Food and Chemical Toxicology 47 (2009) 1139–1145

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Cytotoxicity and genotoxicity of superporous hydrogel containing interpenetrating polymer networks

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ARTICLE INFO

Article history:
Received 31 August 2008
Accepted 27 January 2009

Keywords:
Superporous hydrogel
Interpenetrating polymer networks
Cytotoxicity
Genotoxicity
Drug delivery

ABSTRACT

The superporous hydrogel containing poly(acrylic acid-*co*-acrylamide)/*O*-carboxymethyl chitosan (*O*-CMC) interpenetrating polymer networks (SPH-IPN) that had been developed as an oral delivery vehicle for protein drugs was subject to cytotoxicity and genotoxicity testing, thus evaluating its biological safety in use. In a battery of cytotoxicity assays on RBL-2H3 and Caco-2 cells, the SPH-IPN caused minimal damage towards cell viability, lysosomal activity, and metabolic activity following both direct and indirect treatment. The SPH-IPN did not induce cell apoptosis or DNA breakage in the above cell lines; it did not increase micronucleus (MN) incidence in mouse bone marrow, either. Therefore, the SPH-IPN was preliminarily considered to be biocompatible and might be a safe carrier for protein drugs. In addition, using the HPLC method, residual acrylic acid, acrylamide, and glutaraldehyde in the SPH-IPN were quantified to be 1.4, 2.0, and below 0.2 ppm, respectively. Lack of these low molecular monomers and cross-linker that were mainly responsible for the toxicity provided evidence for the good biocompatibility of the SPH-IPN.

1. Introduction

Superporous hydrogels (SPHs) have been developed as a new category of superabsorbent polymers during the last decade, which are mainly characterized by high porosity, fast swelling and large swelling ratios. However, the swollen SPHs possessed poor mechanical strength, thus limiting their use where high mechanical endurance is demanded (Ornidian et al., 2005).

In our previous study, the full-interpenetrating polymer network (full-IPN) structure was introduced into the SPHs and successfully improved the mechanical properties of the polymers without compromising their porosity and fast swelling abilities (Yin et al., 2007a). The superporous hydrogel containing poly(acrylic acid-*co*-acrylamide)/*O*-carboxymethyl chitosan (*O*-CMC) interpenetrating polymer networks (SPH-IPN) was thereby achieved and developed as a peroral delivery system for protein drugs. It revealed good muco-adhesion, enzymatic inhibition of trypsin and α-chymotrypsin, and enhanced insulin transport in rat intestine (Yin et al., 2008a). Oral administration of insulin-loaded SPH-IPN led to a significant hypoglycaemic effect in normal rats with a relative pharmacological bioavailability of 4.1% (Yin et al., 2008a). Considering its efficacy and potential application, it highlighted the need to perform a systematic biocompatibility study on the SPH-IPN to guarantee the biological safety in use.

In vitro cytotoxicity assays have served to predict human acute toxicity as the initial step in biocompatibility studies. The International Organization for Standardization (ISO) 10993 (1992) has specified various cytotoxicity evaluations for medical devices and materials, based on various endpoints in cell number, morphology, and cellular activity (Eisenbrand et al., 2002; ISO 10993, 1992). However, different assays can lead to different results depending on the test agent, cell type, and the method employed (Weyermann et al., 2005). Therefore, multiple assays with regard to different aspects of cellular activities are deemed necessary for a systematic evaluation of the cytotoxicity of the SPH-IPN. In addition, genotoxicity is an important factor relative to the systemic compatibility of biomedical materials, which means the presence of a DNA reactive component that may result in mutagenicity

Abbreviations: 5-FU, fluorouracil; % Tail DNA, DNA in the tail; AA, acrylic acid; AM, acrylamide; CLSM, confocal laser scanning microscopy; CP, cyclophosphamide; DMEM, Dulbecco's Modified Eagle Medium; DNPH, 2,4-dinitrophenylhydrazine; EB, ethidium bromide; FDA, fluorescein diacetate; full-IPN, full-interpenetrating polymer network; GA, glutaraldehyde; LDH, lactate dehydrogenase; MN, micronucleus; MTT, methyl tetrazolium; NCEs, normochromatic erythrocytes; *O*-CMC, *O*-carboxymethyl chitosan; OTM, olive tail moment; PCEs, polychromatic erythrocytes; PBS, phosphate buffer saline; PI, propidium iodide; SPHs, superporous hydrogels; SPH-IPN, superporous hydrogel containing poly(acrylic acid-*co*-acrylamide)/*O*-carboxymethyl chitosan (*O*-CMC) interpenetrating polymer networks.

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doi:10.1016/j.fct.2009.01.043

and carcinogenicity. ISO 10993 stipulates that all materials that are to be in contact with mucous, bone, or dentinal tissues for longer than 30 days must undergo genotoxicity testing (ISO 10993, 1992). As an oral delivery system for protein drugs, the SPH-IPN keeps chronic contact with the mucosa in the GI tract. Therefore, it is also required to perform the genotoxicity testing.

The present study therefore evaluated the cytotoxicity and genotoxicity of the SPH-IPN, thereby providing preliminary instructions for further studies on systemic toxicities. Caco-2 cells (human colon adenocarcinoma cell lines) and RBL-2H3 cells (rat basophilic leukemia cell lines) were used for *in vitro* cytotoxicity and genotoxicity testing, because the Caco-2 cell line was a commonly used intestinal epithelial model while RBL-2H3 possessed typical fibroblast shape which differed from that of the Caco-2 cell. A battery of cytotoxicity assays related to different aspects of cell damage was performed, allowing a more convincing evaluation. *In vitro* genotoxicity testing was monitored for apoptosis and DNA strand breakage. Micronucleus (MN) studies on mouse bone marrow were conducted for *in vivo* genotoxicity evaluation. Finally, residual acrylic acid (AA), acrylamide (AM), and glutaraldehyde (GA) in the SPH-IPN that were potentially toxic were quantified using the HPLC method to provide evidence for its biocompatibility.

2. Materials and methods

2.1. Materials and cell cultures

Cyclophosphamide (CP) was purchased from Hengrui Medical Co., Ltd. (Jiangsu, China) and fluorouracil (5-FU) from Xudonghaipu Medical Co., Ltd. (Shanghai, China). Methyl tetrazolium (MTT), neutral red, fluorescein diacetate (FDA), and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol (Merck, Darmstadt, Germany) was of HPLC grade. SPH-IPN with monomer/*O*-CMC ratio (w/w) of 0.192 was synthesized in our laboratory as described previously (Yin et al., 2007a). FT-IR, ^{13}C NMR, and DSC spectra confirmed the chemical structure of the SPH-IPN and SEM, light, and CLSM images revealed that the polymers had both the IPN and porous structures (Yin et al., 2007a).

RBL-2H3 and Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal calf serum.

Male Kunming mice (6 weeks old, body weight 20 ± 2 g) were obtained from the Animal Centre of Fudan University, and raised in a SPF laboratory. Animals were given standard food and tap water ad libitum in an air-conditioned room with constant temperature (20 ± 2 °C), relative humidity (55 ± 5%), and a standard light/dark cycle (lights on from 8.00 a.m. to 8.00 p.m.). Animal experiments were performed according to the Guiding Principles for the Care and Use of Experiment Animals in Fudan University.

2.2. Cytotoxicity

2.2.1. Sample preparation and cell treatment

SPH-IPN extract was prepared by incubation of 40 mg of the sterilized polymer with 4 mL of serum-containing DMEM at 37 °C for 72 h, achieving a concentration of 10 mg/mL.

RBL-2H3 and Caco-2 cells were seeded in 96-well plates at a density of 1×10^4 cells per well, and cultured at 37 °C in 5% CO_2 for 24 h, achieving about 5×10^4 cells per well for polymer treatment. For direct polymer-cell contact, 2 mg of the SPH-IPN was fully swollen in DMEM and added to each well. For indirect contact, culture medium was replaced by 200 μl of the polymer extract. Cells were subsequently incubated for a determined period of time prior to following assays. Untreated cells served as a negative control and cells incubated with 50 μl per well of 2 mg/ml sodium deoxycholate for 4 h served as a positive control unless otherwise stated.

2.2.2. "Live/Dead" assay

Following polymer treatment for 24 h, cells were rinsed with phosphate buffer saline (PBS, pH 7.4), fixed with iced methanol, rinsed again with PBS and stained with FDA (20 μg/mL) and PI (0.2 μg/mL) for 5 min. Thereafter, cells were observed with confocal laser scanning microscopy (CLSM, Olympus, Nagano, Japan) at an excitation wavelength of 488 nm and emission wavelength of 520 nm and 630 nm for fluorescein and PI, respectively. Living cells took up FDA, which was hydrolyzed by intracellular esterase to form fluorescein, and thereafter fluoresced green. Dead and dying cells took up membrane impermeable PI, which bound to nucleic acids, and thus fluoresced red.

2.2.3. Lactate dehydrogenase (LDH) leakage assay

After 24 h of polymer-cell contact, the culture medium was aspirated and centrifuged at 3000 rpm for 5 min. The activity of LDH in the supernatant was determined using an LDH kit (Kangte Biotech Co., Zhejiang, China). Tests were performed in sextuplicate for each sample, and results were presented as percentage of total cellular LDH activity, which was measured in cell lysates obtained by treatment with 200 μl of 0.2% TritonX-100 solution.

2.2.4. Neutral red assay

Following 24 h of polymer treatment, the neutral red assay was monitored as described (George and John, 2006). Tests were performed in sextuplicate for each sample, and results were presented as percentage of the negative control values.

2.2.5. Protein assay

Following 24 h of polymer treatment, protein content of the cells was determined as described (George and John, 2006). Tests were performed in sextuplicate for each sample, and results were presented as percentage of the negative control values.

2.2.6. Cell proliferation by MTT assay

Following cell seeding in 96-well plates at a density of 1×10^4 cells per well and subsequent incubation for 12 h, SPH-IPN or SPH-IPN extract were added. Cells were thereafter cultured for 7 days and were subject to MTT assay (Yin et al, 2007b) on day 2, 4 and 7. Cells prior to SPH-IPN treatment served as a control. Tests were performed in sextuplicate for each sample, and the proliferation ratio was defined as the increased proportion of cells compared to the control.

2.3. DNA fragmentation

RBL-2H3 and Caco-2 cells were seeded in flasks (65 cm^2) at a density of 1×10^6 cells per flask, and were cultured for 24 h. Culture medium was replaced by 40 mg of SPH-IPN in 4 mL of DMEM or 4 mL of 10 mg/mL polymer extract for direct and indirect treatment, respectively. CP (100 μg/mL) and 5-FU (500 μg/mL) served as positive controls for RBL-2H3 and Caco-2 cells, respectively. DNA fragmentation after 48-h culture was detected using the reported method (Herrmann et al., 1994). Briefly, following collection and lysis of both floating and adherent cells, genomic DNA was isolated, separated by electrophoresis in 1% agarose gels, and detected by ethidium bromide (EB) under UV light.

2.4. Flow cytometry

Cell treatment was the same as above, and 25 μg/mL of CP and 150 μg/mL of 5-FU served as positive controls for RBL-2H3 and Caco-2 cells, respectively. Floating and adherent cells were collected, and early apoptosis was determined using an AnnexinV-FITC Kit (Jing Mei Biological Co., Ltd., Shanghai, China). Tests were performed in triplicate for each sample, and analyses were performed on a flow cytometer (FACS Caliber, BD, Franklin, NJ, USA) with dedicated software.

2.5. Comet assay

Cell treatment was the same as above and cells exposed to 10 mmol/L $K_2Cr_2O_7$ for 90 min served as a positive control. DNA strand breakage was evaluated using the comet assay (Collins et al., 1993). Briefly, cells were mixed with 0.7% low melting point agarose and layered onto a microscope slide pre-coated with 1% normal melting point agarose. Cells were then subject to lysis at pH 10.0, alkaline unwinding and electrophoresis at pH 13.0. Following EB staining, DNA migration was visualized using a CLSM and analyzed with the CASP software. Fifty cells were scored for each sample, and the percentage of DNA in the tail (% Tail DNA) and the olive tail moment (OTM) were used as DNA damage indicators.

2.6. In vivo MN studies

SPH-IPN extract used here was obtained through extraction with normal saline at ambient temperature for 72 h, and was sterilized through 0.22 μm filtration. The extract was delivered to mice via two intraperitoneal injections spaced 24 h apart, and the injection volume was 0.1 mL per 20 g body weight. CP solution (8 mg/mL) served as a positive control while normal saline served as a negative control. A group of 10 mice was used for each sample. Six hours after the second injection, the animals were sacrificed and bone marrow cells in the femurs were collected. Following smear preparation, fixation in methanol and staining with Giemsa solution, scoring was monitored under a 55i microscope (Nikon, Tokyo, Japan). Two thousand polychromatic erythrocytes (PCEs) per animal were scored to determine the MN frequencies and 200 erythrocytes were examined to calculate the ratio of PCEs to normochromatic erythrocytes (NCEs) (Clarice et al., 2007). NCE cells were pale orange while PCE cells were gray blue. MN appeared round and royal purple, with its diameter 1/5–1/20 of that of the PCE cell. When more than one MN was observed in one PCE, they were scored as one.