IN VITRO CYTOTOXICITY EVALUATION OF FUNCTIONAL PEG-PDMA BLOCK COPOLYMER IN LIVER HEPG2 CELLS

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Abstract. The development of matrices to control the release of drugs into specific sites in the human body is a perspective biomedical application of polymeric materials.The aim of this work was to evaluate the cytotoxicity of a newly synthesized functional block copolymer of composition PEO-b-PDMA for application in nanosized drug delivery systems.
The toxicological effect of the copolymer was studied by in vitro exposure of human liver HepG2 cell line. Toxicity was examined by two methods – MTT test and Neutral red assay following the exposure to the copolymer in the concentration range from 1 – 1000 μg/ml for 24 and 48 h. It was shown that no toxic outcome was observed in the concentration range from 1 – 1000 μg/ml, even after 48h of incubation. The results from the study demonstrated a good safety profile for the investigated hydrophilic PEO-PDMA block copolymer.

Keywords: HepG2, polymer nanomaterials, cytotoxicity

Introduction

Recently, nanotechnology and particularly nanomedicine and nanopharmacy, have undergone dynamic development. Consequently, a significant amount of information on materials, composed of particles with dimensions of the order of nanometers was accumulated. The potential use of polymers for biomedical applications has increased in recent years due to their characteristics, like biocompatibility, flexibility and safety. Current research on polymers focuses on their applications in different areas of medical sciences, including oncology, neurology, vascular tissue engineering, etc. For example, the treatment of the mostly recognized neurodegenerative disorders like Alzheimer's disease, Parkinson's disease and multiple sclerosis, remains a major challenge, because effective drug delivery depends on the ability of the drug formulation to pass the blood-brain barrier (1). Thus, nanotechnology might provide engineered materials with functional organization on the nanometer scale, which are capable of penetrating protective barriers surrounding the central nervous system (2). Polymer nanomaterials could be successfully used in the vascular tissue engineering and diagnosis (3, 4). Another field of investigation and future application of nanocarriers as drug delivery systems might be the treatment of atherosclerosis and restenosis (5). The toxicity of cytostatic drugs, used in oncology, is not limited only to malignant cells and this is the main reason for numerous side effects. So, in order to make the drugs safer, it is important to encapsulate them in target-specific controlled-release polymers (6). Nanoparticles provide good penetration of therapeutic substances within the tissues and cells at a minimized risk and could reduce the multidrug resistance – the main problem for achieving the effective anticancer therapy (7).

The most important application of polymeric materials in the field of pharmacy is the development of matrices to control the release of drugs into specific sites in the body (8).Therefore, there are nanodrugs specifically designed to carry therapeutic molecules

that are directly coupled, functionalized, coated, or entrapped in devices produced by controlled manipulations of size and shape at the nanometer scale (9). Polymeric materials provide a platform on which nanoscaled structures can be developed, and this property can be used in numerous medical applications, from surgical implants to binding matrices of drugs (10, 11).

Poly(N,N-dimethylacrylamide) (PDMA) is a hydrophilic biocompatible polymer well-known for its range of useful properties including remarkable water solubility and strong adhesion on different surfaces [12–14]. Linear as well as crosslinked copolymers and blends based on PDMA find numerous applications in medical and pharmaceutical fields including contact lenses, drug delivery systems [15], DNA sequencing [16,17], scaffolds for in vitro cell culture or tissue engineering [18,19]. That is why the development of novel PDMA-based materials with tailored features is of increasing interest. Functional PDMA copolymers of diverse composition and structure provide prospects for application in emerging areas of medicine and pharmacy. The objective of our research was to evaluate the cytotoxicity of a newly synthesized functional PEG-PDMA block copolymer, hereafter design as **CH68**, by two different methods, MTT test and NR test. In this study, a human liver cell line hepatoblastoma HepG2 cells, was used as a predictive screening tool to assess preliminary liver toxicity.

Materials and methods

Reagents

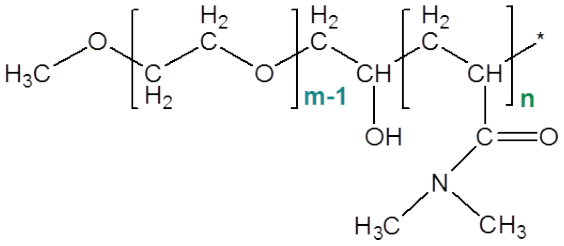
Monomer N,N-Dimethylacrylamide,DMA (99%) and initiator diammonium cerium (IV) nitrate(≥98.5%) were purchased from Sigma-Aldrich (Seelze, Germany). Poly(ethylene glycol) methyl ether (average Mn ~2000) was obtained from Fluka (Buchs, Switzerland). All other solvents used were of standard laboratory reagent grade.

Synthesis of functional PEG-PDMA block copolymer

PEG-PDMA block copolymer was synthesized by means of cerium ion-initiated redox polymerization of N,N-dimethylacrylamide (1 mol/L) when using poly(ethylene glycol) methyl ether as precursor (0.025 mol/L) in deionized water. The reaction mixture was acidified with 1 N nitric acid and purged with nitrogen for 30 min. Then aqueous solution of initiator diammonium cerium (IV) nitrate (0.01 equiv in respect to the PEG precursor) was added.The polymerization was carried out at 35°C under nitrogen atmosphere and constant stirring. After the polymerization completion, the product was first purified by dialysis against deionized water. The purified copolymer aqueous solution was concentrated on a rotary evaporator and finally freeze-dried.

The structure and composition of the obtained copolymer was confirmed by FT-IR and ^{1}H-NMR spectroscopy.

Structure:



Composition: PEO_{45}-*b*-$PDMA_{37}$; molar mass 5700 g/mol (based on ^{1}H-NMR)

Cell cultures

Human hepatoblastoma HepG2 were prepared from the frozen stock cells and kept in a subconfluent state. HepG2 were placed into 75 cm^2 tissue culture flasks and grown at 37^0 C under a humidified 5% CO_2 atmosphere in 90% DMEM with 2 mM – glutamine adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 10% fetal bovine serum, and 2% penicillin-streptomycin (10 000 U/ml penicillin and 10 mg/ml streptomycin) (Gibco BRL). The cells were used for testing within 17 passages after the cells were received.

Cytotoxicity tests

Cell viability was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reductionand by neutral red (NR) test.

MTT-dye reduction assay.

The cell viability, after continuous exposure to the tested polymer CH68 was assessed using the standard MTT-dye (20). Exponentially growing cells were seeded in 96-well microplates (100 µL/well) at a density 1×105 cells and allowed to attach to the surface of the well for 24 h at 37°C. Thereafter the medium was discarded and cells were exposed to various concentrations of the tested copolymers for a period up to 48 h. For each concentration a set of at least 8 wells were used. After the treatment 10 µL MTT solution (10 g/L in PBS) aliquots were added to

each well. The microplates were further incubated for 4 h at 37°C and the MTT-formazan crystals formed were dissolved through addition of 100 µL/well 5% formic acid (in 2-propanol). The absorption was read on a microplate reader at λ=560 nm and the results were expressed as the relative viability of the cells compared with control cells.

Neutral red uptake (NR) assay [21]

At the end of the exposure periods (24h or 48 h) 100µl of Neutral red reagent, prepared in the medium (50 µg/ml) were added to the cells and incubated for 3 h at 37^0C. After removing the NR, 200 µl of fixative solution (50% ethanol, 1% acetic acid and 49% deionized water) were added to extract the dye from the cells. Absorbance was measured at 550 nm on a microplate reader Multiscan Go™, using SkanIt 3.2 Thermo Scientific Software. The results are expressed as relative viability of cells (%), normalized to that of the control cells.

Statistical analysis

Results from the viability tests are expressed as mean values from at least three independent experiments. The cell survival data were normalized as percentage of the untreated control set as 100% viability. The statistical analysis included the Student's t-test whereby values of $P<0.05$ were considered as statistically significant.

Results and Discussion

In this study, the cell viability was assessed after the exposure of HepG2 cells to different concentrations of CH68 by employing two different methods of evaluation: MTT – test and NR assay. Dose-response curves were carried out in the concentration range 1-1000 µg/ml following exposures for 24 and 48 h. Results are presented on **Fig. 1** and **Fig. 2**, respectively.

HepG2 are widely used as model system for determination of toxicity of xenobiotics, hepatic metabolism, processes of liver carcinogenesis, as well as an in vitro system for evaluation of cytoprotective effects of active substances of chemical and natural origin and targeted drug delivery systems. HepG2 cells represent an immortalised cell line, derived from the liver tissue of a 15-year old white American with a well-differentiated hepatocellular carcinoma. The cells are epithelial in morphology, attached, growing in monolayer and small aggregates. HepG2 cells secrete a major part of plasma proteins like albumin, transferrin, and acute phase proteins like fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferring and plasminogen.

MTT-test was performed according to the Mosmann's protocol as described in Materials and Methods section (8). MTT is a water-soluble tetrazolium salt, which is transformed to insoluble purple colored formazan crystals. The formazan is formed by cleavage of the tetrazolium ring by the mitochondrial enzyme succinate dehydrogenase. The formazan accumulates in healthy cells because the cell membrane is impermeable to the formazan products.

The results from the experiments demonstrated a slight decrease in a cell viability of HepG2 after 24h and 48 h of incubation with CH68. The observed decrease was concentration dependent, but it was less than 20% even in the higher concentration of the polymer nanoparticles, indicating the low cytotoxicity of CH68.

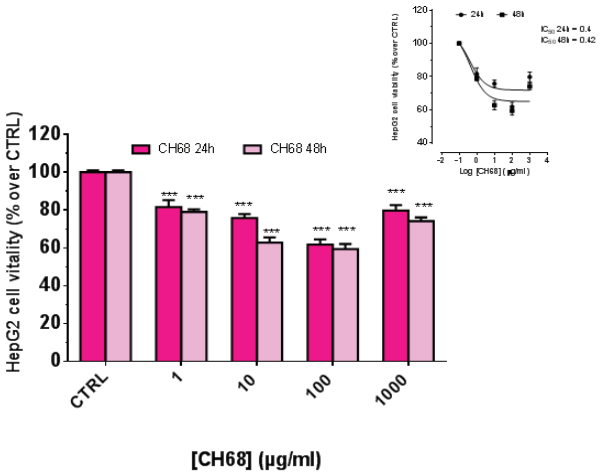


*Fig. 1. Effect of CH68 NPs on cell viability in HepG2 cells. Cells were incubated with 1-1000 µg/ml with CH68 for 24h and 48h. Cell viability was assessed by MTT assays and results are presented as a % of control (untreated) cells. Mean ± SD of three independent experiments performed in triplicate. ***p<0.05.*

In the next experiments, HepG2 cells were incubated with CH68 (1-1000 µg/ml) for 24 and 48 h and analyzed using Neutral red assay. The neutral red (NR) assay was performed according to the protocol decribed by Borenfreund annd Puerner (2).The method is based on the accumulation of the neutral red dye in the lyposomes of viable, uninjured cells. The liver HepG2 cells were found to be non sensitive to the treatment with CH68. The highest concentration of 1

mg/ml CH68 does not cause statistically significant decrease in cell viability, especially after 48h of incubation, where overall decrease in the cell viability was less than 20% in all tested concentrations.

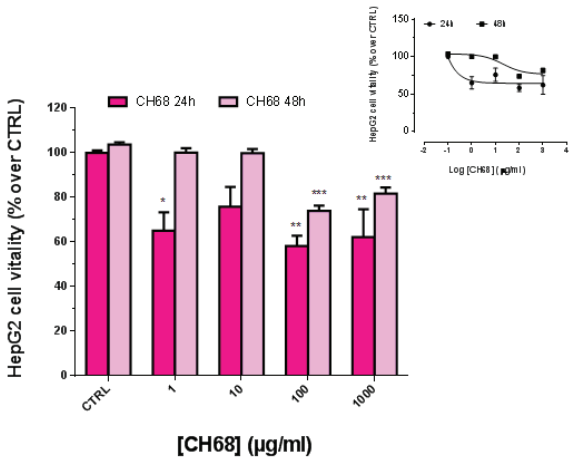


*Fig. 2. Effect of CH68 NPs on cell viability in HepG2 cells assessed by Neutral red assay. Cells were incubated with 1-1000 µg/ml with CH68 for 24h and 48h. The results are presented as % of control (untreated) group; mean ± SD of three independent experiments performed in triplicate. ***p<0.05.*

Conclusions

In the present study we performed an initial toxicological tests *in vitro* on the functional PEO-PDMA block copolymer CH68 in human immortalised liver cells HepG2 using two different test procedures – Neutral red assay and MTT-test. It was shown that no toxic outcome was observed in the concentration range from 1 – 1000 µg/ml, even after 48h of incubation. Nevertheless, more investigations are needed, including large number of diverse cells and toxicological models, in order to fully characterize the safety profil of the polymer nanoparticles, aimed for biomedicinal use as a perspective drug – delivery system.

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