number of particles. Results suggest that, depending on the kind of nanoparticle, the number of particles in the dispersion can negatively influence cell viability in pre-clinical drug development.

1. Introduction

Nanoparticles engineered from a variety of organic and inorganic materials nanostructured systems currently applied in various fields, including drug and gene delivery, biosensors, cancer treatment, diagnostic tools and drug delivery (Kong et al., 2011).

The understanding of the biophysicochemical interfaces between engineered nanostructured materials and biological systems is crucial for the design of more advanced biocompatible and efficient nanodevices addressed for biological applications (Nel et al., 2009; Shang et al., 2014). Moreover, understanding the biophysicochemical interactions is very important from the perspective of safety and pre-clinical development of nanotechnology based systems for drug development and delivery.

The interactions between nanoparticles and biological systems including cells, tissues and organisms, is a dynamic process influenced by the properties of the nanosystem, such as, size, surface of area, shape/angle of curvature, porosity/surface crystallinity, hydrophobicity/hydrophilicity, chemical functionality, surface charge, and composition. On the other hand, the nano-biointerfaces also influenced by the composition of the biological systems (proteins, membranes, phospholipids, organelles, endocytic vesicles, DNA and biological fluids) (Verma and Stellacci, 2010).

Different types of nanostructured systems designed for drug delivery have been extensively investigated over the past decades. These include polymeric nanocapsules (NC) and nanospheres (NS), nanostructured lipid carriers (NLC) and liposomes. Thousands of papers have been published addressing performance evaluation of nanoparticulated drug delivery systems in comparison to the non-nanostructured drug. Most of these studies use cytotoxicity assays as the main tool for measuring and establishing the interaction of the nanoparticle with a biological system (Afshari et al., 2014; Cordewener et al., 2000; Kong et al., 2011).

* Corresponding authors at: Universidade Federal de Goiás, Faculdade de Farmácia, FarmaTec – 5ª Avenida c/Rua 240 s/n, Praça Universitária, Goiânia, GO 74605-170, Brazil. Tel.: +55 62 3609 6039.

E-mail addresses: emlima@ufg.br (E.M. Lima), marizecv@ufg.br (M.C. Valadares).

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Cytotoxicity assays are widely used today, sometimes as the main purpose of the experiment, in preliminary investigation of the toxicity of a nanomaterial (Hillegass et al., 2010). Although several studies regarding cytotoxicity of nanomaterials are available in the literature, the vast majority is related to nonbiodegradable nanoparticles (Love et al., 2012; O'Reilly et al., 2014; Singh and Ramarao, 2012). However, most nanostructured drug delivery systems are based on biocompatible and biodegradable components. In order to obtain reliable data from cytotoxicity studies, carefully designed experiments must take into consideration not only the properties of the nanosystem, but also additional critical issues that may impact its in vitro behavior, such as the concentration of nanoparticle components, the population of the nanoparticles and structure/type of the nanoparticle (Elsaesser and Howard, 2012; Jones and Grainger, 2009). All these parameters can interfere with cellular membrane stability leading to cytotoxicity by promoting an imbalance between intra and extracellular ions, proteins and other vital molecules required to maintain normal cellular functions and properties (Love et al., 2012). Limitations of the techniques currently available and the complexity involved in assessing the toxicity of nanoparticles have also been reported (Arora et al., 2012; Jones and Grainger, 2009; Monteiro-Riviere et al., 2009; Vorup-Jensen and Peer, 2012).

In this work, four different blank nanostructured systems (NC, NS, NLC and liposomes) commonly designed for drug delivery were prepared, characterized for their main parameters (size, size distribution, zeta potential and population number) and their cytotoxicity was evaluated on two distinct cell types: human lymphocytes and Balb/c 3T3 fibroblasts. The ability of these particles in inducing hemolysis was also investigated.

2. Material and methods

2.1. Preparation of nanoparticles

Polymeric nanoparticles were prepared by the nanoprecipitation method (Mendes et al., 2014; Mora-Huertas et al., 2012). For the preparation of polymeric nanospheres (NS), the organic phase was composed of poly-ε-caprolactone (PCL) and sorbitan monostearate dissolved in acetone. For the nanocapsules (NC) capryc/caprylic triglycerid was also added to the organic solution. The aqueous solution was prepared with polysorbate 80 at 0.1%(w/v). The organic solution was poured into the aqueous solution under magnetic stirring, which was maintained for 30 min. The organic solvent was removed under reduced pressure in a rotary evaporator (RV 10, Ika, Germany).

Nanostructured lipid carriers (NLC) were obtained by high pressure homogenization under heat (Kasongo et al., 2012; Souza et al., 2011). The lipid phase was composed of glyceril monostearate, oleic acid and sorbitan monostearate as surfactant. The lipid mixture was heated at 10 °C above the melting point of the solid lipid and then added to the aqueous phase, containing poloxamer 188 (2.5%). After 1 min in an Ultra-turrax T25 digital (Ika, Germany) for at 8000 rpm a pre-emulsion with a volume of 200 mL was formed. The pre-emulsion was submitted to 6 cycles of homogenization at 500 bar in a Panda 2K (GEA Niro Soavi, Italy) to reduce particles size and cooled at room temperature to form the NLC.

Liposomes were prepared by the hydration of the thin lipid film method, followed by extrusion (Gaeti et al., 2015; Olson et al., 1979). Soy phosphatidylcholine was dissolved in chloroform and after the solvent removal by rotary evaporation a thin lipid film was formed and the flask was kept under vacuum (440/2D, Nova Ética, Brazil) for at least one hour to remove residual organic solvent. The thin-film was hydrated with ultrapure water for 1 h, followed by 10 cycles of extrusion (Lipex® Extruder, Northern Lipids) through a 200 nm polycarbonate membrane.

2.2. Nanoparticles characterization

Nanoparticles were characterized by zeta potential (ζ-potential), pH, particle concentration, polydispersity index (PdI) and size distribution, which was determined by two methods: dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA). PdI and mean size were evaluated by DLS in a Zetasizer Nano-S (Malvern Instruments, United Kingdom), which presents the mean intensity scattered by particles in Brownian motion. NTA was accomplished in a NanoSight NS500 (Amesbury, United Kingdom) and was used to evaluate particle size and concentration in the samples. Analysis of pH was done directly in a digital pHmeter Orion 350 PerpHect (Thermo Scientific, USA) and zeta potential was evaluated in a ZetaPlus (Brookhaven Instruments Corporation, USA). All samples were taken directly from their original aqueous dispersion and, except for pH measurements, diluted (10%) with ultrapure water for analysis. NTA measurements were performed in triplicate for each of the 5 batches characterized in the experiment and all results are expressed by the means ± standard deviation (SD) ($n = 5$).

2.3. Cytotoxicity assays

Primary culture of human lymphocytes was obtained by venipuncture from healthy male donors. Equal amounts of peripheral blood and RPMI 1640 medium were mixed and layered over Histopaque® – 1077 density gradient separation solution (1.077 g/ml), which were then centrifuged for 20 min at 2000 rpm and room temperature. The mononuclear cell layer (MCL) was removed, washed and resuspended in RPMI 160 medium, with 10% fetal bovine serum (FBS), antibiotics (penicillin 100 IU/mL; streptomycin 100 μg/mL) and 10 μg/ml of phytohemagglutinin. The cells were cultured for 72 h and after that, 90 μL of the cell suspension (1×10^6 cell/ml) was cultured in 96-well microtiter plates, with and without each of the four types of blank nanocarriers (10 μL) (NS, NC, Liposomes and NLC) for 24 h, in different concentrations (2.1×10^4, 2.1×10^5, 2.1×10^6, 2.1×10^7, 2.1×10^8, 2.1×109, 2.1×10^{10}, 2.1×10^{11} particles/mL). Nanoparticle suspensions were filtered through 0.22 μm Millipore PVDF filters in order to ensure sterility prior to cell culture assays.

Assessment of the cytotoxicity on human lymphocytes was performed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction assay, adapted from Mosmann (1983). After 24 h of incubation 10 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added to each well and incubated for 4 h in the same conditions described above. Supernatants were removed and 100 μL of DMSO were added to each well to solubilize the formazan crystals. The absorbance was measured on a microplate reader at 570 nm in a Stat Fax 2100 Microplate Reader (Awareness Tecnologies, USA).

Balb/c 3T3 fibroblasts were cultured as a monolayer in sterile culture flasks containing DMEM supplemented with 10% FBS. Cells were daily examined by contrast microscope. They were removed from the culture flasks using trypsinization (trypsin/EDTA solution; 0.025%:0.02%) when they reached between 50% and 80% confluence. A cell suspension containing 3×10^4 cells/mL was prepared in DMEM supplemented with 10% FBS and 96-well microtiter plates were seeded and incubated for 24 h for a monolayer with less than 50% confluence. After that period, cells were exposed to eight different concentrations of freshly prepared and diluted nanoparticles (2.1×10^4, 2.1×10^5, 2.1×10^6, 2.1×10^7, 2.1×10^8, 2.1×109, 2.1×10^{10}, 2.1×10^{11} particles/mL) and incubated for 48 h. Neutral red (NR) assay was performed according to the standard protocol of Borenfreund and Puerner (1985) modified by NICEATM (ICCVAM, 2006). After 48 h of exposure, the cells were washed and NR medium was added to all wells and incubated for