Targeted Nanogels: A Versatile Platform for Drug Delivery to Tumors

Eric A. Murphy, Bharat K. Majeti, Rajesh Mukthavaram, Lisette M. Acevedo, Leo A. Barnes, and David A. Cheresh

Abstract

Although nanoparticle-based drug delivery formulations can improve the effectiveness and safety of certain anticancer drugs, many drugs, due to their chemical composition, are unsuitable for nanoparticle loading. Here, we describe a targeted nanogel drug delivery platform that can (i) encapsulate a wide range of drug chemotypes, including biological, small molecule, and cytotoxic agents; (ii) display targeting ligands and polymeric coatings on the surface; (iii) enhance drug retention within the nanogel core after photo-cross-linking; and (iv) retain therapeutic activity after lyophilization allowing for long-term storage. For therapeutic studies, we used integrin αvβ3–targeted lipid-coated nanogels with cross-linked human serum albumin in the core for carrying therapeutic cargoes. These particles exhibited potent activity in tumor cell viability assays with drugs of distinct chemotype, including paclitaxel, docetaxel, bortezomib, 17-AAG, sorafenib, sunitinib, bosutinib, and dasatinib. Treatment of orthotopic breast and pancreas tumors in mice with taxane-loaded nanogels produced a 15-fold improvement in antitumor activity relative to Abraxane by blocking both primary tumor growth and spontaneous metastasis. With a modifiable surface and core, the lipid-coated nanogel represents a platform technology that can be easily adapted for specific drug delivery applications to treat a wide range of malignant diseases. Mol Cancer Ther; 10(6); 972–82. ©2011 AACR.

Introduction

The promise of nanotechnology has fueled research efforts in drug delivery leading to clinical testing of a variety of formulations. The most notable of these drug delivery systems are Doxil (liposomal doxorubicin; refs. 1–3), SMANCS (polymeric conjugate of neocarzinostatin; refs. 4), and Abraxane (albumin-bound paclitaxel nanoparticles; refs. 5, 6), which are approved by the Food and Drug Administration (FDA) as cancer therapeutics. Several other examples of drug delivery systems are in advanced clinical testing for cancer including polymeric micelles such as NK105 and NK012, which deliver paclitaxel and SN-38, respectively, and polymer conjugates such as CT-2103 (Poliglumex), PK1, and CT-2106, which deliver paclitaxel, doxorubicin, and camptothecin, respectively (7). These drug delivery systems use highly potent chemotherapeutics and offer benefit by improving the therapeutic index and reducing the dose-limiting side effects associated with their respective drug cargoes (8, 9). All the nanoparticles that are FDA approved for cancer therapy or in advanced clinical testing rely on the enhanced permeability and retention (EPR) mechanism, which enables passive targeting through the disorganized and leaky vasculature and reduced lymphatic drainage associated with tumors (10). However, incorporating targeting ligands on the surface of nanoparticles enables active targeting of the tumor or neovasculature, which can lead to a further improvement in therapeutic index by reducing toxicity.

Although nanoparticle formulations such as Doxil and Abraxane are highly efficacious, the versatility of these drug delivery systems is limited on the basis of the drug chemotypes that can be loaded, the drug release kinetics, or the feasibility of incorporating surface modifications. For example, liposomal drug delivery systems effectively load drug cargoes such as doxorubicin or vincristine that are amenable to a chemical gradient method (11, 12). The chemical gradient loading requires therapeutics containing a weak base to enable stable loading within the acidic intraliposomal space via formation of a transmembrane pH gradient. However, the majority of amphiphilic drugs must be loaded via passive entrapment, leading to inefficient loading and rapid release rates that limit therapeutic efficacy. Abraxane, conversely, was developed to deliver paclitaxel (hydrophobic drug) but is difficult to functionalize with targeting ligands or surface modifications like other nanoparticle platforms such as carbon nanotubes.
(13), lipid-monolayer-stabilized perfluorocarbon emulsions (14), and polymeric micelles (15). Because Abraxane quickly breaks into smaller 10-nm diameter particles in the blood stream (16), the stability of the albumin–drug interaction in plasma may not be appropriate for non-taxane-based chemotherapeutics.

Combining the advantages of both liposomes and nanoparticles-based albumin vehicles such as Abraxane could offer great advantages for both surface functionality and stable drug loading. Here, we describe lipid-coated nanogels that use a lipid bilayer as a template to control the formation of a cross-linked protein/polymeric core. The lipid bilayer incorporates targeting ligands and polymeric coatings such as polyethylene glycol (PEG), whereas the cross-linked core consists of proteins such as human serum albumin (HSA) or α1-acid glycoprotein, which serve as carriers for the drug cargoes. In this work, we show that integrin αvβ3–targeted, albumin-based nanogels load a wide array of drug chemotypes, and that these nanogels effectively inhibit tumor cell viability in vitro. Importantly, the targeted nanogels loaded with taxanes showed a 15-fold improvement in tumor growth suppression compared with Abraxane in models of pancreatic and breast cancer.

Materials and Methods

Animal studies

All animal procedures were conducted in accordance with all appropriate regulatory standards under protocol #S05018 and approved by the UCSD Institutional Animal Care and Use Committee.

Cell culture

M21 melanomas were maintained under standard culture conditions in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS. The MDA-MB-231/LM2-4 cells were a gift from Dr. Robert Kerbel (Toronto, Canada) and maintained as described previously (17). R40P cells were isolated and cultured from KRasG12D, Ink4a/Arflox/lox mice after 7 weeks; both the mice and tumor cell isolation procedure were described previously (18, 19). The cells were routinely screened for Mycoplasma and tested for pathogens before use in vivo in accordance with UCSD policies. No authentication of the cell lines was done by the authors.

Reagents

Diroleoylphosphatidylethanolamine (DOPE), distearoylphosphatidylcholine (DSPC), distearoylphosphatidylethanolamine (DSPE), and cholesterol were purchased from Avanti Polar Lipids and the succinimidyl ester-PEO₄-maleimide cross-linker was purchased from Pierce. Irgacure 2959 was a gift from Ciba (the LD₅₀ in 28-day oral toxicity experiments in rats was more than 1,000 mg/kg/d, based on a free public report). Paclitaxel, docetaxel, bortezomib, 17-AAG, sunitinib, sorafenib, dasatinib, and bosutinib were purchased from Chemietek. HSA and α1-acid glycoprotein were purchased from Sigma-Aldrich. Hydroxyethyl-methacrylate-polyethylene glycol (HEMA-PEG) was synthesized as described previously (20). 4-Arm PEG acrylate was purchased from Layson Bio, Inc. Water soluble CdSe/ZnS core nanocrystals (5 nm quantum dots) and iron oxide nanoparticles (5 nm) were purchased from NN-Labs. Gold nanoparticles (2 nm, water soluble) were purchased from NANOCS.

Synthesis of peptide–lipid conjugates

The cyclic peptides, cRGDfK and cRADfK (f denotes ω-phenylalanine), were synthesized by using standard Fmoc solid phase chemistry as described previously (21). Coupling of the cyclic peptides to DSPE was carried out as described previously (19).

Nanogel preparation

The lipid formulation of cholesterol, DOPE, DSPC, DSPE-(PEO)₄-cRGDfK, and DSPE-mPEG2000 (6:6:6:1:1 molar ratio) in chloroform were evaporated under argon gas and the dried lipid film was hydrated with a solution containing the monomer, drug, and photoinitiator in phosphate buffer (pH 7.4) for a minimum of 30 minutes. Specific details of the monomeric solutions and drug loading methods are described in the Supplementary Methods. The solution was vortexed for 1 minute to remove any adhering lipid film and sonicated in a bath sonicator (ULTRasonik 28X) for 1 minute at room temperature to produce multilamellar vesicles (MLV). MLVs were then sonicated with a Ti-probe (Branson 450 sonifier) for 2 minutes to produce small unilamellar vesicles (SUV) as indicated by the formation of a translucent solution. To reduce the size of the SUVs, stepwise extrusion was carried out with the final step being extrusion through a polycarbonate filter with 200-nm pore size (Whatman). The nanogels are then purified by size exclusion chromatography on sepharose CL-4B columns to remove free monomers and drug. After purification, the nanogels are exposed to a handheld UV lamp at 365-nm wavelength for 5 minutes at room temperature. Drug concentrations are measured as described in the Supplementary Methods. Hydrodynamic diameter and zeta potential were measured following photo-cross-linking by using a Malvern NanoZS dynamic light scattering instrument.

Cell viability assay

For 2,3-bis[2-methoxy-4-nitro-S-sulfophenyl]H-tetrazolium-5 carboxanilide inner salt (XTT) assays, cells were grown in 96-well plates overnight and all assays were conducted in growth medium with full serum and additives. For studies with free drugs, 10 mmol/L stocks were serially diluted in dimethyl sulfoxide and then further diluted into the medium to give the appropriate concentration while minimizing precipitation associated with serial diluting in medium alone. For studies with nanogels or Abraxane, the nanoparticles...
were diluted in serum-free medium and added to the cells at 4°C for 20 minutes, washed to remove the particles, and the cells returned to 37°C in complete medium with 10% FBS for an additional 72 hours. Inhibitors were added and removed as mentioned earlier or left in the medium for 72 hours. Cell viability was quantified at 450 nm after the addition of 1 mg/mL XTT solution (Sigma-Aldrich) in phenol red-free DMEM medium containing phenoxyethosulfate (Sigma-Aldrich). Dose–response curves were plotted by using GraphPad Prism software and EC50 values were calculated by this program.

Orthotopic breast cancer model

MDA-MB-231/LM2-4 cells were derived from metastatic sites in the lung in an orthotopic breast cancer model as described previously (17). Briefly, 2 million cells were injected in the mammary fat pad no. 4 and tumors were allowed to grow to the size of approximately 70 to 80 mm3 (17, 22). Nanoparticle dosing was initiated via i.v. administration on an every other day dosing schedule. The breast tumors were measured with calipers after drug administration every other day and tumor size was calculated by the standard formula: Size = (Length × Width2)/2. The animals were sacrificed once the volume of control tumors reached 800 mm3. Abraxane was resuspended in sterile saline and dosed i.v. based on its paclitaxel content at 1, 5, or 15 mg/kg on an every other day dosing schedule. Nanogels containing taxanes were injected intravenously, every other day. For targeting studies, BODIPY 630/650–labeled Abraxane was resuspended in sterile saline and dosed i.v. based on its paclitaxel content at 1, 5, or 15 mg/kg on an every other day dosing schedule. Nanogels containing taxanes were injected intravenously, every other day. For targeting studies, BODIPY 630/650–labeled Abraxane was resuspended in sterile saline and dosed i.v. based on its paclitaxel content at 1, 5, or 15 mg/kg on an every other day dosing schedule.

Results

Design and characterization of the nanogel platform

We designed a new platform technology, lipid-coated nanogels, which enable versatile and stable loading of drug cargoes and imaging agents within a cross-linked core. The lipid bilayer serves a dual purpose for (i) introducing targeting ligands and polymer coatings and (ii) providing an optimal template for controlling nanogel size. In this report, we used a previously described in vivo optimized bilayer composition (19) as the template: DSPC, cholesterol, DOPE, DSPE-mPEG2000, and cRGDK-PEO4-DSPE (6:6:6:1:1 molar ratio). The desired monomers (HEMA-PEG, albumin, α1-acid glycoprotein, etc.) containing drug and a photo-initiator (Irgacure 2959; ref. 25) are added to this lipid film and sonicated to form MLVs (Fig. 1A). Extrusion, purification, and photo-cross-linking of the encapsulated monomers create a targeted lipid-coated nanogel, which enables stable loading of a wide array of chemotypes (schematic in Fig. 1A). Nanogel core compositions include proteins such as HSA or α1-acid glycoprotein (1), and polymers such as HEMA-PEG (2) or 4-arm PEG acrylate (3), which show the ability to form gels of defined size, capable of loading hydrophobic drugs, proteins, peptides, nucleic acids, and/or imaging agents (ref. 4; Fig. 1B). The general schematic of the lipid-coated nanogel components is represented in Fig. 1C, and includes targeting ligands and surface coatings such as PEG on the core. Encapsulation of albumin-bound docetaxel forms homogenous nanogels with approximately 90-nm average hydrodynamic diameter following cross-linking as observed by both dynamic light scattering (Supplementary Table S1) and scanning electron microscopy (Fig. 1D). Various core formulations lead to similar diameters and zeta potentials as summarized in Table 1 and loading of different drug chemotypes into the cores does not affect diameter or zeta potential (data not shown). In addition to the versatile loading properties of the lipid-coated nanogels, we showed that photo-cross-linking slows the drug release rate of nanogels loaded with albumin-bound docetaxel when compared with the non–cross-linked version (Fig. 1E). Cross-linking of the core improves docetaxel retention in mouse plasma with 50% and 30% improvement over the non–cross-linked particles at 2 and 4 hours, respectively (Fig. 1E). This finding is important for efficacy studies because most nanoparticles reach the tumor target within this time frame.

Targeted nanogels show versatile drug loading and effective inhibition of tumor cell viability

To show the versatility of the lipid-coated nanogels, we optimized loading of a wide variety of therapeutics including natural products, kinase inhibitors, proteasome inhibitors, and chaperone inhibitors. For tumor targeting, we used a cyclic peptide ligand for integrin αvβ3 that is
widely accepted as a targeting moiety for the tumor neovasculature (26–29). The integrin \( \alpha_v \beta_3 \)-targeted nanogels (RGD) were loaded with paclitaxel (\( \beta \)-tubulin), docetaxel (\( \beta \)-tubulin), bortezomib (26S-proteasome), 17-AAG (Hsp90), sorafenib (VEGFR2/RAF), sunitinib (broad RTK), bosutinib (Src), or dasatinib (Src/Abl), and tested in cell viability assays with M21 human melanoma cells, which express integrin \( \alpha_v \beta_3 \) (Fig. 2A and B). A summary of the encapsulation efficiency for each of these drugs in the HSA-based nanogel core is

![Figure 1. Formation of lipid-coated nanogels. A, the method for controlled photo-cross-linking and formation of the nanogel core. The lipid bilayer is used to encapsulate monomers and a photoinitiator (Irgacure 2959) and this is exposed to UV light to form nanogel cores. B, examples of various monomeric inputs and imaging agents that can be used to form the nanogel core are shown. 1, proteins such as HSA, \( \alpha \)-1-acid glycoprotein, and others serve as hosts for drug binding. Other inputs include polymers such as HEMA-PEG (2) or a 4-arm PEG acrylate (3). 4, to enable tracking with imaging agents, iron oxide nanoparticles, gold nanoparticles, or fluorescent nanocrystals can be encapsulated in the core, provided they are small enough (1–10 nm in diameter). C, a schematic representation of the final nanogel product with a lipid bilayer, presenting targeting ligands and polymeric coatings surrounding the gel core containing drug cargoes. D, scanning electron micrograph of a lipid-coated nanogel with a PEG2000 coating, cyclicRGDfK targeting ligand, and photo-cross-linked HSA-bound docetaxel core. E, nanogels with RGD peptide on the surface and docetaxel-loaded HSA in the cross-linked core were incubated in the presence of 20% plasma for the indicated time points. Docetaxel was extracted and quantified as described in the Supplementary Methods by using high-performance liquid chromatography.](https://www.aacrjournals.org/mct/article-figures/10/6/975/291574/Targeted-Nanogels-for-Drug-Delivery)
taxane-loaded nanogels (RGD-Doc-NG) or targeted, Abraxane for the treatment of breast cancer in patients (30), we compared the effects of taxane-loaded nanogels to Abraxane in a MDA-MB-231 human breast cancer cell line variant LM2-4 (17). In vitro, either targeted, docetaxel-loaded nanogels (RGD-Doc-NG) or targeted, paclitaxel-loaded nanogels (RGD-Pac-NG), with EC50 values of 0.348 and 0.231 μmol/L, respectively, showed a more than 10-fold enhancement in reducing cell viability when compared with Abraxane alone (3.374 μmol/L; Fig. 3A).

After establishing targeting in vitro, the RGD-Doc-NGs were tested for targeting to the tumor vasculature. To assess the effect of the taxane-loaded nanogels on both tumor targeting and tumor growth in vivo we used an MDA-MB-231/LM2-4 orthotopic breast cancer model. Fluorescent RGD-Doc-NGs (vascular targeted) or RAD-Doc-NGs (nontargeted) were i.v. administered at a 1 mg/kg dose of docetaxel for 5 hours, and the tumors were resected and imaged with confocal microscopy to view accumulation of the nanogels (green) in the tumor vasculature (red; Fig. 3B, top). The vascular targeted RGD-Doc-NGs targeted the vascular beds within the tumor and bright, punctate nanogel fluorescence was colocalized with the tumor vessels throughout the tumor area (Fig. 3B). As expected, the RAD-Doc-NGs did not show any significant vascular targeting in the breast tumors and only slight signals were detected in major vessels, presumably from the remaining circulating nanogels in the bloodstream (Fig. 3B). For the efficacy studies, treatment was initiated on day 10 (~70–80 mm3) with targeted, docetaxel-loaded nanogels (RGD-Doc-NG); nontargeted, docetaxel-loaded nanogels (RAD-Doc-NG); or Abraxane. At 1 mg/kg effective taxane dose, Abraxane showed no effect when compared with saline vehicle on day 30 with tumor volumes of approximately 830 mm3 (Fig. 3B, bottom). The untreated RAD-Doc-NG showed intermediate activity with a tumor volume of 190 mm3 on day 30 (Fig. 3B). Importantly, the targeted RGD-Doc-NG suppressed tumor growth significantly with an average tumor volume of 190 mm3 (Fig. 3B). The improved efficacy with the targeted nanogel (RGD-Doc-NG) compared with the nontargeted nanogel (RAD-Doc-NG) shows the benefit of active targeting over passive tumor uptake.

**Comparison of targeted nanogels containing taxanes to Abraxane in breast cancer**

On the basis of the approval of the taxanes and Abraxane for the treatment of breast cancer in patients (30), we compared the effects of taxane-loaded nanogels to Abraxane in a MDA-MB-231 human breast cancer cell line variant LM2-4 (17). In vitro, either targeted, docetaxel-loaded nanogels (RGD-Doc-NG) or targeted, paclitaxel-loaded nanogels (RGD-Pac-NG), with EC50 values of 0.348 and 0.231 μmol/L, respectively, showed a more than 10-fold enhancement in reducing cell viability when compared with Abraxane alone (3.374 μmol/L; Fig. 3A).

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In a second experiment, we compared the effects of targeted RGD-Doc-NG or RGD-Pac-NG to a dose response of Abraxane, ranging from 1 to 15 mg/kg total paclitaxel administration (Fig. 3C). In this study, we observed a more than 5-fold enhancement in tumor growth suppression with 1 mg/kg of RGD-Pac-NG.

<table>
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<th>Core composition</th>
<th>Diameter, nm</th>
<th>Zeta potential, mV</th>
<th>PDI</th>
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<td>4-Arm PEG acrylate</td>
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<td>α1-Acid glycoprotein</td>
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NOTE: All nanogels were prepared with the same lipid formulation as described in the Materials and Methods.

Abbreviation: PDI, polydispersity index.
Targeted Nanogels for Drug Delivery

Figure 2. Targeted-lipid-coated nanogels show efficacy with a wide range of chemotypes in vitro. A-C, M21 melanomas (integrin \( \alpha_v \beta_3 \)-positive) were incubated with cyclic RGDK nanogels (RGD-NG) loaded with various small molecules bound to HSA. After a 20-minute exposure at 4 °C, the nanogels were removed and the cells were incubated at 37 °C for an additional 72 hours before XTT measurements for cell viability. A and B compared the RGD-NGs with a wide range of chemotypes, whereas C measured the effect of targeting integrin \( \alpha_v \beta_3 \) on cell viability of nanogels loaded with albumin-bound docetaxel. RGD is the integrin \( \alpha_v \beta_3 \)-targeting peptide and RAD is the untargeted control peptide ligand. All experiments were carried out in triplicate and are represented as the mean ± SEM.

Representative images show high levels of active caspase-3 (red) in the tumors treated with 15 mg/kg of Abraxane, or 1 mg/kg of either RGD-Pac-NG or RGD-Doc-NG (Fig. 3D). As expected, based on the tumor growth curves, the 1 mg/kg Abraxane group showed little to no induction of apoptosis in the sections examined (Fig. 3D). Tumor vessels (green) are largely surrounded by areas of apoptosis (red) in treatment groups that caused tumor suppression, which is not surprising as this is typical with nanotherapeutics.

Comparison of targeted nanogels containing taxanes to Abraxane in pancreas cancer

Recent clinical trials have reported impressive response rates for pancreatic cancer patients treated with an Abraxane–gemcitabine combination (31). For our studies in pancreas cancer, we evaluated the nanogels by using mouse R40P pancreatic tumor cells, which were originally derived from a spontaneous mouse pancreatic cancer model (18). The RGD-Doc-NG or RGD-Pac-NG (0.096 vs. 0.241 \( \mu \text{mol/L} \), respectively) greatly improved the cellular EC\(_{50}\) relative to Abraxane (2.913 \( \mu \text{mol/L} \)) in cell viability studies (Fig. 4A). Additionally, we assessed the effects of lyophilization and storage on nanogel activity by reconstituting lyophilized nanogels and measuring their capacity to impact the viability of R40P cells. Lyophilized RGD-Doc-NG or RGD-Pac-NG retained the same physical characteristics (hydrodynamic diameter) and activity against R40P cell viability as the freshly prepared nanogels, even 3 weeks after storage in the lyophilized state (Supplementary Fig. S1).

Next, we tested the nanogels in the orthotopic R40P pancreatic cancer model comparing targeted nanogels containing paclitaxel or docetaxel to an Abraxane dose response. Importantly, we previously reported that the R40P cells did not display surface expression of integrin \( \alpha_v \beta_3 \) (19). At 1 mg/kg taxane dose, RGD-Doc-NG or RGD-Pac-NG reduced primary pancreatic tumor growth by 38% or 43%, respectively (Fig. 4B). However, the 1 mg/kg RGD-Doc-NG or RGD-Pac-NG showed similar efficacy to 15 mg/kg Abraxane, leading to a 15-fold improvement in tumor suppression (Fig. 4B). R40P cells are known to metastasize to the hepatic hilar lymph node and resection of the lymph node is a reliable measurement of metastatic rate (19). Treatment with either 1 mg/kg RGD-Doc-NG or RGD-Pac-NG, or 15 mg/kg of Abraxane completely prevented metastasis to the hepatic hilar lymph node (Fig. 4C). Importantly, 1, 5, and 15 mg/kg of Abraxane produced a dose response with respect to controlling metastasis to the lymph node as we measured 40, 25, and 4 mg of burden upon lymph node resection (Fig. 4C). Corresponding with the activity against both the primary tumor and metastasis to the hepatic hilar lymph node, treatment with RGD-Pac-NG, RGD-Doc-NG, or Abraxane at 15 mg/kg induced apoptosis in the primary tumors as measured by staining for active caspase-3 (red; Fig. 4D). The 1 mg/kg Abraxane-treated tumors showed minor amounts of apoptosis as

because 5 mg/kg of paclitaxel in Abraxane did not suppress tumor growth to the same tumor volume (430 vs. 710 mm\(^3\); Fig. 3C). The 1 mg/kg docetaxel in RGD-Doc-NG produced the same result as 15 mg/kg of Abraxane treatment, showing the value of targeting a docetaxel-loaded nanogel to the tumor. Our results suggest that docetaxel-loaded nanoparticles will likely lead to improved efficacy against breast cancer.

At the end of the study, the primary breast tumors were sectioned and stained for active caspase-3 to evaluate the level of apoptosis caused by the nanoparticle treatments.
expected on the basis of the limited suppression of tumor growth (Fig. 4D). Of note, the RGD-Pac-NG treatment induced the highest level of apoptosis relative to the other groups, which may represent an advantage of paclitaxel over docetaxel in this syngeneic pancreatic cancer model. These findings reveal the advantages of our new targeted nanogel system over our previously published targeted-stealth liposome encapsulating doxorubicin (19). A 15-fold improvement in efficacy in the orthotopic pancreatic carcinoma model is expected to correlate with reduced toxicity on the basis of the reduction in taxane concentration required for therapeutic efficacy.

**Discussion**

Although nanoparticles approved for cancer therapy such as Abraxane and Doxil provide benefit by reducing dose-limiting toxicities, most nanoparticles are only capable of loading a limited number of drug cargoes with defined chemical properties. Here, we describe lipid-coated nanogels that can be easily tailored for specific uses by modifying the nanogel core to load various biological agents and small molecule therapeutics, and the surface properties including targeting ligands and polymer coatings for enhancing in vivo efficacy. The lipid bilayer acts as a template for the core and can be extruded to a defined size (100 nm hydrodynamic diameter) before photo-cross-linking to form the nanogel, which enables precise control of nanogel formation. Photo-cross-linking of the nanogel core enhances drug retention (Fig. 1E), thereby improving a major limitation associated with liposomes, micelles, and coblock polymeric systems.

In our studies, we focused on utilizing proteins such as HSA or α1-acid glycoprotein because these plasma proteins are known to bind cancer therapeutics in the bloodstream (32). Using HSA as the host for drug binding, we loaded several different chemotypes into the nanogel core including kinase inhibitors, taxanes, chaperone inhibitors, and proteasome inhibitors to show the versatility of the nanogel platform (Fig. 2A and B; Table 2). Imaging agents such as iron oxide nanoparticles and quantum dots are also easily encapsulated in the nanogel core (Table 1), enabling the possibility of theranostic applications to follow disease outcome during therapy. Another advantage is the ability to tune the nanogel core with various polymers that will alter the release kinetics of the therapeutic cargo. The photo-cross-linking step is amenable to use with polymers capable of forming hydrogels such as HEMA-PEG and 4-arm PEG acrylate, which enable loading of oligonucleotides, growth factors, and enzymes.

A major limitation of many nanoparticle systems is the rapid release of the encapsulated therapeutic. The nanogel system described here may be optimized for each therapeutic by tailoring the core to improve stable drug loading. In this study, we added HSA as a host for hydrophobic compounds and then used a lipid bilayer to encapsulate this solution to form nanoparticles of desired size and provide a template for photo-cross-linking. We also showed that acrylates such as HEMA-PEG or a 4-arm PEG acrylate could be used to form the core of the nanogel (Table 1), and these types of polymers have been used extensively as hydrogels for sustained release applications as reviewed in ref. 33. Additionally, other groups have used novel coblock polymers or hybrid lipid/polymer systems that self-assemble to form structures with a hydrophobic core surrounded by a hydrophilic shell, or vice versa. One novel approach used poly(ethylene oxide)-b-poly (methacrylic acid) and divalent cations to form micelles, and the doxorubicin-loaded ionic cores were chemically cross-linked with cystamine to improve drug retention within the core (34). Additionally, alginate cores have been coated with an anionic surfactant, Aerosol OT, for improved release kinetics of weakly basic drugs such as verapamil and doxorubicin (35). Another integrated approach termed nanocells used a doxorubicin-poly(lactic-co-glycolic acid) as the core that was coated with lipid containing combretastatin A4, which resulted in tumor suppression by delivering an antiangiogenic agent and chemotherapeutic simultaneously (36). Recently, a microfluidic approach was reported for

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**Table 2. Summary of cell viability EC₅₀ values in M21 cells from the curves in Figure 2A and B**
the assembly of homogenous populations of similar hybrid lipid-polymeric systems consisting of a lecithin monolayer surrounding a poly(lactic-co-glycolic acid) hydrophobic core (37), and this is an important development that may greatly improve the production of these systems when compared with traditional emulsion-solvent evaporation techniques. Altogether, both the studies described above and the nanogel system described in this report represent a current movement toward integrated nanoparticle platforms that use a core/shell approach to avoid rapid drug release, while providing a versatile solution to load a wide array of therapeutics and imaging agents.

Unfavorable pharmacokinetics and dose-limiting toxicities are 2 of the most common limitations associated with small molecule chemotherapeutics. Drug-associated toxicities include neuropathy, neutropenia, cardiotoxicity, hypertension, as well as many other serious side effects. Nanoparticle-based drug delivery systems offer a strategy to improve therapeutic efficacy by increasing accumulation of the drug at the disease site and therefore decreasing the toxicity arising from drug exposure to normal tissues. However, the value of actively targeting receptors on either the tumor (folate, EGFR antibody, transferrin) or the tumor neovasculature (integrin αvβ3) remains controversial. All of the major nanotherapeutics

Figure 3. Targeted nanogels containing taxanes suppress orthotopic breast cancer growth. A, XTT cell viability assay for MDA-MB-231/LM2-4 breast cancer cells comparing Abraxane, docetaxel-loaded nanogels (RGD-Doc-NG), and paclitaxel-loaded nanogels (RGD-Pac-NG). The experiment was carried out in triplicate with data represented as the mean ± SEM. B–D, human MDA-MB-231/LM2-4 cells were injected into the mammary fat pad no. 4 of athymic nu/nu mice and primary tumors were established (average size 70–80 mm3) before initiating dosing. B, top, comparison of the RGD-Doc-NG and RAD-Doc-NG vascular targeting in MDA-MB-231/LM2-4 tumors on day 21. RGD-Doc-NGs or RAD-Doc-NGs labeled with 1% BODIPY 630/650-conjugated DSPE in the lipid formulation were i.v. injected (1 mg/kg docetaxel concentration) and the tumors were imaged with confocal microscopy at 5 hours postinjection. Red, rhodamine-labeled G. simplicifolia lectin for staining the endothelium and green represents nanogel binding. Scale bar, 100 μm. Bottom, a taxane dose of 1 mg/kg was compared for the nanogels and Abraxane (Abx). Dosing was via i.v. administration on an every other day dosing schedule until the end of the experiment. Tumors were measured every 2 days. Targeted nanogels carrying HSA-bound docetaxel (RGD-Doc-NG) were compared with either untargeted nanogels (RAD-Doc-NG) or Abraxane. n = 8/group; P < 0.01 for RGD-Doc-NG or RAD-Doc-NG versus PBS control and for RAD-Doc-NG versus RGD-Doc-NG. C, nanogels containing 1 mg/kg docetaxel or paclitaxel were compared with a dose response of Abraxane at 1, 5, and 15 mg/kg of total paclitaxel n = 8/group; P < 0.01 for RGD-Doc-NG, RGD-Pac-NG, or Abx15 versus Abx1. All data points are represented as the mean ± SEM D. Immunofluorescence of sections from the tumors treated in C show induction of apoptosis. Red, anti-active caspase-3 (cells undergoing apoptosis); green, CD31 (blood vessels); and blue, TO-PRO-3 as a nuclear counterstain. Scale bar, 100 μm.
that are FDA approved or in advanced clinical trials for cancer therapy accumulate at the tumor site via the EPR mechanism. Two notable nanoparticles, Doxil (1–3) and Abraxane (5, 6), show passive uptake at the tumor site via the EPR mechanism. However, adding targeting ligands to either liposomal doxorubicin (19, 27) or albumin-bound paclitaxel nanoparticles (38) improves efficacy in preclinical studies. We used a cyclic RGD peptide serving as an integrin αvβ3–targeting ligand that has been validated in several previous studies to selectively target the tumor neovasculature (19, 39–41). Targeted docetaxel-loaded nanogels (RGD-Doc-NG) greatly suppressed the growth of an orthotopic breast cancer model (Fig. 3B), suggesting that active targeting provides a significant benefit. As expected, the untargeted docetaxel-loaded nanogel (RAD-Doc-NG) showed intermediate tumor suppression in the orthotopic breast cancer model, which we assume is mainly from passive targeting through the EPR mechanism because no vascular targeting is observed with the untargeted particles (Fig. 3B). These results support the use of an active targeting approach over relying purely on passive uptake, and the nanogel system can be easily modified to target receptors either on the tumor or the vasculature.

Pancreatic cancer represents a difficult tumor type for drug delivery, based on the highly desmoplastic nature of the tumor as a result of the large deposition of collagen and other extracellular matrix proteins.

Figure 4. Targeted nanogels containing taxanes suppress orthotopic pancreatic cancer growth. A, XTT cell viability assay for R40P pancreatic cancer cells comparing Abraxane, docetaxel-loaded nanogels (RGD-Doc-NG), and paclitaxel-loaded nanogels (RGD-Pac-NG). The experiment was carried out in triplicate with data represented as the mean ± SEM. B and C, murine R40P cells derived from a spontaneous mouse model of pancreatic adenocarcinoma were injected into the pancreas and allowed to establish tumors for 7 days. At this time, mice were dosed by i.v. administration on an every other day dosing schedule. Similar to Fig. 3C, nanogels containing either 1 mg/kg of docetaxel or paclitaxel were compared with a dose response of Abraxane at 1, 5, and 15 mg/kg of paclitaxel in Abraxane. At day 21, primary tumors (B) and hepatic hilar lymph nodes (LN; C) were resected and weighed, n = 9/group. Measurement of the hepatic hilar lymph node corresponds to metastatic burden in this model. P < 0.025 for RGD-Doc-NG, RGD-Pac-NG, and Abx15 versus Abx1 for both B and C. All data points are represented as the mean ± SEM. D, immunofluorescence of sections from the primary tumors treated in B show induction of apoptosis. Red, anti-active caspase-3 (cells undergoing apoptosis); green, CD31 (blood vessels), and blue, TO-PRO-3 as a nuclear counterstain. Scale bar, 100 μm.
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