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Biocompatibility of a self-assembled glycol chitosan nanogel

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ABSTRACT

The research of chitosan-based nanogel for biomedical applications has grown exponentially in the last years; however, its biocompatibility is still insufficiently reported. Hence, the present work provides a thorough study of the biocompatibility of a glycol chitosan (GC) nanogel. The obtained results showed that GC nanogel induced slight decrease on metabolic activity of RAW, 3T3 and HMEC cell cultures, although no effect on cell membrane integrity was verified. The nanogel does not promote cell death by apoptosis and/or necrosis, exception made for the HMEC cell line challenged with the higher GC nanogel concentration. Cell cycle arrest on G1 phase was observed only in the case of RAW cells. Remarkably, the nanogel is poorly internalized by bone marrow derived macrophages and does not trigger the activation of the complement system. GC nanogel blood compatibility was confirmed through haemolysis and whole blood clotting time assays. Overall, the results demonstrated the safety of the use of the GC nanogel as drug delivery system.

1. Introduction

Nanoparticles have been largely researched as drug nanocarriers, yet their interaction with cells and extracellular environment is still poorly explored. The evaluation of the potential effects of drug delivery devices on the biological systems is indeed a crucial requirement in the development of nanomedicines: cytotoxicity, haematocompatibility (haemolysis and complement activation), inflammatory response; biodegradability and potential cytotoxicity of the degradation products, cellular uptake and intracellular fate, *in vivo* biodistribution studies, all assist in allowing a better definition of the biological properties of the novel polymers and their polymeric nanoparticles. (Gaspar and Duncan, 2009; Naahidi et al., 2013; Rodrigues et al., 2012) The so called "biocompatibility" is largely dependent on the physical and chemical properties of the nanoparticles (size, shape and surface characteristics), as well as on the used raw material (Liu et al., 2013; Naahidi et al., 2013).

The use of polymeric nanoparticles based on chitosan has been extensively reported in biomedical applications, due to its interesting characteristics. Non-toxicity, biocompatibility, biodegradability, antibacterial activity, mucoadhesiveness and permeation enhancing properties are among the features that have been reported, the cationic character being responsible for some of them (Bernkop-Schnurch and Dunnhaupt, 2012; Croisier and Jérôme, 2013). Although often claimed as biocompatible, more comprehensive studies are required for a proper understanding of the biological effects of this polymer and of its nanoformulations.

As reported previously, a polymeric nanoparticle made of glycol chitosan (GC), here designated GC nanogel, has been synthesized in our laboratory by chemical grafting hydrophobic chains on the GC backbone, yielding an amphiphilic polymer capable of self-assembling in aqueous environment (Pereira et al., 2013). The present work focuses on the detailed evaluation of the biocompatibility of this nanogel. For this purpose, in vitro cell toxicity of the GC nanogel was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) release assays to gauge the nanogel cytotoxicity. Induction of apoptosis and/or cell cycle arresting was tested through flow cytometry. The complement activation was semi-quantified by western blot, analysing the degradation of the C3 factor. Interaction with murine macrophages was observed through confocal

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microscopy. Finally, the nanogel haemocompatibility was evaluated through haemolysis and whole blood clotting time assays.

2. Experimental

2.1. Materials

Glycol chitosan (GC, G7753), mercapto hexadecanoic acid (MHDA), N-hydroxysulfosuccinimide (NHS) and 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), NADH, pyruvate, haemoglobin from bovine blood and Drabkin's reagent were acquired from Sigma–Aldrich. 5/6-Carboxyfluorescein succinimidyl ester was purchased from Thermo Scientific. Cell Culture reagents and culture medium were purchased from Biochrom.

2.2. Nanogel self-assembling

GC nanogel was synthesized and characterized as previously reported (Pereira et al., 2013). Briefly, GC nanogel was prepared by conjugation of the mercapto hexadecanoic acid (MHDA) to GC, through a carbodiimide reaction. Nanogel dispersions used in the different experiments were obtained after dispersing the lyophilized reaction product in distilled water, under magnetic stirring at 50 °C for 48 h, and passed through a cellulose acetate syringe filter (pore size 0.45 μm).

2.3. Cell cultures

3T3 fibroblasts and mouse leukaemic monocyte macrophage (RAW 264.7) cell lines were maintained in DMEM supplemented with 10% bovine calf serum or fetal bovine serum (FBS), respectively, 100 IU/mL penicillin and 0.1 mg/mL streptomycin. Human microvascular endothelial cells (HMEC) were grown in RPMI-1640 supplemented with 10% FBS, Epidermal Growth Factor (EGF, 10 ng/mL), Hydrocortisone (1 μg/mL), 100 IU/mL penicillin and 0.1 mg/mL streptomycin. All cell lines were cultured as a monolayer in a humidified atmosphere containing 5% CO_2 at 37 °C.

Murine Bone Marrow-Derived Macrophages were collected from femoral and tibial mouse bone marrow using a previously published protocol (Carvalho et al., 2011; Cerca et al., 2011). Briefly, mouse long bones were extracted from the mousse under aseptic conditions and flushed with RPMI-1640. The resulting cell suspension was centrifuged at 500*g* during 10 min. The cell pellet was resuspended in RPMI-1640 supplemented with 10 mM HEPES, 10% heat-inactivated FBS, 60 μg/mL penicillin/streptavidin, 0.005 mM β-mercaptoethanol (RPMI complete medium) and 10% L929 Cell Conditioned Medium (LCCM). To remove adherent bone marrow cells, the cell suspension was incubated overnight at 37 °C and 5% CO_2 atmosphere in a Petri dish. The non-adherent cells were collected, centrifuged at 500*g* (10 min) and seeded in 24 well plates at 5×10^5 cells per well in RPMI complete medium containing 10% of LCCM and incubated at 37 °C in a 5% CO_2 atmosphere. Four days after seeding 10% of LCCM was re-added to the cultures. The culture medium was replaced with fresh RPMI complete medium containing 10% LCCM on day 7. After 10 days in culture, cells were completely differentiated into macrophages.

2.4. In vitro cell toxicity

2.4.1. MTT assay

The cytotoxicity of GC nanogel was evaluated by using the quantitative colorimetric MTT assay. Cells were seeded onto 24-well cell culture plates at a density of 1×10^4 cells per well for 3T3 and RAW and of 2×10^4 for HMEC, and left adhering in 0.5 mL of culture medium overnight. Afterwards, the cells were incubated with nanogel dispersions at 0.1 and 0.5 mg/mL in fresh culture medium containing 25% of distilled water (v/v). Cells cultivated in medium with or without 25% of distilled water (v/v) (without nanogel) were used as controls. After 24, 48 and 72 h the metabolic activity was measured adding MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The tetrazolium salt is reduced by metabolically active cells using mitochondrial succinate dehydrogenase enzymes (Mosmann, 1983). The MTT solution (0.5 mg/mL in PBS) was carefully removed from each well and the resulting dark blue formazan crystals were solubilized in dimethyl sulfoxide and quantified spectrophotometrically at 570 nm. The experiments were performed in triplicate and the metabolic activity results were shown as percentage of the values obtained at 0 h time point.

2.4.2. LDH release assay

Cytotoxicity can also be assessed by the degree of membrane damage. The LDH release assay measures the membrane integrity as function of the amount of cytoplasmic LDH leaked into the culture medium. The conversion of NADH and pyruvate into NAD^+ and lactate catalysed by LDH is the basis of the method (Rodrigues et al., 2012). Cells were seeded in 12-well plate at a density of the 2×10^5 cells per well for 3T3 and HMEC and 1×10^5 for RAW and allowed to settle overnight in 0.5 mL of culture medium. The cells were treated with nanogel dispersions with a concentration of 0.1 and 0.5 mg/mL, in fresh culture medium containing 25% of distilled water (v/v). Untreated cells, exposed to 20% DMSO (v/v) or 25% distilled water (v/v) in culture media were used as controls. At the 24 and 48 h time points the culture medium from each well was collected and centrifuged at 13,000 rpm for 1 min and the cell free supernatant was collected and stored on ice for further extracellular LDH measurement. The respective cells were scraped with the aid of a Tris solution 15 mM and lysed through sonication. Supernatants of centrifuged samples were used to quantify the intracellular LDH. Samples of extracellular (40 μL) or intracellular (10 μL) LDH were plated into a new microplate and 250 μL of the NADH solution 0.31 mM in phosphate buffer 0.05 M, pH7.4 added to each well. Finally, 10 μL of a 8.96 mM pyruvate solution in phosphate buffer (substrate solution) was added and immediately afterwards the variation of the absorbance at 340 nm was read in a microplate spectrophotometer, as to determine the rate of NADH consumption (slope of the line). LDH leakage was expressed as the ratio between extracellular and total LDH, corresponding the inverse value to the cell membrane integrity. Untreated cells were used as a reference for the estimation of the maximum membrane integrity. Each experiment was performed in triplicate.

2.5. Apoptosis assay

The FITC Annexin V Apoptosis Detection Kit was used to determine apoptotic cell membrane changes in 3T3, HMEC and RAW cell lines. Cells (2×10^5/well) were seeded in a 12-well plate and left adhering overnight. The cells were then incubated with nanogel dispersions at 0.1 and 0.5 mg/mL in fresh culture medium containing 25% of distilled water (v/v). A negative control assay was carried out without nanogel (the dilution with 25% of distilled water had no effect on the assay outcome), while H_2O_2 was used as positive control for apoptosis using different incubation times and concentrations according to the cell line (0.5 mM for 6 h – RAW; 0.2 mM for 24 h – HMEC and 5 mM for 3 h – 3T3) (Piao et al., 2011; Wang et al., 2007). After 24 h the cells were treated with 250 μL trypsin/EDTA 0.25%/0.02% in PBS for 2 min at 37 °C. The cell suspension was transferred to flow cytometry sample tubes (Beckman Coulter) and washed twice with cold PBS. Double staining with FITC-Annexin V/PI was performed as recommended by the