Published in final edited form as:

Biomaterials. 2011 August ; 32(23): 5417–5426. doi:10.1016/j.biomaterials.2011.04.006.

Folate-Decorated Nanogels for Targeted Therapy of Ovarian Cancer

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Abstract

Nanogels are comprised of swollen polymer networks and nearly 95 % water and can entrap diverse chemical and biological agents for cancer therapy with very high loading capacities. Here we use diblock copolymer poly(ethylene oxide)-*b*-poly(methacrylic acid) (PEO-*b*-PMA) to form nanogels with the desired degree of cross-linking. The nanogels are further conjugated to folic acid (FA) and loaded with different types of drugs (cisplatin, doxorubicin). For the first time we demonstrate a tumor-specific delivery and superior antitumor effect *in vivo* of an anti-cancer drug using these polyelectrolyte nanogels decorated with folate targeting groups. This reinforces the use of nanogels for the therapy of ovarian and other cancers, where folate receptor (FR) is over-expressed.

1. Introduction

Targeted delivery of drugs to cancer cells has attracted considerable attention in developing new chemotherapeutic modalities. In this field folic acid (FA) has recently emerged as a prominent targeting moiety capable of specific interaction with cells expressing the folate receptor (FR) [1]. FR consists of a high affinity ($K_d \sim 10^{-9}$ – 10^{-10} M) folate binding protein (FBP) attached to the membrane through a glycosylphosphatidylinositol anchor [2]. It is overexpressed in ovarian carcinomas and other human tumors and has little expression in normal tissues [3, 4]. This provides tumor cells with increased amounts of the FA essential

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Supplementary information

Supplementary data associated with this article can be found in the online version.

Author contributions

N.V.N. synthesized all materials, performed the research, analyzed the data, and wrote the manuscript, H.S.O. participated as equal contributor in cell and animal studies and data analysis; A.V.K. designed the research, analyzed data and wrote manuscript; T.K.B. designed the research, analyzed data and wrote the manuscript.

for DNA synthesis and seems to aid in aggressive tumor growth. In patients diagnosed with epithelial ovarian cancer the overexpression of FR isoform α correlates with a higher histological grade and more advanced stage of the disease [5]. The differential expression of FR in ovarian and other cancers makes it an attractive marker and target molecule for diagnosis and therapy of the disease [6]. Several folate-conjugated drugs and imaging agents have reached clinical evaluation stage [7].

The site-specific delivery of drugs to the tumors using FR can be enhanced using high capacity carriers that can simultaneously incorporate multiple drug molecules into one particle and target them to the disease sites. One recent type of high capacity carriers is nanogels composed of water-soluble polymer chains cross-linked within a nanoscale volume [8, 9]. Such nanogels are highly swollen and can incorporate 30 % wt. and more drug molecules through covalent or electrostatic bonding with the nanogel chains. These loading capacities are unusually high and exceed those of liposomes and polymeric micelles [10]. Furthermore, nanogels do not have a dense core or a defined surface and can undergo dramatic volume transitions upon environmental changes. As a result, nanogels have unprecedented capacity for steric stabilization and decreased non-specific interactions, which can be used for example to stabilize colloidal microemulsions [11]. The very same properties could be beneficial for the targeted delivery of nanogels in the body. So far, however, nanogels were not used for this purpose, because extreme softness and flexibility of their hydrated chains have presented a challenge for attachment of targeting groups. This now becomes possible due a controlled template synthesis of nanogels by polyion complexation and cross-linking of doubly hydrophilic block ionomers, such as PEO-*b*-PMA. Using this synthetic approach [12], here we evaluate the drug therapy efficacy of FA decorated nanogels loaded with drugs (cis-dichlorodiamminoplatinum (II) (cisplatin, CDDP) and doxorubicin (DOX)) in an animal model of ovarian cancer.

2. Materials and Methods

2.1. Materials

PEO-*b*-PMA with terminal hydroxyl group in PEO block was from Polymer Source Inc., Canada (Mw/Mn=1.16, PEO 5.5 kDa, PMA 15.5 kDa). Disposable PD-10 desalting columns, Amicon YM-30 centrifugal filters (MWCO 30 kDa, Millipore), CaCl₂, 1,2-ethylenediamine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), ethylenediaminetetraacetic acid (EDTA), folic acid (FA), divinyl sulfone (DVS), folate binding protein (FBP), fluorescein isothiocyanate (FITC), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cis-dichlorodiamminoplatinum (II) (cisplatin, CDDP) and other chemicals were from Sigma-Aldrich (St Louis, MO, USA). Doxorubicin hydrochloride (DOX) was a kind gift from Dong-A Pharmaceutical Company, South Korea. Folate-free RPMI 1640 medium was also from Sigma-Aldrich. Anti-FR α MAb (Mov18/ZEL) were from ALEXIS Biochemicals (Plymouth Meeting, PA, USA). Fetal bovine serum (FBS), RPMI 1640 medium, penicillin, streptomycin, Trypsin-EDTA (0.5% trypsin, 5.3mM EDTA tetra-sodium), Alexa Fluor[®] 488 goat anti-mouse IgG, LysoTracker[™] Red and other biochemicals were from Invitrogen (Carlsbad, CA, USA).

2.2. Synthesis of folate-decorated nanogels

The synthesis of folic acid (FA) conjugated nanogels (FA-nanogels) involved three steps.

2.2.1. Synthesis of nanogels—PEO-*b*-PMA/Ca²⁺ complexes were prepared by mixing PEO-*b*-PMA and CaCl₂ aqueous solutions at a molar ratio of [Ca²⁺]/[COO⁻]=1.1. The concentration of carboxylic groups in the copolymer samples was determined by potentiometric titration. The chains were cross-linked overnight at room temperature (r.t.)

using 1,2-ethylenediamine and EDC ([EDC]/[1,2-ethylenediamine] = 2; [COOH]/[EDC] = 5). The nanogels were dialyzed (MWCO 3.5 kDa) extensively against a) 0.5% aqueous ammonia in the presence of EDTA and b) bi-distilled water.

2.2.2. Synthesis of 1,2-ethylenediamine-nanogels (ED-nanogels)—Nanogels were loaded with CaCl_2 at $[\text{Ca}^{2+}]/[\text{COO}^-]=1$ and then reacted with 3-fold molar excess of DVS (THF) at pH 9.6 upon vigorous mixing for 30 min at r.t.. After termination (pH 6.5, HCl) the unreacted DVS were removed using Amicon YM-30 filters at 3,000 rpm, 10 min. The activated nanogels were reacted with 5-fold excess of 1,2-ethylenediamine at pH 8.5–9.0 (r.t., overnight stirring), and filtered as described above. Free amino groups were assayed colorimetrically by ninhydrin.

2.2.3. Synthesis of FA-nanogels—FA was activated with EDC (2.5–3 eq.) in bi-distilled water (pH 7.4, 5 min stirring in the dark) and then reacted with ED-nanogels at various molar ratios (r.t., stirring overnight, dark). FA-nanogels were purified using PD-10 desalting columns, thoroughly dialyzed against bi-distilled water (MWCO 3.5 kDa) and lyophilized. Conjugated FA was determined by 1) proton nuclear magnetic resonance spectroscopy (^1H NMR) using Varian 500 MHz spectrometer (D_2O , pH 7.0, 25°C) and 2) UV absorbance ($\epsilon_{363}=6,500\text{ M}^{-1}\text{cm}^{-1}$) in phosphate buffered saline (PBS) using SpectraMaxM5 spectrophotometer (Molecular Devices Co., USA). Degree of modification was defined as μmol of FA per mg of nanogel.

2.3. Synthesis of FITC-labeled nanogels

First, fluoresceinthiocarbamyl 1,2-ethylenediamine (FITC-ED) was synthesized as reported before [13]. Briefly, 117 mg (0.3 mmol) of FITC in 10 ml of methanol-triethylamine (TEA) mixture (100:1) was added dropwise over 30 min to 200 mg (1.5 mM) of 1,2-ethylenediamine in the same solvent (50 ml) and reacted for an additional hour. After completion of the reaction the solvents were evaporated in vacuum until dryness. Reddish precipitate was washed several times by methanol, and then dried out in air. Second, FITC-ED was conjugated to PEO-*b*-PMA. Briefly, PEO-*b*-PMA (0.78 mmol carboxylic groups) in dimethylformamide (DMF) (2 ml) was reacted overnight at r.t. with *N*-hydroxysuccinimide (2 mg, 0.0174 mmol) and EDC (4.8 mg, 0.025 mmol). This mixture was then supplemented with 4 mg FITC-ED in DMF (200 μl) and 20 μl TEA and left overnight. FITC-PEO-*b*-PMA was purified by exhaustive dialysis against bi-distilled water (MWCO 3.5 kDa) for 2 days and then used for synthesis of the nanogels as described above. The content of FITC in nanogels was determined by UV spectrometry ($\epsilon_{490}=87800\text{ M}^{-1}\text{cm}^{-1}$) in 50 mM bicarbonate buffer, pH 9.5. Notably, various FA-nanogels were synthesized using the same stock of FITC-labeled nanogels and had practically the same charge and size at pH 7.4.

2.4. Drug loading

Nanogel dispersions were mixed with CDDP (1 mg/ml) (pH 9.0, 37°C , 48 h) or DOX (2 mg/ml) (pH 7.0, r.t., 24 h) at various [drug]/[COOH] ratios. Unbound drug was separated on NAP-10 column and filtration on drug-pretreated Amicon YM-30 filters. Pt (Pt194/Pt195) was assayed on Varian ion coupled plasma-mass spectrometer (ICP-MS, expert software version 2.1 b103) calibrated with Holmium (internal standard) and Pt (2 to 100 ng/ml). Samples were diluted in 0.1 N HCl. DOX was assayed by UV spectrometry ($\epsilon_{485}=10,800\text{ M}^{-1}\text{cm}^{-1}$ in water and $10,400\text{ M}^{-1}\text{cm}^{-1}$ in PBS). Drug loading capacity was calculated as percent ratio of mass of incorporated drug to total mass of drug-loaded nanogels without water. Encapsulation efficiency was determined as percent ratio of incorporated drug to total drug added upon loading.

2.5. Drug release

Drug release (minimum of 300 µg CDDP and 200 µg DOX) was examined in PBS (pH 7.4, 0.14 M NaCl) and acetate buffered saline (ABS, pH 5.5, 0.14 M NaCl) at 37°C using Spectra/Por Float-A-Lyzer G2 dialysis systems (MWCO 3.5–5.0 kDa) and expressed as percent of total vs. time.

2.6. Particle characterization

Particles were characterized by dynamic light scattering (DLS) (zeta-potential (ζ), effective hydrodynamic diameters (D_{eff}), polydispersity indices (PDI)), and atomic force microscopy (AFM) as previously described[12].

2.7. Cell culture

Cells were cultured in folate-depleted RPMI 1640 medium (FD-RPMI) with 2mM glutamine, 10% (v/v) FBS, 100 U/ml penicillin and 0.1mg/ml streptomycin at 37°C, 5% CO₂. This medium contains nearly physiological level of folate[14].

2.8. Flow cytometry

Cells (50,000 cells/well) grown in FD-RPMI media in 24-well plates for 2 days were exposed to nanogels (0–0.5 mg/ml) at 4°C or 37°C for up to 3 h, washed three times in PBS or acid saline (0.15 M NaCl, pH 3.0), trypsinized at 37°C, centrifuged (1,500 rpm, 5 min) and re-suspended in PBS (pH 7.4, 1% BSA). The % gated cells and mean fluorescence intensity (FITC for CDDP- or DOX for DOX-loaded nanogels) were analyzed using Becton Dickinson FACStarPlus. For double staining, cells were exposed to Alexa 680-nanogels for 3 h and then mouse antibody to FR- α Mov18/ZEL and Alexa 488-goat anti-mouse antibody. 10,000 events were acquired in linear mode, gated to exclude debris and dead cells, and visualized in logarithmic mode. The normalized mean fluorescence is a product of % gated and mean fluorescence.

2.9. Confocal microscopy

Cells (50,000 cells/chamber) were grown in Lab-Tek Chambered Cover Glass dishes in FD-RPMI for 2 days and exposed to FITC-labeled nanogels (0.3 mg/ml) for 10 or 45 min at 37°C. For co-localization studies, 100 nM LysotrackerTM-Red was added for the last 10 min of exposure (45 min). After exposure cells were washed three times with PBS and kept in FD-RPMI media prior to visualization by live cell confocal imaging (Carl Zeiss LSM 510).

2.10. In vitro cytotoxicity

Cells seeded in 96-well plates (10,000 cells/well) 24 h before the experiment treated with drugs (0 to 300 µg/ml CDDP or 0 to 100 µg/ml DOX), drug/nanogel or unloaded nanogels for 6, 24 or 48 h and then cultured for additional 24 h (CDDP) or 48 h (DOX) in drug-free media at 37°C. Cytotoxicity was determined by standard colorimetric MTT assay [15] and the IC₅₀ values were calculated using GraphPad Prism Software. In selected studies on DOX cytotoxicity cells were trypsinized, stained with 7-aminoactinomycin D (7-AAD) and analyzed by flow cytometry. Nanogels or FA-nanogels alone did not exhibit a cytotoxic effect.

2.11. Antitumor activity of CDDP-loaded nanogels

Four week old female nude athymic (nu-/nu-) mice (National Cancer Institute) were housed in AAALAC accredited facility. Food and RO water were available *ad libitum* throughout the study. Animals were maintained on a FA deficient diet (Harlan diet TD.00434) for 2 weeks before tumor implantation and during the period of studies and quarantined for 7 days

prior to tumor inoculation. Tumors were initiated by subcutaneous injection of 37°C A2780 cells (5×10^6) on the flanks at two sites above each limb. Mice with 100 to 200 mm³ tumors (4 to 7 mm in each dimension, ca. 10 to 12 days) were randomized (6 groups, 7–8 mice). Treatments were administered via tail vein injections at 4-day intervals. The body weight, and tumor volume were monitored every second day. Tumor volume was calculated as $V = 0.5 \times L \times W^2$, using tumor length (L) and width (W) measured by electronic calipers. All animals were sacrificed by endpoint day 23. Protocols were approved by the Institutional Animal Care and Use Committee.

2.12. Statistics

Statistical comparisons except animal studies were carried out using Student t-test. In animal studies means for tumor volumes and body weights were analyzed using one way analysis of variance (ANOVA) followed by Tukey's test for group-wise comparisons. Survival was analyzed using Kaplan-Meier analysis and compared using the log-rank test. All tests were performed using GraphPad Prism 5.

Additional methods. Detailed methods are available in the supplementary methods online.

3. Results

The synthesis of FA-conjugated nanogels (FA-nanogels) involved three steps: 1) preparation of nanogels with free OH groups at the PEO termini; 2) synthesis of stable intermediate with terminal amino groups; 3) conjugation of the intermediate with activated FA. Each of these steps produced stable intermediates, which can be isolated, characterized and stored (Scheme 1, Fig. S1). The preparation of active FA-nanogels was confirmed by surface plasmon resonance using FBP immobilized onto a dextran-coated gold sensor chip (Fig. S1b). This has shown that FA-nanogels strongly and specifically bind with surface-immobilized FBP while unmodified nanogels do not interact at all.

3.1. Morphology and swelling behavior of folate-decorated nanogels

The morphology of nanogels remained unchanged after their conjugation with FA as determined by AFM in air (Fig. S1c). The mean heights and diameters of “dry” particles were 48.55 ± 2.88 nm and 103.18 ± 9.85 nm for nanogels, and 45.37 ± 2.21 nm and 110.16 ± 3.51 nm for FA-nanogels. The mean volumes were ca. 2.70×10^5 nm³ and 2.90×10^5 nm³, respectively. The same particles in aqueous dispersions were considerably swollen as measured by DLS (Table S1). The swelling degree depended on the pH (Fig. 1a, Fig. S2a) and low molecular mass electrolytes (Table S1). Generally, increase of pH resulted in increase of the particle size and decrease of ζ -potential due to combined effect of ionization of the PMA chains, penetration of the counterions into the nanogel volume and increased osmotic pressure (Fig. 1a,b, Fig. S2a,b). The differences were virtually abolished as the ionic strength increased (Table S1). Interestingly, in the absence of low molecular mass electrolyte the conjugation of folate resulted in a decrease of size and increase of ζ -potential (Fig. 1a). Moreover, the swelling of FA-nanogels was further suppressed as the amount of folate increased (Fig. S2a). At high degrees of modification, e.g. 2 μ mol/mg FA-nanogels practically did not change size upon variation of pH. This may be explained by a side reaction involving chemical cross-linking of the of nanogel chains with bis-activated FA. Alternatively, swelling may be hindered by association of the FA moieties into multimers owing to Hoogsteen-type hydrogen bonds and stacking interactions[16].

3.2. Drug loading in folate-decorated nanogels

FA-nanogels remained stable in PBS in a wide range of concentrations (up to 1.5 % w/v) and exhibited no aggregation at 37°C for two weeks. The dispersion stability was also

maintained after loading the nanogels with the drugs, which bind with PMA chains through coordination (CDDP) or combination of electrostatic and Van der Waals interactions (DOX). In both cases, there was a decrease of the particle size and increase of ζ -potential, which was consistent with the consumption of the carboxylate groups by the drugs (Table S1, Fig. S2c,d). Interestingly, the loading capacity of nanogels was decreased after conjugation of the folate residues by as much as 45% in heavily modified FA-nanogels (e.g. 1.0 μmol folate/mg polymer) (Fig. 1c,d, Fig. S3a,b). This may be due to steric hindrance to drug binding as a result of nanogel condensation. Also, variation of the molar ratio of drug to carboxylic groups of nanogels from 0.25 to 1.5 did not appear to change the drug loading capacity (although the loading efficiency accordingly decreased, Fig. S3c,d). However, at higher drug concentrations the nanogels were destabilized and precipitated (days, Fig. 1c,d and Fig. S3). Nevertheless, we were able to obtain stable drug loaded FA-nanogels with 0.1 to 0.3 μmol folate/mg polymer and drug: polymer weight ratios as much as 1:2. Such nanogels could be lyophilized and re-dispersed in an aqueous buffer albeit with some increase in particle sizes and polydispersity (ca. $D_{\text{eff}} = 152 \pm 8$ nm, PDI 0.05 ± 0.01 vs. $D_{\text{eff}} = 171 \pm 10$ nm, PDI 0.12 ± 0.05 for initial and reconstructed nanogels respectively).

3.3. Drug release from folate-decorated nanogels

For both drugs there was very little difference in the release rates between nanogels and FA-nanogels (Fig. 1), except for heavily modified FA-nanogels (Fig. S3e). As is seen in Figure 1e, f, both drugs were released from the nanogels significantly faster at pH 5.5 (ABS) than at pH 7.4 (PBS). For example, up to 60% of the drug was released from DOX-loaded nanogels during first 5 h at pH 5.5 but not at pH 7.4 (Fig. 1f). The accelerated release at the acidic pH may be due to protonation of carboxylic groups of PMA, which weakens the drug and nanogel coupling. Additionally, in the case of DOX, the release rate may be increased due to increased solubility of this drug at acidic pH [17]. Also, noteworthy that the release rates decreased as the release conversion increased. This may be attributed to 1) different mobility of DOX molecules in external and internal layers of nanogels, or 2) increase in the net negative charge of nanogels, which slows down migration of DOX cations to external solution at high conversions. Notably, CDDP release was much slower than that of DOX (Fig. 1e). This is probably explained by stronger coordination bonding between CDDP and carboxylic groups of nanogels, compared to predominantly electrostatic coupling in case of DOX.

3.4. Cell uptake of folate-decorated nanogels

To determine effect of attachment of folate on cell transport of nanogels we used human ovarian carcinomas A2780 overexpressing FR- α as a FR-positive tumor model and A549 as a FR-negative control (Fig. S4a). As expected, the uptake of FA-nanogels in FR-positive cells greatly exceeded the uptake of the untargeted nanogels (Fig. 2a). Moreover, the amount of cell-associated FA-nanogels gradually decreased in the presence of free folate (Fig. 2b), suggesting that the targeted nanogels interacted with the FR. It should be noted, that cellular uptake of FA-nanogels was unaffected by physiological concentration of folate (about 20 nM in human plasma [18]). Furthermore, even at 0.5 mM folate the uptake of FA-nanogels was nearly 50% of the initial. Only at very high concentration of FA (10 mM) it was suppressed to the level of the untargeted nanogels (20–25%). Since the binding constant of the free folate with FR is as little as 0.01 to 1 nM [1, 6], FA-nanogels displayed much higher affinity to cellular FR than the free ligand. This may be beneficial for targeting in the body environment, where FA-nanogels would compete with the free folate. Interestingly, as previously reported for other nanoformulations [19] there was an optimal amount of folate moieties, 0.1 to 0.2 μmol folate/mg polymer, above which the uptake of FA-nanogels was gradually decreased (Fig. 2c).

Using flow cytometry analysis, we separately determined the total fluorescent cells (“total uptake”) as well as cell fractions (% gated cells) with either internalized or membrane bound nanogels (Table 1). The total uptake of both targeted and untargeted nanogels was reduced and their internalization was essentially abolished at 4°C compared to 37°C, suggesting their temperature-dependent endocytosis. However, the untargeted nanogels practically did not adsorb at the membranes, while the FA-nanogels appeared to be considerably adsorbed at both temperatures. Interestingly, at 37°C the processing of the FA-nanogels with low modification degree (0.15 μmol folate/mg polymer) was shifted towards internalization (88 % cells vs. 7% cells with membrane bound nanogels). Once their internalization was abolished at 4°C their fraction bound with the membrane was increased (ca. 19%). In contrast, the heavily modified FA-nanogels (1.4 μmol folate/mg polymer) were strongly retained in the membrane even at 37°C (30%) and internalized to a lesser extent (55%). This may be due to down-regulation of FR-mediated endocytosis resulting in decreased entry of heavily modified nanogels into endocytic compartments and/or increased recycling of such nanogels to the cell surface[4, 20].

Our previous work demonstrated that following the initial entry in epithelial cancer cells the nanogels transport to the lysosomes [21]. Similarly, the FA-nanogels at 10 min were mainly bound with the cell membrane and at 45 min taken inside the cells (Fig. S4b,c). At this later time point the FA-nanogels co-localized with the lysosomes (Fig. 2d and S4d,e). Although the detailed trafficking itinerary of compounds bound to FR may differ, in many cases their lysosomal destination was reported [6, 22]. This may explain similarities in intracellular localization between untargeted and targeted nanogels.

3.5. Differential delivery and toxicity of drug-loaded FA-nanogels in FR-positive cells

First, we determined whether loading of the drug into targeted nanogels could interfere with their ability to interact with the FR. While this appeared to be a nonissue for DOX, the uptake of CDDP-loaded FA-nanogels in FR overexpressing A2780 cells strongly depended on the sequence of folate attachment and drug loading (Fig. 3a). In particular, if the drug was first loaded, and then nanogel was modified with folate the uptake was greatly increased compared to the untargeted loaded nanogels. In contrast, if CDDP was added to FA-nanogels after attachment of the folate the uptake increased only marginally, most likely due to reaction of CDDP with the folate α -carboxylic groups essential for the FR binding[18]. In case of the A549 cells with low levels of FR the uptake did not depend on whether CDDP was loaded before or after folate attachment and was similar to that of the untargeted nanogels.

Next, we determined whether the drug loaded FA-nanogels could recognize FR-positive cells with differential expression of FR. Indeed, the FA-nanogel/CDDP accumulated mainly in the FR-positive cell subpopulation in A2780 cell culture (Fig. 3b). Its uptake in the FR-negative subpopulation was much lower and similar to that observed in A549 cells. Same trend was observed in the A2780 and A549 co-culture, which clearly suggests that FR-nanogels recognize their target. In contrast, untargeted nanogels did not reveal any selectivity to FR-positive cells. Furthermore, at 4°C when the trans-membrane diffusion of free DOX was suppressed the targeting of FA-nanogel/DOX in A2780 cells was superior to both free drug and untargeted nanogel/DOX (Fig. 3c).

These differences in cellular delivery translated into cytotoxicity (Table 2, Fig. S5). As expected, CDDP activity was greatly decreased after its incorporation into nanogels (Fig. 1e). However, attachment of folate groups resulted in considerable increase of cytotoxicity of FA-nanogels/CDDP. Similar result was obtained for FA-nanogel/DOX in FR-positive cells (Table 2). Although free DOX in A2780 cells was nearly as active as FA-nanogel/DOX, the drastic difference between these drug formats was observed in cells with different

levels of FR-expression. As shown in Figure 3d FA-nanogel/DOX was much less toxic in cells with lower level of FR-expression (A549 and NIH 3T3). In contrast, toxicity of free DOX was comparable in all three lines.

3.6. Antitumor activity of drug-loaded nanogels in mice

The proof-of-concept of *in vivo* delivery and therapy of tumors using FR targeted nanogels was obtained in mice bearing A2780 xenografts. Since targeted nanogels are more likely to benefit delivery of a drug with slower release kinetics we chose CDDP as a model drug. Free CDDP or its nanogel formats were injected 4 times at 4-day intervals at 4 mg/kg CDDP equivalents (corresponding to a maximum tolerated dose (MTD) of free CDDP upon this treatment schedule). All animals were kept on folate-deficient diet, which increased expression of FR in tumors (Fig. S6). This may be more relevant to clinical situation since humans have nearly 10-times less serum folate than mice (20 nM vs. 250 nM) [23]. On 4th day post treatment animals injected with FA-nanogels/CDDP displayed much higher levels of Pt in tumors compared to free CDDP and nanogels/CDDP (Fig. 4a and Table S2). This increase appeared to be due to specific targeting of FR because it was reversed by co-administration of free folate. The increase in Pt accumulation in kidney with FA-nanogels/CDDP, was not accompanied by any increased toxicity. The tissue histopathology did not reveal any adverse changes in kidney, liver or spleen histology in any nanogel treatment group. In contrast, the free drug was toxic resulting in focal renal tubular toxicity and presence of basophilic regenerative tubules (Fig. S7).

The changes in the relative tumor volume, body weight and animal lifespan are shown in Figure 4b-d and Table S3. All CDDP treatments decreased tumor growth rates ($p < 0.05$) compared to the vehicle controls (Fig. 4b). However, FA-nanogels/CDDP treatment resulted in the greatest tumor growth inhibition, increase in the lifespan and no loss of the body weight (Fig. 4c,d). Free FA suppressed the antitumor effect of FA-nanogels/CDDP to the level observed with untargeted nanogels/CDDP, probably, due to saturation of FRs. The worst performance was observed for free CDDP, which resulted in profound loss of body weight (toxicity) and less tumor inhibition than either of the nanogel formulations.

4. Discussion

Pharmaceutical drug delivery has experienced explosive growth in the last decade due to introduction of novel nanoformulations. Following development of liposomes and polymeric micelles, which are approved for clinical use or undergo clinical trials [24], numerous other materials based on polymer-coated drug nanocrystals, insoluble polymer nanoparticles, dendrimers, and polyion complexes have been explored as drug carriers [25]. These materials often contain water-soluble polymer brushes (such as PEG layers) and can change shape in response to mechanical or adhesive forces. However, nanogels are unique among soft materials, since they undergo at least 10-fold volume expansions upon swelling from dry state and nearly 95% of their swollen space consists of water [8, 9].

The specific nanogel type studied here comprises a network of cross-linked polyanion chains bearing carboxylic groups, which become ionized as the pH increases. In response to ionization the counterions are concentrated in the network resulting in increase of the osmotic pressure and swelling [26]. The swelling of nanogels at constant pH also depended on the ionic strength. Thus upon increase of the salt concentration at pH 7.4 the nanogel size decreased, while at pH 5.5 when PMA segments are only partially ionized, upon addition of salt the size increased (Table S1). It is possible, that at low pH increase in the ionic strength results in increase of both ionization and osmotic pressure due to interpenetration of the counterions into the network. At the same time at high pH the PMA chains are completely

ionized and addition of the counterions results in a decrease of electrostatic repulsion due to screening of the charge.

The effect of pH and ionic strength on the nanogels ζ -potential is probably even more complex. According to the Stern theory particles with defined surface form an electrical double layer, consisting of the dense part at the particle surface (the Stern-Helmholtz layer) and diffuse part where the counterions are localized (the Gouy-Chapman layer) [27]. However, in contrast to solid spheres the nanogel particles do not have a defined surface. Thus, the “dense part” of the electrical double layer may spread throughout the volume of the nanogel and its size and capacity may change as a result of swelling. Furthermore, due to the chain conformational flexibility the “interior” carboxylic groups upon ionization may translocate to the exterior and contribute to the ζ -potential decrease. It is interesting that in the absence of elementary salt the increase of pH resulted in decrease of the ζ -potential while at high ionic strength (0.15 M NaCl) the ζ -potential increased and practically did not change upon pH variation. To the best of our knowledge the theory of ζ -potential in nanogels is not sufficiently developed. Nevertheless, based on our results one can suggest that penetration of counterions in nanogel particles can strongly affect both size (swelling) and ζ -potential. Further phenomenological analysis and theoretical understanding of the swelling and electrokinetic behavior of nanogels is needed. In addition to D_{eff} and ζ -potential, which are now conventional measures for pharmaceutical characterization of more rigidly structured nanoformulations, a more detailed swelling analysis may be required for quality control and regulatory approval of nanogel-based products.

This study for the first time demonstrated a possibility of targeted delivery of nanogels and their therapeutic cargo to the cancer cells in the body. Achieving this goal required controlled modification of nanogels with multiple chemical functionalities. Indeed, each nanogel particle carried at least several hundred carboxylic, amino, and hydroxyl groups that could simultaneously participate in chemical reactions and obstruct their direction and yield. Therefore, it is important that we were able to synthesize FA-nanogels in reproducible fashion, and isolate stable intermediates, which can be stored and used in such synthesis for at least several months. All reactions were carried out in mild conditions in aqueous media, since this nanogel type was insoluble in organic solvents.

Another task was optimization of the number of folate groups linked to a nanogel. As the amount of folate moieties became too large the nanogel swelling was affected, the drug loading capacity and dispersion stability decreased, and the cell uptake inhibited. Hence, an optimal number of folate molecules were conjugated to nanogels to maintain good loading, stability and cell uptake. Such optimized FA-nanogels were shown to carry drugs to targeted cell populations expressing FR. It was also important that drug loading did not affect the ability of targeted nanogels to interact with the receptors. Specifically, to avoid inactivation of folate through CDDP reaction with its α -carboxylic group [18] folates were attached after drug loading.

Perhaps, the most important challenge addressed in this study is the delivery of the FA-nanogels to an *in vivo* tumor and demonstration of a superior antitumor activity of the CDDP-loaded targeted nanogel. Surprisingly, similar to the *in vitro* experiments, the *in vivo* tumor delivery and anti-tumor effect were inhibited by the free folate. This suggests that the FA-nanogel displays folate specificity and exhibits elevated activity through a specific mechanism involving *in vivo* FR. In view of the aggressiveness of the tumor model used in our work the extent of the observed tumor inhibition with the folate-targeted nanogels is considerable even compared with successful previous studies on other folate-targeted nanoformulations. In particular, in the control groups the tumors reached 3000 to 3500 mm³ approximately at 13 to 14 day after inoculation. In comparison, in a recent study by Kim et

al. [28] using A2780 xenografts to assess the activity of folate-conjugated polymeric micelles the same tumor growth (without treatment) required twice more time. While previous successes in folate-mediated targeting of drugs have been encouraging [7], a demonstration of a targeted delivery of nanogel-based therapeutic is a major step towards practical use of this distinct and novel delivery system for therapy of cancer. Our group had previously shown that the entry of such materials is restricted in non-cancerous epithelial cells, which possess tight-junctions, but allowed in cancer cells that do not form tight-junctions[21].

Notably, increased accumulation of Pt with nanogel treatment formats was observed in all organs (Table S2). Organs of reticuloendothelial system (liver and spleen) exhibited the highest increase, which is consistent with the reports that these organs are responsible for elimination of various nanoformulations from the circulation [29–31]. The increased accumulation of Pt in the kidneys with FA-nanogels could be a concern for renal toxicity. To the contrary, however, nanogels appeared to decrease the kidney toxicity compared to the free drug. Furthermore, the animals in nanogel groups displayed weight gain and did not show any signs of stress during daily monitoring, while the free drug was obviously more toxic in this regard as well. Earlier studies reported that free CDDP is rapidly eliminated from the circulation by kidneys, causing an acute peak exposure of the renal tubules to the drug, which is the main cause for its renal toxicity [32]. In contrast, CDDP incorporated in the polymeric micelles had a much lower initial renal exposure, but higher exposure at the late term [33]. Notably, no nephrotoxicity was detected with such treatment in comparison with the free CDDP, which was accounted for by the lack of the acute renal exposure to the nanoformulated drug. It is possible that similar phenomenon underlies decreased toxicity of the nanogel formulations in the present study.

5. Conclusions

This study demonstrated possibility of delivery of FR-targeted nanogels and their therapeutic cargo to the cancer cells *in vivo*. Here we use diblock copolymer poly(ethylene oxide)-*b*-poly(methacrylic acid) (PEO-*b*-PMA) for controlled template synthesis of nanogels by polyion complexation and cross-linking of doubly hydrophilic block ionomer. An optimal number of folate molecules were conjugated to nanogels to maintain good anti-cancer drug loading, stability and cellular uptake. Such optimized FA-nanogels were shown to carry their therapeutic cargo to targeted cell populations expressing FR. Finally, we show superior antitumor efficacy of anti-cancer drug, CDDP delivered to a xenograft tumor using these FR-targeted nanogels along with decreased renal toxicity. Altogether, this study demonstrates fundamental possibility for targeted delivery of the nanogel-based anticancer therapeutics and opens a new prospect for clinical development of such nanofomulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the grants from U.S.A. National Institute of Health CA116590 (T.K.B.) and Department of Defense USA MRC 06108004 (A.V.K.). We thank Dr. Frederic C. Laquer for ICP-MS Pt measurements and Dr. Larisa Poluektova for her help with preparation of tissue sections. We acknowledge the assistance of the Nanomaterials Core facility of the Center for Biomedical Research Excellence (CoBRE) Nebraska Center for Nanomedicine supported by the NIH grant RR021937. We also thank Nanoimaging Core, Molecular Interaction Core, Flow Cytometry Research Core (Dr. Charles A. Kuszynski) and Confocal Microscopy Core facilities (University of Nebraska Medical Center).

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Figure 1. Swelling behavior (a,b), drug loading (c,d) and drug release characteristics (e,f) of nanogels

(a) The effective diameter (D_{eff}) and (b) ζ -potential of (▲) nanogel and (■) FA-nanogel as a function of pH. The insert presents AFM imaging of nanogel and reveals characteristic spherical morphology, which did not change after conjugation of the folate (Figure S1). (c,d) Drug loading capacity of nanogels (solid bars) and FA-nanogels (transparent bars) at different molar ratios of (c) CDDP or (d) DOX to carboxylate groups at 37°C. The arrows designate molar ratios of the drug to carboxylate groups, at which (1) FA-nanogels or (2) both nanogels and FA-nanogels become unstable and precipitate over days. (e,f) Drug release profiles for (e) CDDP and (f) DOX in nanogel and FA-nanogel in PBS, pH 7.4 or ABS, pH 5.4 at 37°C: (▲) nanogel, pH 5.5, (■) FA-nanogel, pH 5.5, (Δ) nanogel, pH 7.4, and (□) FA-nanogel, pH 7.4. (A–D) Measurements are made in the absence of a buffer. The degree of modification of FA-nanogel was 0.3 (a, b) or 0.2 (c–f) μmol folate/mg polymer. (e, f) The molar ratios of drug to carboxylate groups upon loading were 0.5 (CDDP) or 0.25 (DOX). Data are mean $\pm$ SD, $n = 5$ (a, b) or 3 (c–f).

Figure 2. Uptake of FA-nanogels by A2780 cell line

FITC-labeled nanogel (▲) and FA-nanogel (■) in A2780 cells as a function of (a) exposure time, (b) concentration of free FA and (c) degree of modification of FA-nanogels. (a, b) FA-nanogels contained 0.2 μmol folate/mg polymer. (b, c) Cells were exposed to FA-nanogels for 3 h. Data are mean ± SEM (n=3). (d) Cellular trafficking of nanogel and FA-nanogel in A2780 cells. The cells were pulsed for 45 min with the FITC-labeled nanogels in the presence of LysoTrackerTM Red (10 min) at 37°C, followed by live cell imaging. In all experiments cells were treated with 0.3 mg/ml of the nanogels.

Figure 3. Cellular delivery and cytotoxicity of nanogel-formulated drugs

(a) Uptake of CDDP-loaded nanogel and FA-nanogel in A2780 and A549 cells: 1 –nanogel/CDDP, 2 – FA-nanogel/CDDP loaded with the drug after attachment of folate; 3 – FA-nanogel/CDDP loaded with the drug prior to attachment of folate. (b) Differential uptake of FA-nanogel/CDDP in FR-positive and FR-negative cell populations in individual cultures and A2780 and A549 co-culture. (c) Uptake of different DOX formats in FR-positive A2780 cells at 4°C and 37°C. (d) Cytotoxicity of DOX formats in A2780, A549 and NIH 3T3 cells. (c, d) 4 – free DOX, 5 –nanogel/DOX, 6 – FA-nanogel/DOX. (a–d). FA-nanogel contained 0.2 μmol folate/mg polymer. The drug to carboxylate groups ratio was 0.5. Cells were treated with 0.3 mg/ml of nanogels for 3 h (a, b); 10 $\mu\text{g/ml}$ DOX equivalent for 1 h (c); or 20 $\mu\text{g/ml}$ DOX equivalent for 24 h (d). Data are mean $\pm$ SEM (n = 3), * p <0.05, ** p < 0.01, NS - not significant.

Figure 4. *In vivo* tumor accumulation and antitumor efficacy of CDDP formats in A2780 human ovarian cancer xenograft-bearing female nude mice

(a) Concentration of Pt in tumors by ICP-MS. Mice were sacrificed at 4th day after receiving four i.v. injections of (1) free CDDP, (2) nanogel/CDDP, (3) FA-nanogel/CDDP and (4) mixture of 1 mg/kg FA and FA-nanogel/CDDP. Data are mean $\pm$ SD, $n = 4-5$ (tumors), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(b)** Tumor growth, **(c)** survival and **(d)** body weight loss after administration of (◇, 1) 5% dextrose, (◆, 2) nanogel alone, (●, 3) CDDP alone, (▲, 4) nanogel/CDDP, (■, 5) FA-nanogel/CDDP and (□, 6) mixture of FA and FA-nanogel/CDDP. Drug formulations were injected in 100 μ l at a dose of 4 mg CDDP equivalents/kg body weight every 4th day as indicated by the arrows. Data are mean $\pm$ SEM, $n = 7-8$, * $p < 0.05$, ** $p < 0.01$, NS - not significant.

Scheme 1.
Synthetic scheme for preparation of FA-nanogel.

Table 1

Uptake of FITC-labeled nanogels and FA-nanogels with different degrees of modification in A2780 cells.[‡]

Formulation	% Gated Cells					
	Total uptake			Internalized		
	37°C	4°C	37°C	4°C	37°C	4°C
nanogel	39.0 ± 2.2	6.5 ± 1.0	37.8 ± 4.1	3.8 ± 0.4	1.1 ± 0.8	2.8 ± 1.5
FA-nanogel †	94.7 ± 2.7	26.4 ± 0.7	88.0 ± 3.1	7.0 ± 4.3	6.7 ± 2.7	19.4 ± 2.5
FA-nanogel §	84.8 ± 2.3	46.1 ± 0.7	55.0 ± 1.4	3.5 ± 0.8	29.8 ± 2.8	42.6 ± 0.4

[‡] Total uptake was defined as % gated cells after washing of the cells with PBS. Cells with internalized nanogels were determined as % gated cells after the acid treatment (acidic saline, pH 3). Fraction of cells in which nanogels were membrane bound but did not internalize were determined as the difference between the total uptake and internalized fractions. In all experiments cells were exposed to 0.3 mg/ml of nanogels for 3h. Degree of modification of FA-nanogels was (†) 0.15 and (§) 1.4 µmol folate/mg polymer.

Table 2

IC₅₀ values (CDDP or DOX equivalents) of different drug formats in A2780 cell line.

Formulation	IC ₅₀ (CDDP equivalents, µg/ml or DOX equivalents, ng/ml) [‡]		
	6 h	24 h	48 h
free CDDP	2.34 ± 0.1	0.67 ± 0.1	0.60 ± 0.1
nanogel/CDDP	100.20 ± 1.0	15.91 ± 2.6	8.39 ± 0.9
FA-nanogel/CDDP [‡]	72.33 ± 1.0	8.53 ± 0.6	5.12 ± 0.2
free DOX	461.20 ± 50.3	54.05 ± 8.0	27.05 ± 3.1
nanogel/DOX	513.80 ± 46.6	42.75 ± 9.6	20.32 ± 1.5
FA-nanogel/DOX	397.35 ± 29.0	30.09 ± 4.4	18.29 ± 1.1

[‡]The IC₅₀ values represent the means ± SEM of at least 4 independent experiments. Each of the nanogel groups differ significantly from the free drug and each other (p < 0.0001).

[‡]Folic acid was conjugated to the nanogel after CDDP loading (see text for more details).