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Formulations of biodegradable Nanogel carriers with 5'-triphosphates of nucleoside analogs that display a reduced cytotoxicity and enhanced drug activity

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Abstract

Therapies including nucleoside analogs are associated with severe toxic side effects and acquirement of drug resistance. We have previously reported the drug delivery in the form of 5'-triphosphates (NTP) encapsulated in cross-linked cationic networks of polyethylenimine (PEI) and PEG/Pluronic® polymers (Nanogels). In this study, Nanogels, containing biodegradable PEI that could easily dissociate in reducing cytosolic environment and form products with minimal toxicity, were synthesized and displayed low cytotoxicity. Toxicity of Nanogels was clearly dependent on the total positive charge of carriers and was 5–6-fold lower for carriers loaded with NTP. Though intracellular ATP level was immediately reduced by ca. 50% following the treatment with Nanogels, it was largely restored 24 h later. Effect of Nanogels on various respiratory components of cells was reversible too, and, therefore, resulted in low immediate cell death. Nanogel alone and formulations with AZT-TP demonstrated a much lower mitochondrial toxicity than AZT. As an example of potential antiviral applications of low-toxic Nanogel carriers, a 5'-triphosphorylated Ribavirin-Nanogel formulation was prepared that demonstrated a 30-fold decrease in effective drug concentration (EC₉₀) and, totally, a 10-fold increase in selectivity index compared to the drug alone in MDCK cells infected with influenza A virus.

Keywords

delivery vehicle; nanogel polymer network; nucleoside analogs 5'-triphosphates; mitochondrial toxicity; influenza A virus

1. Introduction

Application of nanomaterials for drug delivery experiences rapid progress during the last years [1]. Presently used in therapy liposomal drug formulations and other advanced pharmaceutical nanocarriers demonstrate an ample variety of useful properties, such as permanence in the blood; accumulation in pathological areas with compromised vasculature; targeting at certain disease sites due to attached ligands on the surface of nanocarriers; enhanced intracellular penetration with the help of surface-attached cell-penetrating molecules; contrast properties due to the carrier loading with various contrast materials allowing for direct carrier visualization *in vivo*; stimuli-sensitivity allowing for drug release from the carriers under

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certain physiological conditions. Some of those pharmaceutical carriers have already made their way into clinic, while others are still under preclinical development [2].

Hydrophilic microgels demonstrated a great potential for drug delivery and diagnostic applications [3]. We recently developed charged submicron polymer networks for drug delivery called Nanogels [4]. The swollen macroporous network of Nanogels consist of covalently cross-linked polymer molecules, cationic polyethylenimine (PEI) or anionic polyacrylic acid (PAA), and neutral hydrophilic molecules of polyethylene glycol (PEG) or amphiphilic Pluronic® block copolymers [4–7]. Nanogels have sizable drug loading capacity, low buoyant density and high dispersion stability in aqueous media and, enhance the efficacy of therapeutic nucleotides by increasing drug stability, intracellular drug release, and decreasing non-specific drug toxicity.

Nucleoside analogs (N) represent the cornerstone of many antiviral regimens and are widely used in cancer chemotherapy. These drugs are activated by intracellular or viral kinases into the pharmacologically active form of nucleoside 5'-triphosphate (NTP). NTPs incorporated into nucleic acids lead to cell death or inhibit viral replication. The low conversion of nucleoside analogs into NTPs results in the development of drug resistance and high toxicity, ultimately compromising the effectiveness of this therapy [8]. Nucleoside analogs phosphorylated in the cytosolic/nuclear compartment can be imported into mitochondria and hinder DNA replication both in the nucleus and mitochondria [9]. The mitochondrial genome encodes enzymes involved in the respiratory chain, and defective expression of these enzymes results in disruption of cellular aerobic metabolism and cell death. A range of drug or tissue specific cases associated with mitochondrial toxicity, such as peripheral neuropathy, myopathy, pancreatitis, fat atrophy, lactic acidosis and hepatic steatosis has been documented. The drug effects on mitochondria have been intensively studied since it was shown that several pyrimidine nucleoside analogs used in antiviral therapy damaged mitochondrial DNA and thereby interfered with mitochondrial function [10–12]. Several analogs such as 3'-azido-2', 3'-dideoxythymidine (AZT) can be incorporated into mitochondrial DNA and are known to cause mitochondrial toxicity after prolonged treatment in patients [13]. Potentially, drug carriers will be able to reduce a non-specific drug biodistribution and the associated toxic effects.

Short term exposure *in vitro* to some NA increases lactate production and mitochondrial toxicity [14]. Nanogel formulations with cytotoxic NTP decrease effective drug doses (200–240 times), but concern about inherent toxicity of the carrier remains [15]. Toxicity is an inherent property of most polycations and increases with the total charge of the carrier molecule. In addition to acute toxicity, cationic carriers released after drug delivery may accumulate in many tissues and cells [16]. Accumulation of non-degradable materials in tissues may pose a problem, due to unknown effects of long-term toxicity [17]. Therefore, biocompatible polymers, which can be easily digested and eliminated by the host, would be more advantageous. An attractive strategy is the chemical engineering of polycations performed by introduction of cleavable disulfide bridges is [18]. Disulfide bonds are known to be cleaved in the reductive environment of endo/lysosomal compartments [19] or by cytosolic glutathione [20].

In the present study, we evaluated toxicity of drug-loaded Nanogels with major emphasis on respiratory chain components, such as intracellular ATP, mitochondrial membrane potential (MMP) and lactate dehydrogenase (LDH), and compared cell viability assays for drug or drug formulations. We also described the preparation of Nanogels with biodegradable structure demonstrating reduced inherent cytotoxicity. Nanogel formulations seem to significantly reduce adverse effects of cytotoxic nucleoside analogs on mitochondrial function. Efficacy of Nanogel-encapsulated antiviral drugs was additionally confirmed by using 5'-triphosphate of

Ribavirin against influenza A virus in cell culture model, where it showed significantly better therapeutic efficacy, a lower EC₉₀ and higher selectivity index (SI) than the parental drug.

2. Materials and Methods

2.1. Materials

All solvents and reagents, except specially mentioned, were purchased from Sigma-Aldrich (St. Louis, MO) at highest available quality grade and used without purification. 5'-Triphosphorylated nucleoside analogs, AZT and Ribavirin, were synthesized by the recently introduced one-pot chemical method and purified by HPLC to an average purity of 90% [15].

2.2. Nanogel Synthesis

Synthesis of NG(PEG) and NG(F68) was performed by the emulsification-solvent evaporation method described previously [7]. NG(F127) was obtained using micellar approach [21]. Biodegradable Nanogels have been synthesized by the same methods but using biodegradable PEI instead of regular branched PEI (MW 25,000). In all procedures the input weight ratio of activated polymers to PEI was equal to 3. Real polymer ratios determined by elemental analysis of nitrogen in the purified Nanogel samples well correlated with the input amounts of polymers. Nanogels were purified by centrifugation (30 min, 2,500 rpm) to remove large particles and by extensive dialysis against diluted aqueous ammonia (x1/1000) in membrane tubes with the MWCO 25,000, and then lyophilized.

2.3. Synthesis of biodegradable PEI

Branched PEI (MW 2,000) was purified by gel permeation chromatography on the column with Sephadex G-25 (2.5 × 60 cm) in diluted aqueous ammonia (x1/1000). Major fractions in the middle of the PEI-positive peak were collected and concentrated *in vacuo*. The purified PEI (2 g) was dissolved in 10 ml of 50% aqueous DMF and treated dropwise with a solution of dimethyl-3,3'-dithiobispropionimidate 2HCl (DTBP, 310 mg) in 10 ml of 50% aqueous DMF. The reaction mixture was stirred for 2 h at 25°C and then dialyzed overnight in a membrane tube with MWCO 3,400 against water. The high-MW PEI component eluted before the position of isolated PEI (MW 2,000) fractions was separated in previously used conditions. Yields were usually higher than 70% by weight. Average MW of the product was determined by viscosimetry using different commercial PEI standards as reported previously [22].

2.4. Drug-Nanogel Complexes

Drug-Nanogel complexes were prepared using corresponding NTP or cytidine 5'-triphosphate (CTP) as a model drug [7]. Initially, solution of sodium salt of NTP or CTP was passed through a short column with Dowex 50×6 in H⁺ form to convert into an acidic form. Nanogels dispersion in amine form (pH ~10) was titrated using the obtained NTP/CTP solution until pH 7.5, then concentrated *in vacuo*, and passed through NAP-20 column and lyophilized. NTP/CTP content in dried formulations was determined spectrophotometrically at 260 nm.

2.5. Cell Cultures

Human breast carcinoma MCF-7 and Madin-Darby canine kidney MDCK (NBL-2) cells were obtained from the ATCC (Rockville, MD). Cell lines were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with nonessential amino acids, 2 mM of L-glutamine, 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml). All culture media were obtained from Gibco (Fisher Scientific, Pittsburgh, PA). Influenza A virus A/PR/8/34 (ATCC) was propagated in MDCK cells in the presence of 0.3% trypsin.

2.6. Cytotoxicity assays

Cytotoxicity of Nanogels were assessed in standard thiazolyl blue tetrazolium bromide (MTT) assay [23] following 24h-incubation with cells as described in our earlier publication [7]. For comparison of CC₅₀ values obtained from MTT assay, Sulforhodamine B (SRB) cytotoxicity assay was used [24]. SRB assay was performed with minor modifications using serial dilutions of NG(PEG) (0.0001–1mg/ml) in the complete media were incubated with MCF-7 cells for 24 h at 37°C and then washed twice with PBS before cytotoxicity analysis. In brief, 70 µl of 0.4% (w/v) solution of SRB in 1% acetic acid was added to each well and 96-well plate was incubated for 20 min at 25°C. SRB was removed and the plates washed 5 times with 1% acetic acid before air drying. Cell-bound SRB was solubilized with 200 µl of unbuffered 10 mM Tris-base solution in the shaking plates for 10 min at 25°C. Absorbance was read in a microplate reader at 492 nm and data were normalized by subtracting the background absorbance at 620 nm. The CC₅₀ values were calculated using the Prism software.

2.7. Intracellular ATP level

Intracellular ATP level in metabolically active cells was quantified using CellTiter-Glo® luminescent cell viability assay kit from Promega (Madison, WI). In brief, 5,000 MCF-7 cells were seeded in multiwell plates in culture medium and allowed to attach overnight. Cells were treated with Nanogels at different concentrations (0.05–1.5 mg/ml) for 2 h at 37°C and 5% CO₂. At the end of incubation, 100 µl of CellTiter-Glo® reagent was added to each well, mixed for 2 min, and luminescence was recorded using MicroTek Flx-800 microplate reader.

2.8. Lactate dehydrogenase (LDH) measurement

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme released upon cell lysis. To assess the cytotoxicity of Nanogels alone or loaded with drug, the released LDH was measured in coupled enzyme assay using Cytotox 96® assay kit from Promega (Madison, WI). Briefly, 10,000 cells were seeded in 96-well plates and allowed to attach overnight. Cells were treated with Nanogels at different concentrations for 4 h at 37°C and 5% CO₂. At the end of incubation 100 µl of supernatants were transferred to another multiwell plate, treated with 50 µl of reconstituted substrate mix and incubated for 30 min at 25°C in the darkness. Absorbance was recorded at 490 nm following the addition of the stop solution. Maximal LDH release in control experiments performed with 0.3% H₂O₂ was set as 100% cytotoxicity. Cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental LDH release (OD}_{490})}{\text{Maximal LDH release (OD}_{490})}$$

2.9. Mitochondrial Membrane Potential (MMP)

To examine the mitochondrial toxicity of various Nanogel formulations, the MCF-7 cells were treated with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanine iodide (JC-1, Molecular Probes), a cationic, lipophilic dual fluorescence dye that exhibits potential-dependent accumulation in mitochondria. Approximately 1×10^7 MCF-7 cells were incubated with various concentrations of drugs and drug-loaded Nanogel NG(PEG) in assay buffer for 2 h at 37°C. Control cells were also incubated in assay buffer. For the negative control cells were incubated for 5 min with 2 µl of 50 mM solution of carbonylcyanide m-chlorophenylhydrazone (CCCP) in DMSO. MMP was measured using 2.5 µl of 200 µM solution of dual-emission potential-sensitive probe, JC-1 (final concentration of 0.5 µM), added to all cell groups and incubated for 30 min at 37°C and 5% CO₂. At the end of incubation period cells were centrifuged at 1600 rpm for 10 min and re-suspended in PBS. The fluorescence of labeled cells was analyzed by flow cytometry on Becton Dickinson FAC Star Plus three-laser flow cytometer equipped with a Miltenyl Auto MACs automated magnetic cell separation system at λ_{ex} 488 nm. In the experiment with AZT, AZT-TP and AZT-TP/NG(PEG) formulation, an

equimolar drug concentration (0.5mM) was used and cells were treated and analyzed as described above.

2.10. Influenza A virus inhibition

In this microplate-based assay cytopathic effect of influenza A virus resulted in cell death. Cell survival increase directly depended on the efficacy of antiviral drug and was measured using MTT assay. Drug efficacy was calculated by comparing cell survival in non-treated and drug-treated infected MDCK cells.

For initial virus titration, 2×10^5 MDCK cells per well in 96-well plates were cultivated for 48 h. Influenza A virus suspension medium containing $1/100$ dilution of 0.05% TPCK trypsin (0.5mg/ml PBS) in DMEM without serum was used. Previously prepared virus samples were serially diluted $1/2$ to $1/128$ in the virus suspension medium. Cells were washed with 200 μ l of sterile PBS and inoculated with 100 μ l of each viral dilution in quadruplicates. Plates were incubated for 4 days at 35°C. Then, 10 μ l of MTT (5 mg/ml) in PBS was added and plates were incubated for 4 h at 35°C and 5% CO₂. 100 μ l of isopropanol was added and plates incubated for 18 h at 37°C. Absorbance was measured at 540 nm using a microplate reader. TCID₅₀ values were calculated using the equation: $-\log \text{TCID}_{50} = 0.3 - 0.3 (S - 0.5)$, where S - number of dilutions before no more virus was detected.

In the virus inhibition assay, 2×10^5 MDCK cells per well in 96-well plates were cultivated for 48 h. Cells were washed in plates with 200 μ l of sterile PBS and supplemented with 100 μ l of drug or drug formulations serially diluted in culture medium in quadruplicates and incubate for 2 h at 35°C and 5%CO₂. Then, cells were inoculated with 90 μ l of virus solution (50 TCID₅₀) obtained by $1/2$ dilutions of initial viral samples in virus suspension medium. A separate plate was used for incubation with tested solutions without virus to measure cytotoxicity. Plates were incubated at 35°C for 4 days, a half of solution volume was removed from each well, and samples were analyzed by MTT assay as described above. Effective concentration for 90% inhibition of virus-induced cytopathic effect (EC₉₀) and drug/formulation cytotoxicity concentration (CC₉₀) were measured and used to calculate the selectivity index $SI = CC_{90}/EC_{90}$.

3. Results and discussion

3.1. Synthesis and properties of biodegradable Nanogel carriers

Structures of obtained Nanogels are shown in Figure 1, and some of their characteristics are presented in Table 1. In all synthesis, freshly activated by 1,1'-carbonyldiimidazole PEG/Pluronic® polymer components were taken into the reaction with PEI at weight ratio 3:1. Total nitrogen content in purified Nanogels was determined by elemental analysis (MHW Laboratories, Phoenix, AZ) and by titration with 2,4,6-trinitrobenzenesulfonic acid (TNBS), using branched PEI (MW 25 kDa) for calibration curve, and was in good agreement with the input amounts of all components.

Biodegradable polycation PEI_{ss} was obtained by intermolecular binding the segments of branched PEI (MW 2 kDa) with disulfide bridges in the result of reaction with bifunctional cross-linking reagent DTBP. It was isolated using gel permeation chromatography with yields usually higher than 70%. Molecular weight of the segmented polycations was determined by viscosimetry using standard solutions of commercial PEI with labeled MW. Calculated MW of the PEI_{ss} samples was found to be 28 ± 2 kDa. This polycations was then used in the preparation of biodegradable Nanogels (NG_{ss}) the same way as commercial PEI (MW 25 kDa).

Nanogels NG_{ss} demonstrated low polydispersity by size exclusion HPLC analysis with refractive index detection. Treatment with dithiothreitol (DTT, 20 mM, 1h, 37°C) resulted in

ca. 70% degradation of the NGss network and formation of products with significantly lower MW (10–12 kDa) (Figure 2A). At the same time, *in vitro* degradation of Nanogels in PBS at 37°C was slow and characterized by a half-life of 8.5 ± 0.5 days (Figure 2B).

3.2. Comparative cytotoxicity

Cytotoxicity of NG (PEG) was compared in MCF-7 cell culture using a MTT as well as SRB cell viability assays. SRB assay is an NIH-recommended method for determination of cytotoxic effects of anticancer drugs, especially, when drugs in question are capable of inducing mitochondrial toxicity. In SRB assay, the amount of incorporated dye is directly proportional to the number of living cells. MTT assay is also a widely used method to assess cell viability based on the dye reduction by mitochondrial dehydrogenases in living cells to a blue-colored formazan precipitate. However, cytotoxic compounds damaging mitochondria might decrease the reduction of MTT to formazan. The obtained by SRB assay CC_{50} value for NG(PEG) was 0.06 mg/ml, while MTT assay gave very close value of 0.08 mg/ml under the same conditions. The rightward shift in CC_{50} value in case of SRB assay could be partially due to the detergent-like effect of amphiphilic polymeric carrier and partial detachment of even healthy cells from the plate. In MTT assay, cells can overcome during the proliferation period any initial toxicity caused by Nanogels and, therefore, are less prone to interfering effect of polymeric drug carriers. Based on these finding, MTT assay, decisively more competitive in our research, was used for determination of cytotoxicity of Nanogels and their formulations.

Cytotoxicity of biodegradable and regular Nanogels, as well as their formulations with CTP, was compared in MCF-7 cells at 24 h-incubation time. Carriers with segmented network (NGss) were generally in several times less toxic than Nanogels with regular network (Table 1). It is noteworthy that CTP-loaded carriers demonstrated also a significantly lower toxicity compared to unloaded Nanogels. Between Nanogels, having different neutral polymers in the network, cytotoxicity was mostly determined by the polymer/PEI ratio and by the nature of the neutral polymer itself. Our data show some slightly higher cytotoxicity of the Pluronic®-based compared to PEG-based Nanogels.

3.3. Intracellular ATP levels

Cellular ATP is primarily produced in mitochondria. To examine effect of Nanogels on intracellular levels of ATP and mitochondrial functioning, MCF-7 cells were exposed for 2 h to various concentrations near cytotoxic doses of Nanogels. ATP levels were quantified using CellTiter-Glo® luminescent cell viability assay kit. The observed intracellular ATP levels following treatment with NG (PEG), NG(F68) and NG(F127) as compared to untreated cells are presented in Figure 3A, while CTP-loaded Nanogels are compared in Figure 3B. 50% reduction of ATP level was observed in cells treated with 0.07 mg/ml of NG (PEG) and NG (F68), whereas 0.1 mg/ml of NG(F127) required to produce the similar effect. These values are very close to IC_{50} values obtained by independent methods. In case of CTP- loaded Nanogels, the same 50% reduction of ATP level was observed at 10, 4 and 5 times higher concentrations of NG(PEG), NG(F68) and NG(F127), respectively, also in a good agreement with MTT cytotoxicity results (Fig. 3B).

The ability of the cells to recover from initial shock and restore ATP levels following removal of Nanogel from the culture medium was tested in the next experiment. MCF-7 cells were exposed initially for 2 h to several concentrations of Nanogels. After removal of Nanogel treatment solutions, cells were cultured in fresh media for additional 4 h or 24 h- periods. Registration of ATP levels at these time points demonstrated restoration of ATP levels up to approximately 80% of the initial ATP level in 24 h-period (Figure 4). Moreover, the restoration ability of cells was governed by Nanogel concentrations. At concentrations 5 and 10 times higher than CC_{50} , only 20 and 10% of ATP levels was restored in 24 h, respectively. These

results indicate that Nanogels at nontoxic concentrations do not cause any irreversible damage to mitochondrial machinery.

3.4. Release of lactate dehydrogenase

LDH is a stable cytosolic enzyme which is released upon cell lysis, so that increased levels of LDH are directly proportional to the extent of damage caused by toxic agent to the cells. LDH assay was performed in MCF-7 cells to test cytotoxicity of Nanogels related to the damage of external cellular membrane. Cells were treated with unloaded and CTP-loaded NG(PEG) and NG(F68) at wide range of concentrations for 4 h. In the same experiments, cells were also treated with 30% hydrogen peroxide to obtain maximum release of LDH associated with 100% damage to cells. LDH assay also revealed that loaded Nanogels were significantly less toxic than unloaded carriers (Fig. 5). Unloaded Nanogels, both NG(PEG) and NG(F68), demonstrated 50% cytotoxicity at concentration of 0.25 mg/ml. Alternatively, CTP-loaded Nanogels exhibited 50% toxicity for NG(PEG) at more than 4 times higher concentration and for NG(F68) at more than 3 times higher concentration than unloaded carriers. Evidently, membrane-related toxicity of studied Nanogels was less pronounced compared to other cytotoxic mechanisms. A reduced toxicity of CTP-loaded Nanogels again clearly indicates that intrinsic toxicity of polycationic Nanogels can be lowered by neutralizing the surface charge of the carriers.

3.5. Mitochondrial Membrane Potential

To examine the effect of NAs with known mitochondrial toxicity on the organelle viability, a mitochondrial membrane potential ($\Delta\psi_m$ or MMP) was measured in MCF-7 cells treated with 3'-deoxy-3'-azidothymidine (AZT), AZT-TP alone and encapsulated in NG(PEG). MMP is a key indicator of cellular viability, as it reflects the pumping of H^+ across the inner mitochondrial membrane during the process of electron transport and oxidative phosphorylation, which is a driving force behind ATP, consequently, the loss of MMP will ultimately lead to cytotoxicity or erratic damage to other cellular functions. In this assay cells are treated with JC-1, a cationic, lipophilic dual fluorescence dye that exhibits potential-dependent accumulation in mitochondria [25]. The dye allows for a dual measurement of dye concentration that does not require the measurement of a nuclear or cytoplasmic reference value. Formation of the J-aggregates is associated with a Stoke's shift in emission from 527 nm for the monomer to 590 nm for the J-aggregate, which require comparison between different cellular compartments [26]. Calculation of the 527:590 nm fluorescence ratios eliminates many of the potential pitfalls of fluorescent dye measurements. Mitochondrial depolarization during drug treatment is determined by decrease in the red/green fluorescence intensity ratio. The ratio is associated with mitochondrial toxicity due to change in membrane potential irrespective of mitochondria size, shape and density in the sample. Because the ratio can be calculated on a pixel-for-pixel basis, it eliminates the possibility that changes in the volume of the emitter account for changes observed for one experimental condition relative to another.

Nanogel NG(PEG) did not affect the MMP in MCF-7 cells with respect to both time (data not shown) and concentration (Fig 6A). The cells were treated with 3 different concentrations of CTP-loaded NG(PEG) for 2 h with no signs of MMP depolarization under used conditions, but cell treatment with a mitochondrial uncoupler CCCP resulted in MMP depolarization as indicated by 80% decrease in the fluorescence ratio as compared to control cells.

In order to examine and differentiate the effect of activated NTP drug alone or encapsulated in Nanogel NG(PEG) compared to nucleoside analog AZT, cells were treated with equimolar drug concentrations for 2 and 6 h under the assay conditions. Cells treated with encapsulated NTP initially showed an increase in red/green fluorescence ratio as compared to control cells indicating MMP hyperpolarization (Fig. 6B), but at a longer treatment there was no abnormality

in MMP. These data clearly demonstrated that drug encapsulated in Nanogels has no mitochondrial toxicity. No MMP depolarization was also observed when cells were treated with AZT-TP alone, while cells treated with AZT showed statistically significant change in red/green fluorescence ratio. This effect was even more pronounced with an increase of treatment time to 6h (Fig. 6B). AZT treatment resulted in the MMP depolarization decrease by 25–40% compared to the control cells.

3.6. Antiviral effect of drug-Nanogel formulation

Ribavirin is well-known drug used for chemotherapy of many respiratory infections including influenza A virus [27]. Application of this drug is limited to children due to severe toxic side effects associated with adults' reproductive function. Unfortunately, ribavirin has a low selectivity index (SI). It was shown that major mechanisms of ribavirin action include interference with viral RNA capping, viral polymerases and accumulation of lethal mutations (error catastrophe) after incorporating the ribavirin 5'-triphosphate (RTP) into viral RNA.

Ribavirin 5'-triphosphate was chemically synthesized from non-protected nucleoside analog using a tris(imidazolyl)phosphate phosphorylated reagent in one-pot procedure. This compound was isolated with 55% yield using combination of reverse phase and anion exchange chromatography and analyzed as described earlier [15]. Encapsulation of RTP into Nanogel NG(PEGss) resulted in drug formulation with 20% of drug by weight.

The cytotoxicity and anti-influenza activity of RTP and RTP/Nanogel formulation were determined in the culture of Madin-Darby canine kidney (MDCK) cells using microplate-based cytotoxicity (MTT) assay [28]. Cytopathic effect of influenza A virus at various drug concentrations was determined by comparison of the formazan formation in infected (A_i) and non-infected (A) cells and expressed as a difference of color intensities $\Delta = (A - A_i)$ vs. drug concentration (Figure 7). Ribavirin and RTP alone demonstrated only moderate antiviral efficacy and the ratio between cytotoxic and inhibitory concentrations (selectivity index) was equal to 3–4. RTP/Nanogel formulation contained 10% (wt) of Ribavirin was less cytotoxic to MDCK cells, while antiviral drug concentration (EC_{90}) was reduced ca. 30-fold as compared to the drug alone. The therapeutic index was also ameliorated in more than 10 times, reaching a value of 38 for the studied RTP/Nanogel formulation.

4. Conclusions

Optimization of many therapies including NAs may largely profit from the development of efficient drug delivery carriers. Since principal mechanisms for activation of NAs are associated with intracellular phosphorylation by host or viral kinases, drug delivery of phosphorylated species will result in greater therapeutic efficacy. Previously, we suggested for this purpose an application of polycationic drug carriers, Nanogels, which were capable to form relatively stable polyionic complexes with nucleoside 5'-triphosphates [7]. Principal shortcomings of Nanogel carriers are related to their intrinsic toxicity associated with polycationic nature and low biodegradability of the carrier. While antiproliferative and cytotoxic applications of drug-Nanogel formulations evidently do not depend significantly on these properties, antiviral drug formulations have to display a lower toxicity in order to qualify for a competitive alternative to non-loaded antiviral drugs. The only way to enhance weak points of Nanogel is through modifying its structure.

Chemical engineering of Nanogels was performed earlier in neutral parts of the polymer network. Introduction of Pluronic® block copolymers allowed us to reduce particle size and enhance affinity of carriers to the cellular membrane [21]. The second component of Nanogel, a high molecular PEI, used initially for chemical synthesis of these carriers was clearly not an optimal choice and demonstrated high toxicity to many cell types. Recently, a low molecular

PEI and various reducible polycations were proposed as comparable by activity and low toxic alternative to the high molecular PEI [7]. In addition, drug release from Nanogels can be also ineffective because of carrier's slow degradation kinetics. Suggested in the paper novel biodegradable Nanogel carriers for encapsulation of NTP will be among the cationic drug delivery systems with the lowest known cytotoxicity [29,30].

And finally, NTP-loaded Nanogel formulations demonstrated significantly reduced cytotoxicity and, moreover, some of the observed toxic effects were reversible for the most part. Mechanism of drug release from NTP-loaded carriers includes association of PEI in the Nanogel structure with negatively charged components of the cellular membrane or cytosol [15]. According to this mechanism the carrier will definitely remain in neutralized and, therefore, low toxic form. Important implication of the NTP encapsulation in Nanogels was also a remarkable reduction in mitochondrial toxicity of nucleoside analogs as shown by preserving MMP in treated organelles. Earlier observed an enhancement of cytotoxicity for NTP-Nanogel formulations was attributed mostly to intracellular effect of the NTP chain terminators on nucleic acids replication. Evidently, the fast consumption of NTP in the cellular pool was major factor of its much lower activity against mitochondrial DNA. Reduced toxicity of modified Nanogels together with enhanced inhibition of viral replication provides an opportunity for design of novel targeted antivirals.

Currently, there is no effective treatment using NAs against respiratory viruses such as influenza. Application of one of few clinically used drugs, Ribavirin, is restricted for treatment of acute pulmonary infections among children due to severe side effects on reproductive function. This drug is relatively low effective and has a very small therapeutic index. We investigated here how encapsulation of Ribavirin 5'-triphosphate (RTP) in Nanogel can help to ameliorate the drug properties. Cytotoxicity of RTP-Nanogel formulation containing 20% of bound RTP was 1.5 mg/ml at used treatment protocol. While cytotoxic concentrations of free drugs, Ribavirin and RTP, were lower (0.5–0.75 mg/ml), their effective antiviral concentrations were relatively high, in the range of 0.12–0.25 mg/ml. Similar inhibitory effect for RTP-Nanogel formulation was observed, nevertheless, at much lower concentration of 0.04 mg/ml. So, a definite 10-fold increase of SI was observed and remarkable 30-fold decrease of effective drug concentration by Ribavirin content as well. Based on the result of these experiments, we are developing now optimal by other parameters drug-Nanogel formulations for aerosol delivery into the lung for treatment of viral infections.

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Fig 1.
 Nanogel structures with Pluronic-based network and regular PEI (A) and with biodegradable PEI-based network (B).

Fig 2. Profiles of the size-exclusion HPLC of biodegradable Nanogels NG(PEGss)(A) and NG (F68ss)(B) performed before (1) and after degradation in the presence of DTT (2). Average amount of NG left after degradation: $33 \pm 2\%$. (C) Degradation of regular Nanogel NG(PEG) during incubation in PBS at 25°C. Results are obtained from size-exclusion HPLC analysis.

Fig 3. Intracellular ATP levels of MCF-7 cells treated with CTP loaded and unloaded Nanogels for 2h. The data are means of three measurements $\pm$ SEM.

Fig 4. Restoration of initial intracellular ATP levels in human breast carcinoma MCF-7 cells after replacement of CTP loaded and unloaded Nanogel treatment solution with fresh media. The data are means of three measurements $\pm$ SEM.

Fig 5.

Cytotoxicity of CTP loaded and unloaded Nanogels in human breast carcinoma MCF-7 cells assessed by release of Lactate dehydrogenase (LDH). Cells were treated for 4h and data are means of five measurements $\pm$ SEM.

Fig 6.

Effect of various Nanogel formulations on mitochondrial membrane potential in breast carcinoma MCF-7 cell line, cells were treated with different concentrations of NG (PEG) for 2h (A); cells treated with NA, NTP and NTP encapsulated in NG(PEG) for 2, 6 and 24 hr (B). The data are means of three measurements $\pm$ SEM.

Fig 7.

Antiviral effect of Ribavirin drugs and RTP-Nanogel formulation in MDCK cells infected with influenza A virus. Cytopathic effect (Δ) of virus was initially determined (A) and dose-dependent curves obtained using 96-well microplate MTT assay (B). These curves were used to determine an effective inhibitory concentration (EC₉₀) and cytotoxicity (CC₉₀). Selectivity index (SI) was calculated as CC₉₀/EC₉₀.

Table 1

Properties of Nanogels with regular (NG) and biodegradable network (NGss)

Nanogel	Nitrogen content, $\mu\text{mol}/\text{mg}$ [*]	Nanogel diameter, nm	Diameter of NTP-loaded Nanogel, nm	Cytotoxicity, $(\text{CC}_{50}, \mu\text{g}/\text{ml})$ [†]
NG(PEG)	6.5	152 $\pm$ 6	84 $\pm$ 3	70
NG(PEGss)	6.1	100 $\pm$ 8	58 $\pm$ 2	250
NG(F127)	5.8	132 $\pm$ 4	69 $\pm$ 1	80
NG(F127ss)	5.5	187 $\pm$ 6	112 $\pm$ 3	190
NG(F68)	6.2	176 $\pm$ 4	74 $\pm$ 0.5	95
NG(F68ss)	6.4	120 $\pm$ 5	67 $\pm$ 2	250
PEI 25kDa	23	n/a	n/a	5
PEIss	21.4	n/a	n/a	36

^{*} All Nanogels have nearly the same PEI content, 25 $\pm$ 4% (wt), determined by elemental analysis.

[†] Based on MTT assay after 24h-incubation of ATP-loaded Nanogels with human breast carcinoma MCF-7 cells.