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In vitro and *in vivo* toxicity evaluation of cationic PDMAEMA-PCL-PDMAEMA micelles as a carrier of curcumin

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ABSTRACT

Polymeric micelles have attracted significant attention because of their potential application as promising drug-delivery systems. In the present study cationic micelles, based on triblock copolymer poly(-dimethylaminoethyl methacrylate) - poly(e-caprolactone) - poly(dimethylaminoethyl methacrylate) were prepared and loaded with curcumin. *In vitro* cytotoxicity of empty and curcumin loaded polymer micelles was investigated on two cell culture models, human hepatoma cell line HEP G2 and freshly isolated rat hepatocytes, following their viability and lactate dehydrogenase (LDH) leakage. MTT dye reduction assay and LDH release study showed that empty cationic micelles did not cause significant changes in cell viability and membrane integrity at the concentration range from 10.0 to 80.0 μg/ml. Our special attention was focused on the effects of empty and curcumin loaded micelles on oxidative stress markers malondialdehyde (MDA) and reduced glutathione (GSH). The increase in the micelles concentration to 100 μg/ml was accompanied by GSH depletion and increased levels of MDA production in isolated rat hepatocytes. The *in vivo* toxicity of polymeric micelles was examined in male *Wistar* rats. The results showed that neither single (7.5 mg/kg, i.p.), nor repeated (3.5 mg/kg, i.p., 14 days) exposure to empty or curcumin loaded polymeric micelles induced any toxicity changes, e.g. hematopoietic and liver tissue damages.

1. Introduction

With the growing application of polymeric materials as drug carriers the concerns of their safety are increasing, but the current understanding on the toxicity of these carriers, especially the newly synthesized is very limited. Among various polymers, cationic polymers are attractive nanocarriers mainly because of their potential in gene delivery (Lin et al., 2008; Layman et al., 2009; Qiao et al., 2010). In addition, cationic polymers could lead to improved interactions between nanoparticulate systems and cell membranes. However, various studies have reported the toxicity of some cationic polymers (Fischer et al., 2003; Lv et al., 2006). According to some studies, the high concentration of amino groups can result in high cytotoxicity, whereas the presence of poly(-ethylene glycol) (PEG) reduces the relative concentration of amino groups and cytotoxicity of polycationic carriers (Van de Wetering et al., 1997; Van de Wetering et al., 1998). Zhang et al. evaluated cytotoxicity of branched polyethylenimine and linear poly(ethylene glycol monomethyl ether)-block-poly(ε-caprolactone)-block-poly(2-(dimethylamino)ethyl methacrylate) (mPEG-PCL-PDMAEMA) copolymer on two cell lines (Zhang et al., 2010). The study reported less toxicity of mPEG-PCL-PDMAEMA copolymer compared to branched PEI, probably due to the presence of mPEG and PCL. In another study, copolymerization of PDMAEMA with methacrylated chondroitin sulphate led to significantly improved cell viability compared to PDMAEMA (Lo et al., 2013).

Poly(2- (dimethylamino)ethyl methacrylate) is an example for cationic polymer widely studied as carrier of gene and drug delivery systems (Verbaan et al., 2005; Karanikolopoulos et al., 2010).

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The copolymerization with hydrophobic polymers leads to the achievement of amphiphilic copolymers (Motala-Timol and Jhurry, 2007; Mespouille et al., 2008). Preparation of drug delivery micelles based on such PDMAEMA-copolymers has been regarded as attractive tendency (San Miguel et al., 2008; Zhu et al., 2010). Recently, micelles based on copolymer comprising two short outer PDMAEMA blocks and a relatively longer middle poly(ε-caprolactone) (PCL) block were developed as curcumin delivery platform possessing improved antioxidant and cytotoxic activity (Yoncheva et al., 2015).

However, the study has just initiated evaluation of toxicity of this block copolymer carrier. Curcumin is an attractive polyphenol compound possessing beneficial pharmacological effects, e.g. antioxidant, anti-inflammatory, antibacterial, anticancer etc. However, the poor solubility and instability of curcumin (e.g. at pH above 7.0) require its formulation in various nanoparticulate systems. Indeed, these systems might be able to overcome these limitations as well as to be safe for application. A recent study reported toxicological evaluation and relevance of polymeric Eudragit S nanoparticles loaded with curcumin as oral drug delivery system (Dandekar et al., 2010). Thus, a systemic evaluation of this type of polymeric carriers would be of great importance for further application in clinical therapeutic areas. Liver is a target tissue for polymeric nanoparticles administered by different routes, e.g. oral and parenteral (Lenaerts et al., 1984; Fawaz et al., 1993). In the liver, nanoparticles are taken up by Kupffer cells by phagocytosis, which might activate reactive oxygen species formation and oxidative stress reactions in the hepatocytes, or tissue inflammatory responses (Fernandez-Urrusuno et al., 1995). Therefore, it is important to assess *in vitro* and *in vivo* toxicity of the nanoparticles in liver.

The aim of the present study was *in vitro* and *in vivo* evaluation of toxicity of empty and curcumin loaded micelles based on a $PDMAEMA_9$-PCL_{70}-$PDMAEMA_9$ copolymer. Since our previous study revealed an improvement of curcumin activities by loading in micelles based on this copolymer, the present study continued with toxicological evaluation of micellar system. *In vitro* studies were performed in two liver cell models, in particular human hepatocellular carcinoma cell line (HEP G2) and primary isolated rat hepatocytes, aiming to evaluate cell viability and membrane integrity. *In vivo* toxicity of the formulated micelles was explored by observation of their effects on oxidative stress markers malondialdehyde (MDA) and reduced glutathione (GSH) in rat liver after single and repeated i.p. administration. Haematology analysis, serum biochemical assays and histological observations were also performed in order to evaluate toxicological profile of micelles in details.

2. Materials and methods

2.1. Materials

HEPES, collagenase from *Clostridium histolyticum* type IV, albumin, bovine serum fraction V (minimum 98%), EGTA, 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol) (TBA), curcumin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were provided by Sigma Aldrich (Germany). NaCl, KCl, D-glucose, $NaHCO_3$, $CaCl_2 \cdot 2H_2O$ and 2,2′-dinitro-5,5′-dithiodibenzoic acid (DTNB) were supplied by Merck (Germany). Pentobarbital sodium was from Sanofi (France); KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ were purchased from Scharlau Chemie SA (Spain) and Fluka AG (Germany), respectively. Trichloroacetic acid (TCA) was from Valerus (Bulgaria). All other reagents were analytical grade and used without further purification. The copolymer ($PDMAEMA_9$–PCL_{70}–$PDMAEMA_9$) was synthesized by atom transfer radical polymerization (ATRP) of DMAEMA in methanol initiated by a bi-functional PCL macroinitiator based on commercial PCL-diol as described in our previous paper (Yoncheva et al., 2015). Number-average molar mass Mn = 10 800 g/mol; PDI 1.35.

2.2. Preparation and physicochemical characterization of the of empty and curcumin loaded polymeric micelles

PDMAEMA-PCL-PDMAEMA micelles were prepared by dialysis method as previously established (Yoncheva et al., 2015). Briefly, the copolymer (2 mg/ml) was dissolved in dioxane and after that purified water was slowly added to the solution. Then, the micellar dispersion was dialyzed against water (MWCO 6000–8000 g/mol, Spectrum Labs) during 4 h with a refreshment of the external water every 1 h.

For preparation of curcumin loaded micelles, curcumin was added into the copolymer solution in dioxane, incubated for 30 min under stirring (700 rpm) and further procedure followed all stages described above.

The size, polydispersity index and zeta-potential of micelles were evaluated by photon correlation spectroscopy and electrophoretic laser Doppler velocimetry (Zetamaster analyser, Malvern Instruments, UK). The dispersions were measured at 25 °C with a scattering angle of 90°.

2.3. Cell line and cultivation

Human hepatocellular carcinoma cells HEP G2 were purchased from DSMZ, Braunschweig, Germany. Cells were kept at 37 °C in a fully humidified atmosphere with 5% CO_2 in the logarithmic growth phase through trypsinization twice a week. Cultivation medium consisted of 90% RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 2 mM L-glutamine. Confluent cultures were split 1:2 to 1:4 every 3–6 days using trypsin/EDTA. Initially after towing HEP-G2 cells were cultured with 20% FBS.

2.3.1. MTT-dye reduction assay

HEP G2 cells were seeded in 96-well microplates at a density 2×10^4 cells/well and allowed to attach to the well surface for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. After incubation, different concentrations of empty, curcumin loaded PDMAEMA-PCL-PDMAEMA micelles and free curcumin were added to cells, and incubated for a period of 24 h and 48 h. For each concentration a set of at least 6 wells were used. After the treatment, 10 μl MTT solution (10 mg/ml in PBS) aliquots were added to each well. The microplates were further incubated for 3 h at 37 °C and the obtained formazan crystals were dissolved by the addition of 110 μl 5% formic acid in 2-propanol per well. The absorbance was measured at 560 nm using an Labexim ELISA reader.

2.3.2. Lactate dehydrogenase (LDH) leakage

Stock dispersions of the empty, curcumin loaded PDMAEMA-PCL-PDMAEMA micelles and free curcumin were freshly prepared and diluted with growth medium in order to obtain different concentrations. Thereafter the micelles were added to cells (2×10^4 cells/well), and incubated for periods of 24 h and 48 h. Six wells were used for each concentration. LDH leakage from the cells was determined using a commercial LDH cytotoxicity detection kit according to the manufacturer's protocols (Clontech, USA). LDH activity was assessed in the conditioned media and the amounts detected were calculated as a percentage of the solvent–treated control (GraphPad Prizm Software).