Pharmaceutical Research, Vol. 23, No. 5, May 2006 (©2006)
DOI: 10.1007/s11095-006-9788-5

Research Paper

Comparison of Nanogel Drug Carriers and their Formulations with Nucleoside 5′-Triphosphates

Serguei V. Vinogradov,[1,2] Ekta Kohli,[1] and Arin D. Zeman[1]

Received October 17, 2005; accepted December 22, 2005

Purpose. The aim of the study is to synthesize and characterize nanogel carriers composed of amphiphilic polymers and cationic polyethylenimine for encapsulation and delivery of cytotoxic nucleoside analogs 5′-triphosphates (NTPs) into cancer cells.
Methods. Nanogels were synthesized by a novel micellar approach and compared with carriers prepared by the emulsification/evaporation method. Complexes of nanogels with NTP were prepared; particle size and *in vitro* drug release were characterized. Resistance of the nanogel-encapsulated NTP to enzymatic hydrolysis was analyzed by ion-pair high-performance liquid chromatography. Binding to isolated cellular membranes, cellular accumulation and cytotoxicity were compared using breast carcinoma cell lines CL-66, MCF-7, and MDA-MB-231. *In vivo* biodistribution of the ^{3}H-labeled NTP encapsulated in different types of nanogels was evaluated in comparison to the injected NTP alone.
Results. Nanogels with a particle size of 100–300 nm in the unloaded form and less than 140 nm in the NTP-loaded form were prepared. An *in vitro* release of NTP was >50% during the first 24 h. Nanogel formulations ensured increased NTP drug stability against enzymatic hydrolysis as compared to the drug alone. Pluronic®-based nanogels NG(F68), NG(F127), NG(P85), and NGM(P123) demonstrated 2–2.5 times enhanced interaction with cellular membranes and association with various cancer cells compared to NG(PEG). Among them, NG(F68) and NG(F127) exhibited the lowest cytotoxicity. Injection of nanogel-formulated NTP significantly modulated the drug accumulation in different mouse organs.
Conclusions. Nanogels composed of Pluronic® F68 and P123 were shown to display certain advanced properties compared to NG(PEG) as a drug delivery system for NTP analogs. Formulations of nucleoside analogs in active NTP form with these nanogels will improve the delivery of these cytotoxic drugs to cancer cells and the therapeutic potential of this anticancer chemotherapy.

KEY WORDS: breast cancer cells; cellular association; drug release; nanogels; organ biodistribution; synthesis.

INTRODUCTION

A recent therapeutic strategy in anticancer treatment is the application of polymer-based nanosized drug delivery systems, such as micelles, biodegradable nanoparticles, liposomes, and microgels (1). These nanocarriers can reduce nonspecific systemic toxicity of antiproliferative drugs and allow a significant amount of drug to be delivered into targeted cancer cells. An important step in drug delivery is the interaction of drug carrier with targeted cells followed by drug release from the carrier. Rational design of the nanocarriers can considerably improve this process. Given the growing number of anticancer drugs, nucleoside analogs (NA) remain among the most effective therapeutic agents despite their toxicity and the multitude of drug resistance mechanisms (2). Nucleoside analogs act as antimetabolites that interfere with nucleic acid synthesis and can exert cytotoxic activity either by incorporating into DNA or RNA or by modifying the metabolism of physiological nucleosides. These agents are generally S-phase-specific and show an antiproliferative effect in actively dividing cancer cells. These compounds also possess unique drug-targeted cancer cell interactions that help explain their different spectra of activity. The biological activity of NA depends primarily on their ability to be converted by intracellular kinases into the corresponding 5′-mono-, di-, and triphosphates (3). Consequently, the cellular kinases' substrate specificity restricts the potential biological activity of NA. In addition, the long-term administration of nucleoside-based drugs can result in decreased kinase activity, thus reducing their efficacy, as seen in

[1] Center for Drug Delivery and Nanomedicine, and College of Pharmacy, University of Nebraska Medical Center, Omaha, Nebraska 68198, USA.
[2] To whom correspondence should be addressed. (e-mail: vinograd@unmc.edu)

ABBREVIATIONS: CMC, critical micellization concentration; CTP, cytidine 5′-triphosphate; FBS, fetal bovine serum; HLB, hydrophilic–lipophilic balance; MWCO, molecular weight cutoff; NA, nucleoside analogs; NMP, nucleoside 5′-monophosphate; NTP, nucleoside 5′-triphosphate; PEG, poly(ethylene glycol); PEI, polyethylenimine; PEO, poly(ethylene oxide); PPO, poly(propylene oxide).

0724-8741/06/0500-0920/0

azidothymidine (AZT), where reduced antiviral activity was a result of decreased activity of the prerequisite first phosphorylating enzyme (4,5). Recent progress in understanding NA drug uptake, metabolism, and interaction with cellular targets concludes that the phosphorylation steps are the key factors in their antiproliferative activity and, in turn, strongly depend on nucleoside structure and cellular type.

In principle, the problem of initial cellular phosphorylation of NA could be overcome by the use of nucleoside 5′-monophosphate (NMP) or their derivatives. Unfortunately, NMPs are not metabolically stable *in vivo*, and they are incapable of crossing cellular membranes because their molecules carry a negative charge at physiological pH. To address this challenge, several "pronucleotide" prodrug approaches have been devised. Amino acid phosphoramidates of NMP have shown promise as potential "pronucleotides" on the assumption that these derivatives will be taken up by tumor tissues and converted *in vivo* to the corresponding NMP (6). Cell extract studies have provided preliminary evidence of bioactivation, but this is still a gray area in research (7). The "pronucleotide" concept can resolve some of the problems associated with intracellular activation of nucleoside analogs; for example, some derivatives of the 5′-triphosphate of antiviral agent AZT showed higher activity and lower toxicity than AZT (8). However, the "pronucleotide" approach does not solve problems associated with intracellular delivery of phosphorylated nucleoside analogs. Cellular uptake of polyanionic phosphorylated NA is usually an ineffective process, making this step decisive for the final drug activity.

One of the ways to enhance drug uptake and bioavailability is an application of efficient drug delivery systems. Recent efforts were directed toward the development of delivery systems similar to liposomes, which can deliver NA or protect their prodrugs against rapid metabolic activation (9). As an example, encapsulation of a series of 4-(*N*)-acylated prodrugs of gemcitabine in liposomes can be mentioned (10). However, many small water-soluble molecules, such as ara-C, a NA related to gemcitabine, and 5-fluorouridine, diffused rapidly through the liposome bilayer, thus limiting the shelf life and clinical usefulness of these liposomal drug formulations (11,12). These findings strongly suggest the need to develop a new efficient drug delivery system for NA.

Recently, we proposed a novel type of formulation for cytotoxic NA in their active nucleoside 5′-triphosphate (NTPs) form based on a nanogel polymeric network composed of branched polyethylenimine (PEI) and polyethylene glycol (PEG)/Pluronic® molecules (13,14). NTPs not only cause termination of chain elongation by DNA polymerases but also induce S-phase-specific apoptosis (15). Administration of NTP as a therapeutic agent has several advantages over NA themselves, including bypass of the key cellular phosphorylation step, direct availability of the drug in active form for inhibition of DNA (RNA) polymerases in proliferating cancer cells, and reduced dosage and nonspecific toxicity. Major obstacles in administrating NTP systemically are drug instability and rapid degradation in the blood stream. Formulation of NTP with cationic polymer molecules of nanogels can significantly increase drug stability (13,16). The complex structure can differ by molecular architecture, as well as by lipophilic properties. The nanogel network binds NTP through ionic interactions forming compact particles, where the drug is protected by the polymer envelope from enzymatic activities and interaction with serum proteins. Moreover, NTP–nanogel complexes are neutral or positively charged, so their cellular accumulation can be significantly enhanced following the initial contact with the cellular membrane. An important property of nanogels is the low buoyant density of the drug formulations that enhances their dispersion stability (16). There are a number of advantages of drug–nanogel formulations over other delivery systems, such as polylactic-*co*-glycolic acid (PLGA) nanoparticles, liposomes, and polymer micelles. The drug–nanogel formulations are prepared by simple mixing of the aqueous solution of NTP and the dispersion of cationic nanogels, thus avoiding the complicated procedures of synthesis of nanoparticles in the presence of encapsulating drug or liposome preparation. The labile NTP cannot be formulated into PLGA nanoparticles by *in situ* polymerization. High drug loading in the drug–nanogel formulations can provide an increased local drug concentration following its delivery to a targeted site, resolving many of the problems associated with development of drug resistance to NA. The drug–nanogel formulations can be lyophilized and stored at room temperature, a major advantage for therapeutic applications. The polymer envelope surrounding the NTP complexed with PEI backbone of nanogels can greatly reduce exposure of the NTP to the degradative biological environment after systemic administration. Efficient accumulation in cancer cells or tumors may be achieved by vectorizing the nanogels with ligands specific to surface determinants on cancer cells. Importantly, the nanogel-encapsulated NTPs are able to cross cellular and endosomal membrane barriers because of enhanced interaction with the membrane itself, a property that can be modified by a proper design of nanogels and their polymer composition. Therefore, these nanogel carriers can be a structural basis in the development of drug delivery systems for chemotherapy using cytotoxic NA.

In the present study, we have synthesized and characterized a series of nanogel carriers composed of the cross-linked amphiphilic Pluronic® block copolymers and cationic branched PEI for the delivery of 5′-triphosphorylated NA into the cancer cells (Fig. 1). The individual Pluronic® consists of hydrophilic poly(oxyethylene) (PEO) and lipophilic poly(oxypropylene) (PPO) segments of various length and is characterized by a different hydrophilic–lipophilic balance (HLB). NTP–nanogel complexes were obtained, and their membranotropic properties were evaluated *in vitro*. The present study examines binding of nanogels with the cellular membrane and drug release from the carriers. These findings provide a rationale for quantitative analysis of structure-activity relationships and allow us to identify the prospective candidates for further development of the nanogel-based drug delivery systems.

MATERIALS AND METHODS

Materials

All solvents and reagents, except specially mentioned, were purchased from Sigma-Aldrich (St. Louis, MO, USA) at

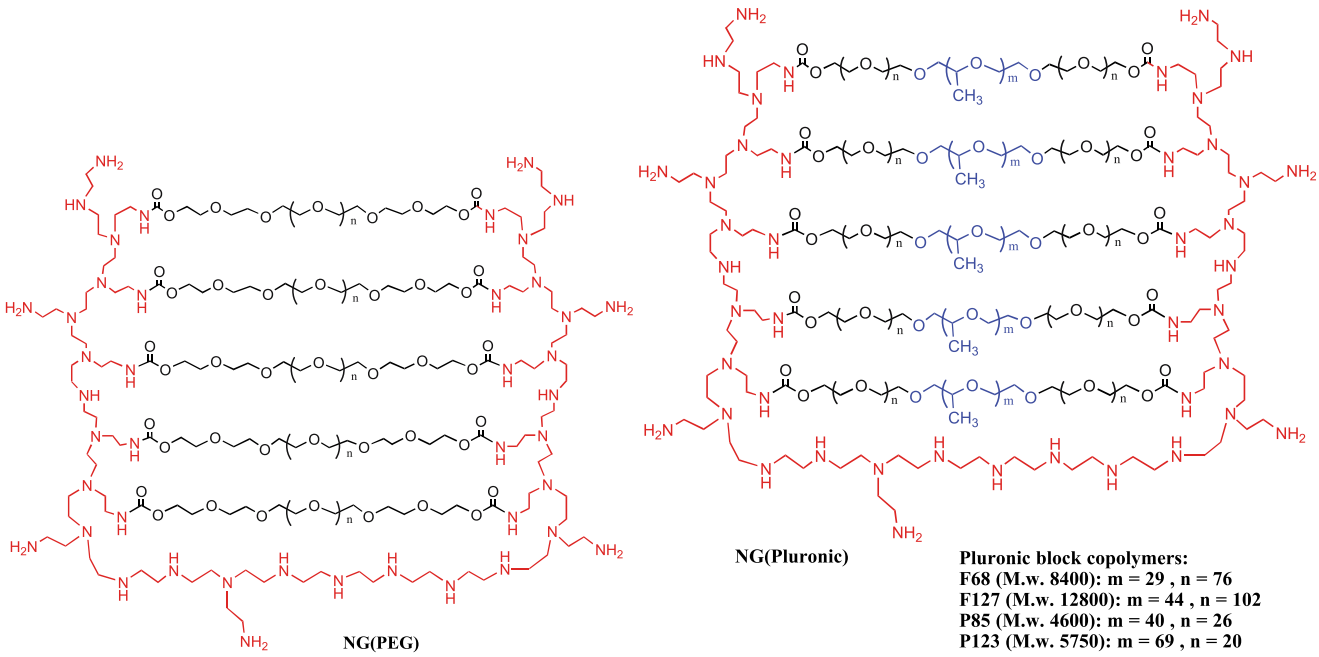

Fig. 1. Schematic presentation of nanogel structure built of (PEG)-*cl*-PEI (left) or Pluronic®-*cl*-PEI conjugates (right). PEG = poly(ethylene glycol); PEI = polyethylenimine.

the highest purity grade. Pluronic® block copolymers were a generous gift from BASF Corp. (Parsippany, NJ, USA). ^{3}H-Succinimidyl propionate, specific activity 40 Ci/mmol, was purchased from ARC, Inc. (St. Louis, MO, USA). BODIPY® FL ATP was purchased from Molecular Probes (Eugene, OR, USA). SpectraPor dialysis membrane tubes with various molecular weight cutoffs (MWCO) were from Fisher Scientific (Pittsburgh, PA, USA).

Synthesis of Nanogels

Both ends of PEG (MW 4.6 and 8 kDa) and Pluronic® (F127, F68, P85, or P123) were activated by reaction with 10-fold molar excess of 1,1′-carbonyldiimidazole in dry acetonitrile (40°C, 2 h). Activated products were purified by dialysis (MWCO 2 kDa) twice for 4 h against 10% ethanol at 4°C. These solutions were concentrated *in vacuo* and lyophilized. Commercial PEI (MW 25 kDa) was purified by gel-permeation chromatography using Sephacryl S200 column (2.5 × 60 cm) and water as an eluent, and a weight diagram was obtained for the content of all fractions. Fractions of PEI with intermediate MW (more than 50% of loaded PEI by weight) were pooled and directly used in nanogel synthesis. Synthesis of nanogels, NG(PEG), NG(F68), NG(F127), and NG(P85), was performed using the "emulsification–solvent evaporation" method as described previously (13,14).

Another micellar approach was used to synthesize nanogels NGM(F127), NGM(P85), and NGM(P123). In this method, aqueous 1% (w/v) PEI solution was added dropwise into an equal volume of aqueous 2% solution of the freshly activated Pluronic® under a vigorous stirring to form polymer micelles. The reaction of PEI with activated ends of polymer micelles was continued overnight at 25°C. Finally, the same volume of aqueous 4% solution of the activated PEG was added to the reaction mixture to cross-link the PEI envelope surrounding the polymer micelles. The stirring was continued for another 24 h at 25°C. The formed nanogel dispersions were purified by dialysis (MWCO 50 kDa) twice during 24 h against 10% ethanol containing 0.02% aqueous ammonia at 25°C and lyophilized.

For analysis of the polymer/PEI ratio in nanogels, 5% solutions were prepared in D_2O and filtered, and ^{1}H nuclear magnetic resonance (NMR) spectra (with integration) were registered at 25°C in the range of 0–6 ppm using the Varian 300-MHz spectrometer. Elemental analysis (M-H-W Laboratories, Phoenix, AZ, USA) was used to measure the total nitrogen content in nanogel preparations.

Rhodamine-Labeled Nanogels

Dispersion of nanogels (10% w/v) in 2 ml of 0.1 M sodium carbonate buffer, pH 9, was treated with 1% solution of rhodamine isothiocyanate (0.2 ml) in dimethylformamide (DMF) overnight at 25°C. The rhodamine-labeled nanogels were isolated by gel filtration using NAP-10 columns (GE-Amersham, Parsippany, NJ, USA) in 10% ethanol and were lyophilized. Fluorescent spectral characteristics of the rhodamine-labeled nanogels (λ_{ex} = 549 nm and λ_{em} = 577 nm) were measured in phosphate-buffered saline (PBS), pH 7.4, using a Shimadzu RF5000 spectrofluorimeter. Linear calibration curves for each nanogel were obtained in the range of 1–500 μg/ml.

Tritium-Labeled Nanogels

All ^{3}H-labeled nanogels used in the present study were obtained as described earlier (17). Briefly, 10% (w/v) dispersion of nanogels in 1 ml of anhydrous acetonitrile containing 2% (v/v) of triethylamine was treated with a solution of ^{3}H-succinimidyl propionate (100 μCi) in ethyl acetate

overnight at 25°C. Organic solvents were removed in the flow of nitrogen, and ^{3}H-labeled nanogels were isolated using NAP-10 column in 10% ethanol. Fractions containing nanogel were collected, analyzed by liquid scintillation counting, and lyophilized. Specific activity of the labeled nanogels was ca. 8 μCi/mg.

Nanogel–Drug Complexes

Nanogel–drug complexes were prepared using cytidine 5′-triphosphate (CTP) as a model nucleoside-based drug. Drug loading was performed by simple mixing of the solution of a sodium salt of CTP with different nanogel dispersions in phosphate buffer at pH 7.4 and the N/P ratio (total amino group concentration in nanogel solution to phosphate group concentration) equal to 3. Complexes were allowed to form for at least 30 min. Excess unbound CTP was removed by short dialysis (MWCO 2 kDa, 4 h, 4°C), and the obtained formulations were freeze-dried (See Supplementary *Formulations A* for this article at http://dx.doi.org/10.1007/s11095-006-9788-5 and is accessible for authorized users). In the second approach, nanogels were converted into a free amino form by overnight dialysis against 0.02% aqueous ammonia. The nanogel dispersion was concentrated *in vacuo*, filtered, and redissolved in water. Sodium salt of CTP was dissolved in water and passed through a short column with Dowex 50 × 6 in H^+ form to obtain a free acid form of CTP. The column was then washed by water (2 × 15 ml), and the eluate was used directly for titration of nanogel dispersions until pH 7.4. The obtained dispersions of the CTP-loaded nanogels (*Formulations B*) were freeze-dried. CTP content in the dispersions was then determined spectrophotometrically at 260 nm.

Electron Microscopy

Electron-contrasted nanogels were prepared from aqueous dispersions (5 mg/ml) mixed with 1 mM cupric nitrate in 10 mM Tris buffer, pH 7.4, at the molar Cu^{2+}/PEI ratio 10, which is equivalent to about 10% of PEI saturation by Cu^{2+} cations. Aqueous CTP solution (0.1 M; 5 μl per 1 mg of nanogel) was added 30 min later, and the mixture was incubated for 1 h at 25°C to obtain nanogel–CTP complexes. Low-molecular weight components were removed during dialysis (MWCO 2000 Da) against water for 4 h, and complexes were freeze-dried. Samples were resuspended by sonication in water (concentration 1%) prior the transmission electron microscopic (TEM) analysis, placed on the grid, and directly investigated using a Philips 410LS TEM equipped with an AMT digital imaging system [University of Nebraska Medical Center (UNMC) Electron Microscopy Core].

Cell Culturing

Human breast carcinoma MCF-7 and MDA-MD-231 cells were obtained from the ATCC (Rockville, MD, USA), and murine breast carcinoma CL-66 cell line was kindly provided by Dr. Rakesh Singh (UNMC). Cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with nonessential amino acids, 2 mM of L-glutamine, 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml). All culture media were obtained from Gibco (Fisher Scientific). Cancer cells were seeded at a density of 10,000 cells per well in 96-well plates or 50,000 cells per well in 24 wells or Bioptech plate for confocal microscopy and were allowed to grow overnight at 37°C and 5% CO_2 for reattachment.

Interaction of Nanogels with Isolated Cellular Membranes

Crude cellular membranes were isolated according to the procedure of Hamada *et al.* with some modifications (18). Briefly, approximately 2×10^8 harvested MCF-7 cells were washed with ice-cold PBS, and the cell pellet was resuspended in hypotonic lysis buffer (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 1.5 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride; 1×10^7 cells in 1 ml). The swollen cells were disrupted with 20 strokes in a tightly fitting homogenizer, and nuclei were removed by centrifugation at 1200 × *g* for 10 min at 4°C. The nuclei-free supernatant was centrifuged at 110,000 × *g* for 1 h at 4°C, and the pellet was used as a crude membrane fraction. The membrane fraction was suspended in a hypotonic lysis buffer containing 50% (w/v) glycerol and stored at −80°C. The total protein content in the membranes was determined using the Pierce bicinchoninic acid (BCA) protein assay.

Binding of ^{3}H-labeled nanogels to isolated cellular membranes was performed as described earlier (13). Briefly, isolated membrane aliquots (1 mg/ml of total protein) were treated with 70 μg of the ^{3}H-labeled nanogels and incubated at 37°C for different time intervals (0.5, 1, 2, and 4 h). Membranes were separated from nanogels in the samples by centrifugation at 12,000 rpm. The membrane pellet was dissolved in 500 μl PBS, and 10 μl of solution was used for the radioactive counting. All measurements were performed in triplicate.

In Vitro Drug Release

Nanogel–CTP complexes (*Formulations A*) were divided into two equal parts with cellular membranes (1 mg/ml protein) added to one part. The mixtures were placed into dialysis tubes (MWCO 2 kDa) and dialyzed against PBS (pH 7.4) at 25°C. At each time point, 50-μl samples were removed, diluted 20-fold, and their ultraviolet (UV) absorbance was determined at 260 nm.

Enzymatic Hydrolysis

Dispersions (0.1%, w/v) of nanogel–CTP complexes (*Formulations B*) were prepared in the Ringer's solution, pH 8.2. Ninety-five microliters of each solution was incubated with or without 5 μl (2 units) of alkaline phosphatase at 37°C for 10 min. Samples were analyzed by the ion-pair high-performance liquid chromatography (HPLC) using Vydac C18 column (5 μm, 0.46 × 15 cm) at a flow rate of 1 ml/min. Elution with buffer A (40 mM KH_2PO_4, 0.2% tetrabutylammonium hydroxide, pH 7.0) and buffer B (30% acetonitrile, 40 mM KH_2PO_4, 0.2% tetrabutylammonium hydroxide, pH

7.0) in gradient mode (100% of the buffer B in 20 min) was used in this analysis.

Cellular Accumulation

Cellular accumulation of rhodamine-labeled nanogel was examined in breast carcinoma cell lines as described earlier (19). Cells were treated with rhodamine-labeled nanogels (0.01 mg/ml) in the serum-free assay buffer for 2 h at 37°C. After treatment, cells were washed with ice-cold PBS containing 1% BSA and then solubilized in 1% Triton X-100 solution. Fluorescence associated with the cells was measured at λ_{ex} = 549 nm and λ_{em} = 577 nm, and obtained values were normalized by protein content (Pierce BCA protein assay) according to the manufacturer's protocol. All experiments were repeated in triplicate.

Cytotoxicity Analysis

Cytotoxicity of CTP-loaded nanogels was determined using the standard MTT assay (20). Briefly, serial dilutions of nanogel–CTP complexes in the assay buffer [122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.2 mM $Mg(SO_4)_2$, 1.4 mM $CaCl_2$, 0.4 mM K_2HPO_4, 10 mM glucose, and 10 mM HEPES] were incubated with the cells for 24 h at 37°C. Samples were washed with 100 μl of assay buffer and grown for 3 days in a full culture medium changed daily. Then, 25 μl of MTT solution (5 mg/ml) was added, and cells were incubated for 2 h at 37°C in the dark. The medium and indicator dye were washed out with PBS, and cells were lysed with 20% sodium dodecyl sulfate in 50% aqueous DMF (100 μl) overnight at 37°C. Absorbance was measured at 550 nm, and cell viability was expressed as a percentage of the initial absorbance values obtained for control nontreated cells. The IC_{50} values were calculated for six to eight parallels using the GraphPad Prism software.

Tissue Biodistribution Study

In animal study described below, cage size and animal care conformed to the Institutional Animal Care and Use Committee guidelines. Female Balb/c mice (Charles River Laboratories, Wilmington, MA, USA), 6–8 weeks of age, weight 20–23 g, were assigned to one of five groups (five animals per cage per group). Animals of the first group received single doses of 2 μCi of ^{3}H-CTP and 0.2 mg of cold CTP (Sigma-Aldrich) in 100 μl of HBS intravenously via tail vein. Other groups received injections of 1 mg of nanogel formulations containing 2 μCi of ^{3}H-CTP and 0.2 mg of cold CTP in 100 μl of HBS. Dispersion of nanogels in HBS was initially mixed with ^{3}H-CTP, incubated for 30 min on ice, then mixed with cold CTP solution, and again incubated for 30 min. This solution was spun for 1 min at 12,000 × *g* and directly used for injections. Samples of plasma and selected tissues were collected 90 min postdose. The following tissues were collected: liver, lung, kidney, spleen, and brain, weighed and homogenized in tissue solubilizer (0.5–0.7 ml) prior to analysis. Homogenized tissue or plasma samples (100 μl) were placed in scintillation cocktail, and radioactive counts were measured. Drug accumulation in tissues (%ID/g) was calculated as mean ± SEM.

RESULTS

Synthesis of Nanogels

For preparation of nanogels, we used two synthetic approaches. The first was described previously and consisted in the conjugation of polymers in heterogeneous phase of the dichloromethane-in-water emulsion (16). To simplify the whole synthetic procedure and reduce utilization of the environmentally hazardous organic solvent, we proposed a novel approach to preparation of nanogels. This second approach is based on the micelle-forming property of amphiphilic block copolymers Pluronic® (Poloxamer) having a PEO–PPO–PEO structure. Many of these block copolymers are able to form polymeric micelles at concentrations as low as 0.01 mg/ml. The synthetic procedure consisted of three simple steps: (1) activation of both ends of Pluronic® molecules and dispersion in water at a concentration higher than the critical micellization concentration of this type of Pluronic®; (2) interaction of the polymeric micelles with PEI; and (3) cross-linking and modification of the Pluronic®–PEI micelles with activated PEG molecules as shown in Fig. 2. The whole synthesis is a one-pot procedure performed by sequential addition of aqueous solutions of nanogel components. We have obtained three types of nanogels using the micellar approach, NGM(F127), NGM(P85), and NGM(P123), and their properties, together with properties of nanogels obtained by the emulsification/evaporation method, e.g., NG(PEG), NG(F68), NG(F127), NG(P85), and NG(P123), are all listed in Table I. The average obtained polymer-to-PEI weight ratio was about 7 with initial Pluronic®-to-PEI ratio equal to 2. The crude nanogels were purified by gel permeation chromatography using Sephacryl S-200 to remove very large particles, small molecular

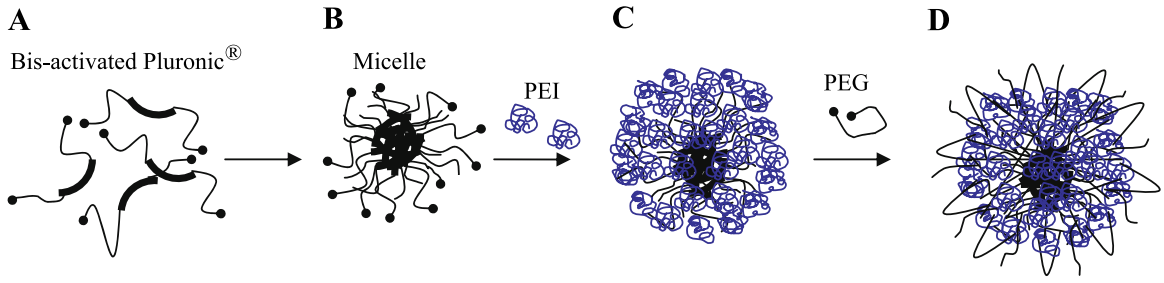

Fig. 2. Nanogel synthesis using the micellar approach. Activated Pluronic® block copolymers (A) formed micelles (B) in aqueous solutions, which could be covered with a layer of PEI (C) cross-linked by activated PEG molecules (D).

Table I. Composition and Particle Size of Nanogels

Nanogel	Total N (μmol/mg)[a]	Weight ratio polymer/PEI[a]	Hydrodynamic diameter (nm)	
			Unloaded[b]	CTP-loaded[c]
NG(PEG)	3.5	5.6	152 ± 6	84 ± 3
NG(F127)	2.3	9.1	113 ± 0	70 ± 2
NG(F68)	2.7	7.6	176 ± 4	74 ± 0.5
NG(P85)	2.6	7.9	297 ± 4	120 ± 1
NGM(F127)	3.0	6.6	132 ± 4	69 ± 1
NGM(P85)	2.2	9.5	270 ± 3	144 ± 4
NGM(P123)	2.7	7.6	102 ± 1	69 ± 0.5

CTP = cytidine 5′-triphosphate; PEG = poly(ethylene glycol).
[a] Data obtained by elemental analysis. Weight ratio was calculated based on the total nitrogen (N) content for PEI: 23.2 μmol/mg (theor.).
[b] Nanogels in phosphate-buffered saline (PBS), at 1 mg/ml.

conjugates, and initial polymer components. The obtained white solid lyophilized material was readily swollen and dispersed in water forming transparent particles with hydrodynamic diameters between 100 and 300 nm. Mass recovery was usually higher than 60–70%.

Complexes with Nucleoside 5′-Triphosphate

Nanogel solutions were directly mixed with solution of nucleoside 5′-triphosphate (CTP); fast formation of ionic complexes between PEI backbone of nanogels and CTP occurred spontaneously, which was demonstrated by a significant 2- to 2.5-fold reduction of the observed hydrodynamic diameter of nanogels (Table I). The size of unloaded nanogel usually showed no visible dependence on the nature of polymer used for the synthesis and was in the range of 100–300 nm. The size of nanogels obtained by micellar approach also fell in this range. By contrast, CTP-loaded nanogels displayed diameters mostly lower than 100 nm, and only the Pluronic® P85-based nanogel showed a larger diameter in the range of 120–140 nm. These complexes lyophilized after a short dialysis and could be then stored in dry form at 4°C for many months without any signs of significant degradation of nucleoside 5′-triphosphate (data not shown).

TEM demonstrated the formation of spherical particles following the CTP binding with Cu^{2+}-stained nanogels and compaction of CTP–PEI complexes into the structures with a dense core (Fig. 3). The only difference observed for nanogels composed of the lipophilic Pluronic® P123 and synthesized using micellar approach was their distinct morphology characterized by a higher peripheral electron density. Evidently, hydrophilic PEG chains, which are not stained by Cu^{2+} cations, surround the compact core of CTP–PEI complexes, stabilize their aqueous dispersion, and shield them from interactions with macromolecules. However, lipophilic segments of Pluronic®-based nanogels evidently become more efficiently exposed following formation of a dense core to enhance the efficacy of nanogel interactions with cellular membranes.

Protection of the encapsulated CTP against enzymatic degradation was studied using ion-pair HPLC analysis of nucleotides. Complexes of CTP with nanogels immediately dissociated in the presence of ion-pairing tetra(*n*-butyl)ammonium cations following the injection of nanogel formulations in the column, where nucleoside and nucleotides could be separated as discrete peaks. Nanogel-formulated CTP showed an enhanced resistance to hydrolysis by the intestinal alkaline phosphatase. Free CTP was digested more than three times as much as encapsulated CTP in the enzyme assay (Table II). The total balance of other nucleotides was also improved in the case of encapsulated CTP; this is an additional advantage because any type of phosphorylated NA can be active during the treatment of cancer cells.

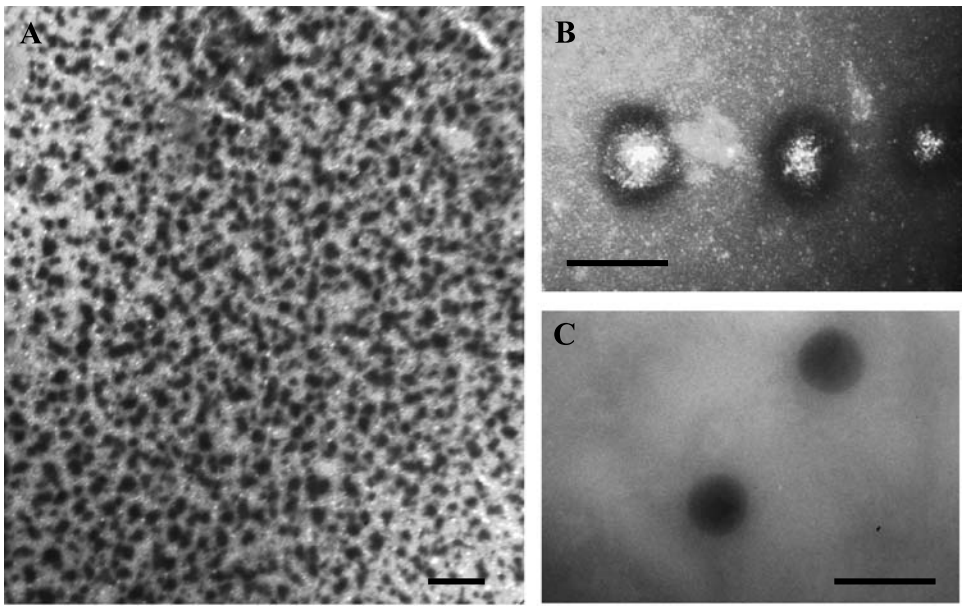

Fig. 3. Transmission electron microscopy of CTP-loaded nanogels contrasted by Cu^{2+} cations: NG(F127) (A, bar = 500 nm), NGM(P123) (B, bar = 100 nm), and NG(F68) (C, bar = 100 nm). CTP = cytidine 5′-triphosphate.

Table II. Protection of CTP Formulations with Nanogels[a]

Nanogel	Initial complex	Phosphatase-treated
NG(PEG)	93.5	38.0 ± 2.5
NG(F127)	93.0	26.6 ± 1.9
NG(F68)	92.0	28.5 ± 1.4
NG(P85)	88.7	34.5 ± 0.7
NGM(P123)	91.7	29.0 ± 1.1
CTP without NG	92.1	10.1 ± 0.6

[a] Based on ion-pair high-performance liquid chromatography (HPLC) data. The CTP peak areas are presented as a percentile of total integrated HPLC areas (n = 3, SEM).

Nucleoside 5′-triphosphates are small molecules that have three negative charges at physiological conditions. We studied the *in vitro* drug release of CTP from the complex with nanogels in PBS, pH 7.4, using an equilibrium dialysis approach at 25°C. Released CTP and products of its hydrolysis freely diffused from the incubated cell through the dialysis membrane in the equilibrating buffer, whereas nanogel-complexed drug remained inside. Change in the quantity of nanogel-loaded drug was measured by UV absorbance directly from the cell at different time points. We observed an initial phase of relatively fast drug release during the first 24 h, when about 50–70% of the drug was released from the nanogel. During the slow second phase, from 24 to 48 h, the drug release was five times slower than the initial phase. NMR spectra of nanogels demonstrated that the PEI backbone of nanogels was composed of primary, secondary, and tertiary amino groups with molar ratio 1:2:1, and only primary (pK_a 9.2) and secondary (pK_a 6.5) amines could be protonated at pH 7.4 (14). Evidently, binding of these amino groups with the phosphate groups of CTP is different; for example, the binding of phosphate group with primary amino groups may be stronger than with secondary amino groups, which means that one third of the encapsulated drug could be bound more strongly to the nanogel backbone as compared to the remaining two thirds of the drug. This might partly explain the partial stability of the encapsulated CTP to enzymatic hydrolysis (Table II).

Affinity to Cellular Membranes

The lipophilic properties of Pluronic® block copolymers primarily depend on the length of poly(oxypropylene) block. In this paper, we explored how nanogels composed of different Pluronic® molecules and synthesized by two different procedures bind cellular membranes of cancer cells. To evaluate separately the degree of binding with cellular membrane and internalization of nanogels, we first studied the interaction of nanogels with isolated cellular membranes. For binding studies, cellular membranes were isolated from MCF-7 cells and incubated with ^{3}H-labeled nanogels at 37°C for different time periods. Membrane-bound nanogel was precipitated by centrifugation, and radioactivity associated with the membrane was counted. Binding of nanogels was expressed as μg/mg of membrane protein (Fig. 4). The binding curves showed saturation after about 2 h of incubation, and binding efficiency of different nanogels increased in the following order: NG(PEG) < NG(F127) < NG(P85) < NG(F68) = NGM (P123). Mostly, this trend reflects increasing length of the PPO block in corresponding Pluronic®-based nanogels. Initial phase of binding (0–0.5 h) was fast and mostly independent of the polymer composition of nanogels.

Cellular Accumulation

In this part, we compared cellular accumulation of rhodamine-labeled nanogels in various types of breast carcinoma cells, mouse CL-66 and human MCF-7, and MDA-MB231 lines. Cells were treated with nontoxic concentrations of rhodamine-labeled nanogels (0.005–0.01 mg/ml) for 2 h and were then lysed. The cell-associated fluorescence was measured and normalized by the protein content in each sample. Nanogel association with cells was highest for NG(F68), NG(P85), and NGM(P123), intermediate for NG(F127) and NGM(P85), and lowest for NG(PEG) and NGM(F127) (Fig. 4). MDA-MB-231 and CL-66 demonstrated lower levels of nanogel association compared to MCF-7 cells, but the observed general trend was the same. Cellular association of nanogels is an integral process that includes binding with cellular membrane, internalization, and accumulation in the cytosol. The percentage of associated nanogels that internalized can be calculated by subtraction of the nanogel amount bound to the isolated cellular membranes at the same time points. This internalization was higher for nanogels composed of Pluronic® with lower HLB, e.g., 16–24 μg/mg for NGM(P123), NG(P85), and NG(F68) and 10–15 μg/mg for NG(PEG) and NG(F127), and constituted about 50% of the total quantity of cell-associated nanogels following 2-h incubation. The difference between membrane-bound and internalized parts of nanogels was even higher at time point 4 h. In general, these data provide evidence of an

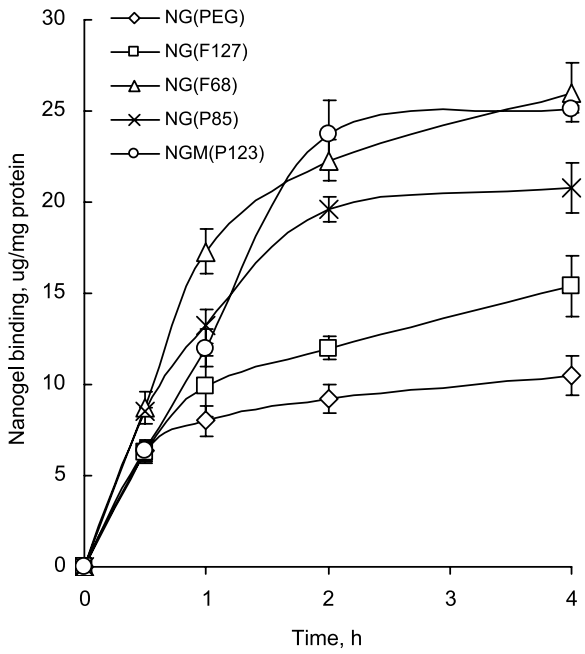

Fig. 4. Binding of nanogels to MCF-7 isolated cellular membranes. The same amounts of cellular membranes (1 mg/ml protein) and ^{3}H-labeled nanogels were used in each experiment. The data are the means of three measurements ± SEM.

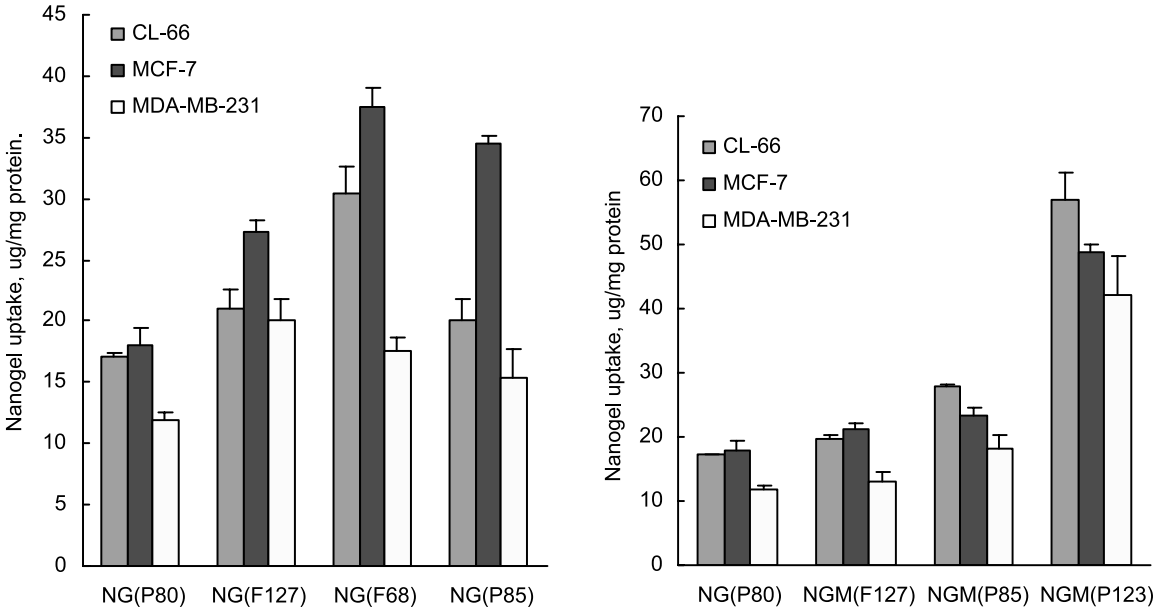

Fig. 5. Cellular uptake of rhodamine-labeled nanogels in different breast carcinoma cell lines following 2-h incubation with CTP-loaded nanogel formulations (0.01 mg/ml). The data are means of three measurements ± SEM.

efficient drug release from the studied drug–nanogel formulations following binding of nanogels with cellular membranes and internalization into cancer cells (Fig. 5).

Cytotoxicity

Application of nanogels for delivery of cytotoxic NTP makes nanogel cytotoxicity an additional factor in increasing the efficacy of drug–nanogel formulations. However, nonspecific cytotoxicity of nanogels caused by accumulation in various tissues and organs remains an important question. In general, drug carriers with lower levels of intrinsic cytotoxicity are regarded as more advantageous. Therefore, in this study, we compared cytotoxicity of various nanogels loaded with nontoxic CTP as a model drug in two human breast carcinoma cell lines, MCF-7 and MDA-MB-231 (Table III). Cytotoxicity of nanogels was significantly lower than cytotoxicity of PEI itself. Hydrophilic Pluronic®-based nanogels, such as NG(F127), NG(F68), and NGM(F127), were two to three times less cytotoxic compared to lipophilic Pluronic®-based nanogels. Also, nanogels obtained by micellar approach demonstrated a comparable cytotoxicity with nanogels obtained by standard method. The metastatic MDA-MB-231 cell line was more sensitive to the nanogel treatment, and observed IC_{50} values were about 6–20 times lower compared to MCF-7 cell line. These results suggest that application of drug–nanogel formulations may be especially effective in the treatment of advanced metastatic types of breast cancer.

Table III. Cytotoxicity of the CTP-Loaded Nanogels in Human Breast Carcinoma Cells[a]

Nanogel	MCF-7	MDA-MB-231
NG(PEG)	0.26	0.04
NG(F127)	0.06	0.003
NG(F68)	0.08	0.005
NG(P85)	0.03	0.002
NGM(P123)	0.03	0.002
NGM(P85)	0.025	0.003
NGM(F127)	0.08	0.009
PEI(25 kDa)	0.002	0.0001

[a] IC_{50} values (mg/ml) at 24-h treatment.

Mouse Tissue Biodistribution

Injected doses of drug-loaded nanogels (50 mg/kg) were well tolerated by animals. Obtained data on the biodistribution of drug (^{3}H-CTP) alone and drug formulations with nanogels NG(PEG), NG(F68), NG(F127), and NGM(P123) are shown in Fig. 6. Evidently, ^{3}H-CTP is rapidly degraded in circulation to the ^{3}H-cytidine, and accumulation data for the free injected drug are related to this product. Pyrimidine nucleoside transporters are widely distributed in the body. The drug was quickly removed from circulation and mainly distributed in liver, spleen, and kidney. We observed 50–90% increase in %ID/g in serum only for two nanogels: NG(F127) and NGM(P123). Drug retention in the first passing organ (liver) was slightly higher for more lipophilic Pluronic®-based nanogels. Reticuloendothelial system macrophages and Kupfer cells in the liver effectively consume large particles, whereas relatively small loaded nanogels demonstrate low retention. High accumulation levels for nanogel NGM(P123) were observed in the spleen, kidney, and brain. Spleen passes large volumes of blood and effectively removes hydrophilic particles from circulation. Transport to the spleen was found to be significantly increased for NGM(P123), the most lipophilic of studied nanogels. The similar trend was observed in the lung and kidney that are also known as efficient accumulators of cationic polymers. NG(PEG)

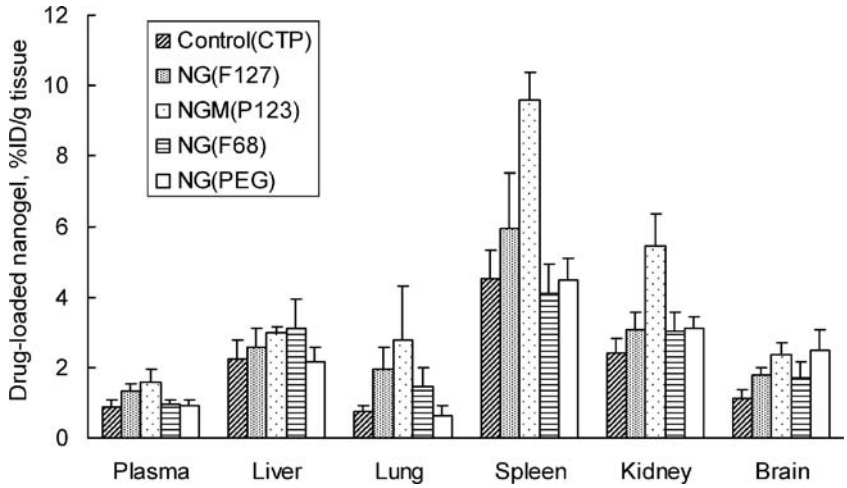

Fig. 6. Tissue distribution of ^{3}H-CTP in mice 90 min following injection of free drug and drug formulations with nanogels NG(PEG), NG(F68), NG(F127), and NGM(P123). Data were given as mean ± SEM for $n = 5$.

demonstrated low levels of retention in all these organs. Brain accumulation was 50–120% higher for all nanogels compared to the free drug, with NG(PEG) and NGM(P123) as the most effective carriers.

DISCUSSION

Our data demonstrate that hydrophilic polymers of Pluronic®/Poloxamer type can be used for the preparation of PEI-based nanogels, which substantially enhances the cell-binding affinity of these drug carriers. Many PEI-based delivery systems were able to induce efficient escape of the encapsulated polynucleotide drugs from endosomes into the cytosol. We showed previously the ability of nanogel to deliver NTP into the cells in an intact form (13). An anticipated mode of drug release from nanogel formulations encapsulating nucleoside analogs in 5′-triphosphate form includes interaction of nanogels with cellular membranes before or after endocytosis. The polymer properties can significantly influence binding properties of polymeric drug carriers. Optimization of the polymeric composition of nanogels to increase cellular membrane affinity and cytosolic delivery of NTP was the major focus of this study.

Clearly, the PEI part of nanogels should remain unchanged to maintain the NTP binding capacity and important buffering property of nanogel carriers. Recently, we studied and compared conjugates of short PEI molecules with different Pluronic® block copolymers for drug formulation and intracellular/transcellular delivery of antisense oligonucleotides (17). A significant dependence on Pluronic® type and HLB was observed in cellular association and transcellular transport of these conjugated carriers. Hydrophobic Pluronic® P123 was able to significantly enhance cellular association of oligonucleotides with cancer cells, whereas Pluronic® P85 with an intermediate HLB was shown to be more efficient in transcellular transport. In the development of systemic drug delivery systems, it is important for the carrier to have a minimal interaction with serum proteins. PEGylation of the surface of drug carriers has been shown to be the most successful way to minimize this interaction. In this paper, we compared properties of nanogels composed of Pluronic® and PEG in physicochemical studies, in *in vitro* studies using breast carcinoma cell lines, and in *in vivo* experiments. There should be an optimal HLB balance between lipophilic PPO blocks that are responsible for interaction with cellular membrane and hydrophilic PEO blocks that shield the polymer structure from serum proteins. We demonstrated in these experiments how the polymer structure of nanogels influences the membrane binding, internalization, drug release *in vitro*, and tissue biodistribution *in vivo*. Based on these data, some promising nanogel candidates for delivery of NTP among nanocarriers composed of large hydrophilic Pluronic®/Poloxamers were identified.

Successful candidates for delivery of anticancer cytotoxic drugs should comply with the following requirements: (1) low nonspecific toxicity of the drug carrier at therapeutic doses; (2) efficient association and internalization into cancer cells; and (3) fast drug release from carrier to ensure efficient killing of cancer cells and prevention of drug resistance. Nanogels composed of PEG and PEI showed drastically lower cytotoxicity compared with the corresponding PEI. The polymer-to-PEI weight ratio was mainly responsible for cytotoxicity of nanogels (14). At ratio higher than 5, nanogels exhibit the lowest cytotoxicity. We prepared a set of novel nanogels with the polymer-to-PEI ratio higher than 5. These two studied nanogels, NG(F127) and NG(F68), demonstrated the lowest cytotoxicity in breast carcinoma cells. (Note: Pluronic® F127 also carries the name of Poloxamer 407 and Pluronic® F68—Poloxamer 188, approved by FDA for *in vivo* applications.) Nanogels composed of more lipophilic nanogels, such as NG(P85) and NGM(P123), were more cytotoxic. All studied nanogels demonstrated practically identical *in vitro* drug release profiles and protection of encapsulated NTP against enzymatic degradation. Nevertheless, these nanogels demonstrated completely different membranotropic properties. Even the most hydrophilic Pluronic®-based nanogels bound isolated cellular membranes more efficiently than PEG-based nano-

gels. We also observed a similar trend in experiments with proliferating breast carcinoma cells. It was found that nanogels NG(F68), NG(F127), NG(P85), and NGM(P123) associated with cancer cells much better than NG(PEG). Based on the properties of these nanogels, we believe that NG(F68) and NGM(P123) can be considered the best candidates for drug delivery of NTP analogs. NG(F127) and NG(P85) could be considered as secondary carrier candidates.

Our data on the intracellular release of encapsulated drug also suggest that these candidate nanogel carriers are capable of efficient NTP drug release in the cytosol. This is a very important property because these carriers are evidently capable of releasing part of the encapsulated drug following their interactions with cellular membranes. Triggered drug release is a feature of advanced drug delivery systems and usually occurs in response to temperature change, ultrasound, irradiation, or enzymatic activity (21–24). We earlier postulated the existence of enhanced drug release from nanogels because of the interaction with cellular membranes, and recently, this point of view received experimental confirmation (13). The actual drug release mechanism will ultimately depend on the interaction of nanogels' PEI backbone with membrane phospholipids. Therefore, membranotropic properties of neutral polymers in the nanogel structure will provide additional benefits in the form of fast drug release in the cytosol. This ability to release the encapsulated drug following the interaction of a drug carrier with targeted cells remains the most important factor in choosing an optimal drug carrier. Our studies will help define optimal drug carrier formulations.

Comparison of drug tissue biodistribution following an i.v. injection of some selected drug-loaded nanogels demonstrated a higher affinity of Pluronic®-based nanogels to many organs compared to the drug alone or PEG-based nanogel. However, except for NGM(P123), there was no significant asymmetry in the biodistribution of these carriers. Based on these results, two nanogels, NG(F127) and NG(F68), can be selected as the most promising candidates for development of novel vectorized nanocarriers for systemic administration of anticancer/antiviral nucleoside analogs. Postsynthetic modification of nanogels with peptide ligands connected through a PEG linker will provide a specific targeting and additional surface shielding, making better their circulation and biodistribution properties.

An important feature of Pluronic® as a polymer for synthesis of nanogels is its strong mucoadhesive property (25–28). Mucoadhesive polymers have a number of advantages compared to other biocompatible polymers. Mucoadhesion increases the contact between drug-loaded nanogels and the mucous surface of many cell types and can significantly enhance the cell permeability of drug. Application of drug delivery systems composed of mucoadhesive polymers resulted in prolonged residence time and protection of the encapsulated active compounds from enzymatic degradation. This is especially important for drug delivery to epithelial cells in respiratory and digestive tracts. We believe that this property of nanogels can be useful for the development of more efficient formulations against cancers related to these organs.

Rational design of systemic drug delivery systems, where the polymer architecture is only the first step, may include also surface modifications and proper vectorization of these carriers with targeting ligands. The present study may be particularly useful in the determination of nanogel candidates for subsequent vectorization of these carriers with cancer-specific peptide ligands and application to *in vivo* studies. Investigation of the vectorized nanogels and their biodistribution in cancer models will follow.

CONCLUSIONS

A novel approach for nanogel synthesis is proposed based on the micelle-forming properties of Pluronic® molecules. The size of unloaded nanogels was in the range of 100–300 nm, showing no strong dependency on the nature of polymer and approach used for the synthesis. Nucleotide drug-loaded nanogels displayed diameters around 100 nm; only the Pluronic® P85-based nanogel showed diameters in the range of 120–140 nm. Nanogel-formulated CTP showed an enhanced resistance to hydrolysis by the intestinal alkaline phosphatase. *In vitro* drug release from nanogel formulations was also similar among various nanogels. The membrane-binding properties of different nanogels increased in the following order: NG(PEG) < NG(F127) < NG(P85) < NG(F68) = NGM(P123). Nanogel association with breast carcinoma cell lines was also the highest with NG(F68), NG(P85), and NGM(P123); NG(F127) and NGM(P85) showed intermediate association levels, and NG(PEG) and NGM(F127) displayed the least efficient binding. Hydrophilic Pluronic®-based nanogels, such as NG(F127), NG(F68), and NGM(F127), were several times less cytotoxic compared to lipophilic Pluronic®-based nanogels. Therefore, nanogels composed of hydrophilic Poloxamers (Pluronic® F68 and F127) and displaying advanced properties compared to NG(PEG) may be considered as prospective candidates for encapsulation of therapeutic nucleoside analogs. Drug delivery systems based on Poloxamer nanogels for formulation of active NTP form of nucleoside analogs can be developed into novel therapeutic antiviral or anticancer drugs and approaches to targeted chemotherapy.

AKNOWLEDGMENTS

This work was financially supported by NIH R01 grant CA102791 (S.V.V.). The authors are extremely grateful to Drs. Elena Batrakova and William Chaney for helpful discussion and Michael Jacobsen for valuable assistance in the preparation of the manuscript.

REFERENCES

1. R. Duncan. The dawning era of polymer therapeutics. *Nat. Rev., Drug Discov.* **2**:347–360 (2003).
2. C. M. Galmarini, J. R. Mackey, and C. Dumontet. Nucleoside analogues and nucleobases in cancer treatment. *Lancet Oncol.* **3**:415–424 (2002).
3. J. Balzarini. Metabolism and mechanism of antiretroviral action of purine and pyrimidine derivatives. *Pharm. World Sci.* **16**:113–126 (1994).
4. G. Antonelli, O. Turriziani, A. Verri, P. Narciso, F. Ferri, G. D'Offizi, and F. Dianzani. Long-term exposure to zidovudine

affects *in vitro* and *in vivo* the efficiency of phosphorylation of thymidine kinase. *AIDS Res. Hum. Retrovir.* **12**:223–228 (1996).
5. G. Hoever, B. Groeschel, P. Chandra, H. W. Doerr, and J. Cinatl. The mechanism of 3′-azido-2′,3′-dideoxythymidine resistance to human lymphoid cells. *Int. J. Mol. Med.* **11**:743–747 (2003).
6. C. R. Wagner, V. V. Iyer, and E. J. McIntee. Pronucleotides: toward the *in vivo* delivery of antiviral and anticancer nucleotides. *Med. Res. Rev.* **20**:417–451 (2000).
7. E. J. McIntee, R. P. Remmel, R. F. Schinazi, T. W. Abraham, and C. R. Wagner. Probing the mechanism of action and decomposition of amino acid phosphomonoester amidates of antiviral nucleoside prodrugs. *J. Med. Chem.* **40**:3323–3331 (1997).
8. G. M. van Wijk, K. Y. Hostetler, E. Kroneman, D. D. Richman, C. N. Sridhar, R. Kumar, and H. van den Bosch. Synthesis and antiviral activity of 3′-azido-3′-deoxythymidine triphosphate distearoylglycerol: a novel phospholipid conjugate of the anti-HIV agent AZT. *Chem. Phys. Lipids* **70**:213–222 (1994).
9. L. Cattel, M. Ceruti, and F. Dosio. From conventional to stealth liposomes: a new frontier in cancer chemotherapy. *Tumori* **89**:237–249 (2003).
10. M. L. Immordino, P. Brusa, F. Rocco, S. Arpicco, M. Ceruti, and L. Cattel. Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing lipophilic gemcitabine prodrugs. *J. Control Release* **100**:331–346 (2004).
11. P. Crosasso, P. Brusa, F. Dosio, S. Arpicco, D. Pacchioni, F. Schuber, and L. Cattel. Antitumoral activity of liposomes and immunoliposomes containing 5-fluorouridine prodrugs. *J. Pharm. Sci.* **86**:832–839 (1997).
12. K. R. Patel and J. D. Baldeschwieler. Treatment of intravenously implanted Lewis lung carcinoma with liposome-encapsulated cytosine arabinoside and non-specific immunotherapy. *Int. J. Cancer* **34**:415–420 (1984).
13. S. V. Vinogradov, E. Kohli, and A. D. Zeman. Cross-linked polymeric nanogel formulations of 5′-triphosphates of nucleoside analogues: role of the cellular membrane in drug release. *Mol. Pharm.* **2**:449–461 (2005).
14. S. V. Vinogradov, A. D. Zeman, E. V. Batrakova, and A. V. Kabanov. Polyplex nanogel formulations for drug delivery of cytotoxic nucleoside analogs. *J. Control Release* **107**:143–157 (2005).
15. S. Grant. Ara-C: cellular and molecular pharmacology. *Adv. Cancer Res.* **72**:197–233 (1998).
16. S. V. Vinogradov, T. K. Bronich, and A. V. Kabanov. Nanosized cationic hydrogels for drug delivery: preparation, properties and interactions with cells. *Adv. Drug Deliv. Rev.* **54**:135–147 (2002).
17. S. V. Vinogradov, E. V. Batrakova, and A. V. Kabanov. Nanogels for oligonucleotide delivery to the brain. *Bioconjug. Chem.* **15**:50–60 (2004).
18. H. Hamada and T. Tsuruo. Characterization of the ATPase activity of the Mr 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug resistance in K562/ADM cells. *Cancer Res.* **48**:4926–4932 (1988).
19. S. Vinogradov, E. Batrakova, S. Li, and A. Kabanov. Polyion complex micelles with protein-modified corona for receptor-mediated delivery of oligonucleotides into cells. *Bioconjug. Chem.* **10**:851–860 (1999).
20. T. Mosmann. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**:55–63 (1983).
21. Y. S. Kaneko, K.T. Okano. Temperature-responsive hydrogels as intelligent materials, Biorelated Polymers and Gels, Academic Press, New York, 1998.
22. N. Kashyap, N. Kumar, and M. N. Kumar. Hydrogels for pharmaceutical and biomedical applications. *Crit. Rev. Ther. Drug Carr. Syst.* **22**:107–149 (2005).
23. N. A. Peppas, K. M. Wood, and J. O. Blanchette. Hydrogels for oral delivery of therapeutic proteins. *Expert Opin. Biol. Ther.* **4**:881–887 (2004).
24. A. V. Kabanov, P. Lemieux, S. Vinogradov, and V. Alakhov. Pluronic block copolymers: novel functional molecules for gene therapy. *Adv. Drug Deliv. Rev.* **54**:223–233 (2002).
25. V. Alakhov, G. Pietrzynski, K. Patel, A. Kabanov, L. Bromberg, and T. A. Hatton. Pluronic block copolymers and pluronic poly(acrylic acid) microgels in oral delivery of megestrol acetate. *J. Pharm. Pharmacol.* **56**:1233–1241 (2004).
26. L. Bromberg, M. Temchenko, V. Alakhov, and T. A. Hatton. Bioadhesive properties and rheology of polyether-modified poly(acrylic acid) hydrogels. *Int. J. Pharm.* **282**:45–60 (2004).
27. J. Cleary, L. Bromberg, and E. Magner. Adhesion of polyether-modified poly(acrylic acid) to mucin. *Langmuir* **20**:9755–9762 (2004).
28. F. Tirnaksiz and J. R. Robinson. Rheological, mucoadhesive and release properties of pluronic F-127 gel and pluronic F-127/polycarbophil mixed gel systems. *Pharmazie* **60**:518–523 (2005).