Enhanced cytotoxicity of anticancer drug delivered by novel nanoscale polymeric carrier

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Abstract. We compared in vitro action of highly toxic anticancer drug doxorubicin under its delivery to the mammalian tumor cells in free form and after encapsulation in novel bio-functionalized nanoscale polymeric carrier. Such encapsulation was found to enhance significantly drug uptake by the targeted cells, as well as its cytotoxic action. 10 times higher cytotoxicity of the carrier-immobilized doxorubicin comparing to its free form was demonstrated by direct cell counting, and 5 times higher cytotoxicity of encapsulated doxorubicin was shown by FACS analysis. The polymeric carrier itself did not possess significant toxicity in vitro or in vivo (laboratory mice). The carrier protected against negative side effects of doxorubicin in mice with experimental NK/Ly lymphoma. The life duration of tumor-bearing animals treated with doxorubicin-carrier complex was significantly longer than life duration in animals treated with free doxorubicin. Besides, the effective treatment dose of the carrier-delivered doxorubicin in tumor-bearing mice was 10 times lower than such dose of free doxorubicin. Thus, novel nanoscale polymers possess high potential as drug carrier.

1. Introduction

Rapidly developing production and growing scales of application of nanocomposites in different fields of industry, agriculture, medicine, and common human life also predict potential dangers of these materials for human and environment. In order to improve the biological characteristics of novel nanocomposites, they are frequently covered with a polymeric shell. Such coating was found to improve the bio-compatibility of the nanoparticles containing very toxic anticancer drugs, as well as to enhance the antineoplastic activity of those drugs [1]. There are also other reasons for using the nanocomposites with specific functionalized polymer coating, such as: 1) extremely high toxicity of many drugs for selected normal tissues and organs; 2) low water solubility of many drugs that often requires applying higher doses of these drugs in order to achieve positive treatment effects; 3) natural and acquired drug resistance in many tumor cells; 4) un-capability of many drugs to cross the existing biological barriers in the treated organism, for example blood-brain barrier. Since most anticancer agents cannot discriminate between tumor and normal cells, they can
accumulate non-selectively in healthy tissues resulting in severe clinical toxicities. Doxorubicin (Dox) is one of the most effective and widely used anticancer agents whose clinical application is of potential danger because of frequent development of acute or chronic cardiotoxicity. Doxil is a PEGylated liposomic nano-container of Dox that is known to lack most of the cardiotoxicity of that drug [2].

In this study, we compared in vitro action of anticancer drug Dox when delivered in free form or by the developed nanoscale polymer coated with bio-functionalized shell. The results of bio-evaluation of novel polymeric nanoscale carrier VEP-GMA that was additionally functionalized with PEG and phospholipid in Dox delivery in vitro into the mammalian tumor cells are presented. Significant enhancement of the cytotoxic action of Dox in vitro and in vivo was demonstrated when this anticancer drug was delivered by the developed carrier, while the carrier itself did not possess significant toxicity towards tumor cells in vitro or toxic action in the laboratory mice. Similar nanoscale polymer BG-2 was also shown to be non-mutagenic in Ames test.

2. Materials and methods

*Synthesis of the nanoscale polymers* was performed by radical copolymerization of vinyl pyrrolidone (NVP), 5-(tertbutylperoxy)-5-methyl-1-hexene-3-yne (VEP) and dimethyl aminoethyl methacrylate (DMAEM) (molar ratio = 1:1:1) in the dimethyl formamide solution, as described [3, 4]. A schematic structure of the obtained polymeric compound VEP-GMA is presented in Fig. 1 A.

![Figure 1](image)

**Figure 1.** Structure of the oligomeric carrier functionalized with polyethylene glycol VEP-GMA-PEG (A) and the oligoelectrolyte polymeric carrier BG-2 (B).
The size of phospholipid-containing nanoscale polymers VEP-GMA-PEG and BG-2 (see below) was measured by a dynamic light scattering on a Zetasizer Nano ZS (Malvern Instruments GmbH, Stuttgart, Germany) and photon correlation spectra using the NIBS (Non-Invasive Back Scatter) technology at 25°C. Their diameter (dynamic light scattering) was shown to be 58 nm and 19 nm, correspondingly.

For preparing of the nanoscaled PEG- and phosphatidyl choline-containing Dox delivery system (PC-Dox), 100 mg of PC were dissolved in 1.0 ml DMSO. 50 mg of L-α-phosphatidylcholine (PL) from egg yolk (Fluka, Germany) were dissolved in 1.0 ml chloroform. Then chloroform was evaporated to form a thin film of phosphatidylcholine. The polymer solution was added to the phosphatidylcholine film and incubated for 30 min. Notably, only 3.0±0.3 mg of phosphatidylcholine was incorporated into the total amount of PC. 3.0 mg of Dox (Arterium, Ukraine) were dissolved in 0.5 ml DMSO. The resulting solutions of Dox and polymer with phosphatidylcholine were mixed and added drop by drop to 8.5 ml water containing 0.9 % NaCl. Subsequently, the solution was sonicated for 20 seconds. Average diameter of the nanocomposites with immobilized doxorubicin was 28 nm (dynamic light scattering). A hypothetical schematic structure of phospholipid (PL)-containing PC-Dox is presented in Figure 1 A. PC-Dox was analyzed by UV-VIS and fluorescence spectroscopy, as well as surface tension measurements indicating the formation of Dox-containing polymeric system. In addition, drug release measurements showed that the nanoparticles were stable for at least one month without significant drug release.

The novel carrier BG-2 is a copolymer of a comb-like structure that combines an oligoelectrolyte chain of the anionic type as a backbone with 1 to 3 of grafted side chains of the cationic type (Figure 1 B). This oligoelectrolyte polymer was synthesized by controlled radical polymerization initiated by the oligoperoxide metal complex (OMC) in a polar organic media. Synthesis of the comb-like oligoelectrolyte polymer was carried out as follows: a monomer mixture of dimethyl aminoethyl methacrylate (DMAEM) (from 17.13 to 28.18 g, with 28.18 g being optimal) and 5-(tertbutylperoxy)-5-methyl-1-hexen-3-yne (VEP) (from 3.19 to 17.13 g, with 3.19 g being optimal) was injected into round bottom glass reactor equipped with impeller mixer and backflow condenser. Then a solution of OMC (1.66 g) in ethanol or dimethyl formamide (67 g) was added with stirring. The temperature of incubation was increased to 333 K, and the reactor was actively maintained at this temperature for 8 hrs under Argon flow. The monomer conversion was controlled by using a gravimetric technique; after achievement of the desired conversion efficiency (i.e., 60 %), the solvent was evaporated under vacuum. The synthesized comb-like polyelectrolyte was purified by multiple precipitations from acetone solution into hexane, and dried till constant weight under vacuum. The diameter of its particles equaled 19 nm (dynamic light scattering).

Cell culture. The murine leukemia cell model L1210 was purchased from the American Type Culture Collection (Manassas, VA). The cell line was cultured in RPMI-1640 medium (Sigma-Aldrich, Austria). Culture media was supplemented with 10% fetal bovine serum (Sigma-Aldrich, Austria).

In Vivo Studies. Animal experiments were conducted in accordance with the requirements of the European Convention on protection of vertebrate animals (November 13, 1987) and The Law of Ukraine “On Protection of Animals From Cruel Behavior” (March 28, 2006). BALB/c mice were obtained from R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine). Animals were kept in a controlled environment in the conventional cages. NK/Ly lymphoma in BALB/c mice (8-10-
week old female, 20–22 g) was used as an experimental tumor model. Animals were injected intraperitoneally with 20–25 mln NK/Ly lymphoma cells, and chemotherapy treatment was performed by the intraperitoneal injection of doxorubicin or its complex with the polymeric carrier (total volume did not exceed 0.2 ml after drug dissolving in saline). Injections were conducted every day for 8 days, starting from the 2nd day after tumor cell inoculation. Treatment doses are noted in Figure 3. In control, mice were injected with saline using the same treatment scheme. Tumor growth was monitored by animal weighting.

**Cytotoxicity Assay.** Mammalian cells used in the study were plated (2x10^3 cells in 100 µl/well) in 96-well plates (IWAKI) and allowed to recover for 24 h. Drugs were added in another 100 µl growth medium and cells exposed for the indicated time periods. After 24 h or 48 h drug treatment, the proportion of viable cells was determined by MTT assay following the manufacturer’s recommendations (EZ4U, Biomedica, Vienna, Austria).

**Ames testing for mutagenicity** was conducted for the BG-2 nanopolymer. TA 98 and TA 100 strains of *Salmonella typhimurium* bacteria were tested in the presence of this polymer with or without the microsomal activation by S9 rat liver homogenate. The ratio of numbers of the revertants produced per plate in the presence of various concentrations of BG-2 to control was measured. Positive controls were benzidine (100 µg/plate), nitrosoguanidine (1 mg/plate) in the presence of the metabolic activation and sodium azide (1.5 µg/plate) in the absence of S9 [5].

**Western blot analysis.** After 24 h exposure of cell to studied substances, cellular proteins were isolated, resolved by electrophoresis in SDS-PAAAG, and transferred onto a polyvinylidenedifluoride membrane for Western blotting, as described [6]. The following antibodies were used in this study at a 1:1,000 dilution: anti-cleaved PARP and anti-cleaved caspase 3 (both polyclonal rabbit and from Cell Signaling Technology, Beverly, MA). Equal loading of each lane was evaluated by the immunoblotting of the same membrane with anti-β-actin monoclonal mouse AC-15 (Sigma). Secondary peroxidase-labelled antibodies were from Pierce and used at working dilution of 1:10,000.

**Statistical analysis.** The results were expressed as means ± standard deviation and significant difference at p<0.05.

3. Results and Discussion

In this study, two versions (VEP-GMA-PEG and BG-2) of novel polymeric nanoscale carrier were studied, and VEP-GMA-PEG was used for the delivery of anticancer drug doxorubicin (Dox). It was found that such encapsulation allows achieving cytotoxic effect (24 hrs) of this drug towards the mammalian tumor cells *in vitro* in 10 times less concentration than for achieving such effect at using free drug - 0.1 µg/ml of the encapsulated Dox versus 1 µg/ml of free Dox (Fig. 2 A). In 48 hrs, the inhibitory effect of such drug complexes towards L1210 leukemia cells was even more pronounced (Fig. 2 B). The encapsulated drug was much faster up-taken by the targeted cells, accumulated in their nucleus, and its cytotoxic effects were accompanied by typical pro-apoptotic changes. There were no significant effects on cell survival and proliferation caused by the developed polymeric drug-free carriers *in vitro*. No visible negative side effects were found in BALB/c mice injected with the drug-free nanopolymers (data not presented).

The results of the bacterial reversion assay (Ames test) with using two concentrations (0.1% and 0.01%) of BG-2 oligoelectrolyte did not demonstrate its mutagenicity. According to the EPA and GenPharmTox guidelines, the mutagenic potential of Ames test item is considered to be significant when the mutant frequency (experiment versus control) equals or
is above 2.0 [5]. It is probable if the quotient ranges from 1.7 to 1.9, while the lack of mutagenicity is assumed if the quotients range from 1.0 (and lower) to 1.6. It was found that almost all quotients were below 1.0, comparing to positive controls: benzidine = 2.54, nitrozoguanidine = 2.69, and sodium azide = 2.14 - all exceeding the critical value of 2.0. Therefore, no mutagenic activity was observed in any of BG-2 type oligoelectrolyte samples. Although an increase in induction factor can be seen in presence of S9 homogenate (+S9), there was no statistical significance in genotoxic potentials of any of the samples comparing to the control value.

Figure 2. Treatment of murine leukemia cells of L1210 line with free doxorubicin (Dox) and doxorubicin-carrier complexes (PC-Dox) for 24 (A) and 48 (B) hrs (the ordinate notes a decrease in cell number relative to cell number in the control, 100%), n=20. (*P<0.05, **P<0.01).

Higher effectiveness of the antiproliferative and cytotoxic action the PC-Dox comparing with such effects of free Dox was confirmed in other mammalian tumor cell lines.

Fig. 3 presents the results of studying the effect of application of novel polymeric carrier for doxorubicin delivery at treatment of NK/Ly lymphoma in BALB/c mice. Treatment for 8 days with free doxorubicin in daily dose of 0.1 mg per gram of animal weight, led to only a transient stop of tumor growth and prolongation of survival of tumor-bearing mice from medially 21 to 39 days. At the delivery of this drug by the developed carrier, all tumor-bearing mice survived over 60 day observation period, and the treated animals were classified as “cured”. Thus, application of novel polymeric carrier allows increasing the effectiveness of doxorubicin delivery and action towards murine lymphoma, comparing with the action of free form of this drug.
Doxorubicin is known to intercalate DNA causing inhibition of its biosynthesis and it also inhibits topoisomerase II activity. That leads to cell stop in G2/M phase of cell cycle [7, 8]. Taking into consideration the above described ability of the nanocarrier to enhance doxorubicin’s cytotoxicity, we have compared the impact of free Dox and its complex with nanocarrier on cell cycling in murine L1210 leukemia cells. As one can see (Fig. 4, 5), Dox significantly and dose-dependently affects cell cycling, namely induces L1210 cells’ arrest in G2/M phase (from 15.7% in control to 26.8% at 0.05 µg/ml Dox dose). 5 times higher dose (0.25 µg/ml) of this drug caused a complete blocking of cell cycle progression in L1210 cells, resulting in 81.95% cells arrested in G2/M phase (Fig. 4, 5). Dox immobilization on the nanocarrier led to even more significant changes in cell cycling – we observed almost complete inhibition of cell cycle progression (83.56% cells were arrested in G2/M phase) at 0.1 µM of Dox that was immobilized on the nanocarrier. At higher concentrations of Dox (0.25 µg/ml) encapsulated in the nanocarrier, such effect was preserved, although the ratio of G2/M-stopped cells was lower (56%). Such decrease in this ration can be explained by stronger induction of apoptosis by the Dox/PC complex which leads to changes in the pattern of cell cycle phases in treated cells. Thus, the enhancement of cytotoxic action of Dox by its encapsulation in novel polymeric carrier has been confirmed by the results of FACS analysis in treated murine leukemia L1210 cells.

![Figure 3](image_url)

**Figure 3.** Survival of NK/Ly lymphoma-bearing mice depending on the treatment course. Dox – doxorubicin, PC – polymeric carrier.
**Figure 4.** Comparison of impact of free doxorubicin and doxorubicin/carrier complex on cell cycling in murine leukemia L1210 cells (24 h treatment). 1 – control (C); 2 – polymeric carrier (PC); 3 – Dx 0.05 µg/ml; 4 – Dx 0.25 µg/ml; 5 – Dx (0.05 µg/ml)+NC; 6 – Dx (0.25 µg/ml)+NC.

**Figure 5.** Quantification of the impact of free doxorubicin and doxorubicin/carrier complex on cell cycling in murine leukemia L1210 cells (24 h treatment). 1 – control (C); 2 – polymeric carrier (PC); 3 – Dx 0.05 µg/ml; 4 – Dx 0.25 µg/ml; 5 – Dx (0.05 µg/ml)+NC; 6 – Dx (0.25 µg/ml)+NC.
For studying the mechanisms of enhanced efficiency of the applied drug delivery system at the molecular level, Western-blot analysis of the expression of proapoptotic proteins – caspase-3 and PARP-1 (DNA repairation enzyme) was carried out. Murine leukemia L1210 cells were treated for 6 and 12 hrs with the Dox-carrier or free Dox used in 0.1 μg/ml and 1 μg/ml concentrations of Dox. The results of Western-blot analysis of the effector caspase-3 and its protein substrate PARP-1 are shown in the Fig. 6. We have found that the PC-Dox treatment of L1210 cells for 6 h caused activation of the effector caspase-3 and cleavage of PARP-1. Free Dox activated these processes much later – only in 12 hrs of the drug treatment.

![Figure 6](image_url)

**Figure 6.** Western-blot analysis of expression of the effector caspase-3 and its protein substrate PARP-1 in murine leukemia L1210 cells treated for 6 h and 12 h with free doxorubicin (Dx) or its complex with the polymeric carrier (PC).

**4. Conclusions.** Encapsulation of the anticancer drug doxorubicin in novel polymeric nanocomposite enhances delivery of this drug into the targeted mammalian cells, as well as its cytotoxic action towards tumor cells. It also protects of negative side effects of this drug in mice organism. The developed polymeric carriers do not possess their own cytotoxicity *in vitro* and *in vivo.*

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