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Targeted Nanogels: A Versatile Platform for Drug Delivery to Tumors

Eric A. Murphy^{†,1}, Bharat K. Majeti^{†,1}, Rajesh Mukthavaram¹, Lisette M. Acevedo¹, Leo A. Barnes¹, and David A. Cheresh^{1,*}

¹UCSD NanoTumor Center, Department of Pathology, Moores Cancer Center, University of California at San Diego, 3855 Health Sciences Drive, La Jolla, California, 92093, USA

Abstract

While nanoparticle-based drug delivery formulations can improve the effectiveness and safety of certain anti-cancer drugs, many drugs, due to their chemical composition, are unsuitable for nanoparticle loading. Here, we describe a targeted nanogel drug delivery platform that can 1) encapsulate a wide range of drug chemotypes including: biological, small molecule, and cytotoxic agents 2) display targeting ligands and polymeric coatings on the surface, 3) enhance drug retention within the nanogel core after photo-crosslinking, and 4) retain therapeutic activity after lyophilization allowing for long term storage. For therapeutic studies, we utilized integrin $\alpha\beta 3$ -targeted lipid-coated nanogels with crosslinked 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 anti-tumor 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.

Keywords

Breast cancer; Pancreatic cancer; Novel drug delivery systems; Kinase and phosphatase inhibitors; Tubulin-targeted agents; Nanoparticles; Nanogels

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 (1–3), SMANCS, polymeric conjugate of neocarzinostatin (4), and Abraxane®, albumin bound paclitaxel nanoparticles (5, 6), which are all FDA approved 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). The drug delivery systems mentioned above utilize highly potent chemotherapeutics and offer benefit by improving the therapeutic

*Correspondence should be addressed to David A. Cheresh, 3855 Health Sciences Drive #0803, La Jolla, California, 92093, USA
dcheresh@ucsd.edu Phone: 858-822-2232; Fax: 858-822-2630.

[†]These authors contributed equally to this work.

index and reducing the dose-limiting side effects associated with their respective drug cargoes (8, 9). All of the nanoparticles that are FDA approved for cancer therapy or in advanced clinical testing rely on the enhanced permeability and retention mechanism (EPR), 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 based on 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). Since 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 nanoparticle-based albumin vehicles like Abraxane could offer great advantages for both surface functionality and stable drug loading. Here, we describe lipid-coated nanogels that utilize a lipid bilayer as a template to control the formation of a crosslinked protein/polymeric core. The lipid bilayer incorporates targeting ligands and polymeric coatings such as polyethylene glycol, while the crosslinked core consists of proteins such as human serum albumin (HSA) or α 1-acid glycoprotein, which serve as carriers for the drug cargoes. In this work, we show that integrin α v β 3 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 demonstrated a 15-fold improvement in tumor growth suppression compared to 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 DMEM supplemented with 10% FBS. The MDA-MB-231/LM2-4 cells were a gift from Dr. Robert Kerbel (Toronto, Canada) and maintained as previously described (17). R40P cells were isolated and cultured from a spontaneous pancreatic tumor in *Pdx1-Cre, LSL-KRas^{G12D}, Ink4a/Arf^{lox/lox}* mice after 7 weeks and both the mice and tumor cell isolation procedure were previously described (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 performed by the authors.

Reagents

Dioleoylphosphatidylethanolamine (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 above 1000 mg/kg/d based on a free public report). Paclitaxel, docetaxel, bortezomib, 17-AAG, sunitinib, sorafenib, dasatinib, and bosutinib were purchased from Chemietek. Human serum albumin and α 1-acid glycoprotein were purchased from Sigma-Aldrich. HEMA-PEG (hydroxyethylmethacrylate-polyethylene glycol) was synthesized as previously described (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 N-N labs. Gold nanoparticles (2 nm, water soluble) were purchased from NANOCs.

Synthesis of peptide-lipid conjugates

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

Nanogel Preparation

The lipid formulation of Cholesterol:DOPE:DSPC:DSPE-(PEO)₄-cRGDfK: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 min. 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 28 $\times$) for 1 min 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 (SUVs) as indicated by the formation of a translucent solution. To reduce the size of the SUVs, stepwise extrusion was performed 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 min at RT. Drug concentrations are measured as described in the Supplementary Methods. Hydrodynamic diameter and zeta potential were measured following photo-crosslinking using a Malvern NanoZS dynamic light scattering instrument.

Cell viability assay

For 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 mM stocks were serially diluted in DMSO 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 min., washed to remove the particles, and the cells returned to 37°C in complete medium with 10% FBS for an additional 72 h. Inhibitors were added and removed as above or left in the medium for 72 h. 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 phenoxymethosulfate (Sigma-Aldrich). Dose-response curves were plotted using GraphPad Prism software and EC₅₀s were calculated using 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 (#4) and tumors were allowed to grow to the size of ~ 70–80 mm (17, 22). Nanoparticle dosing was initiated via iv administration on a qod schedule. The breast tumors were measured with calipers after drug administration every other day and tumor size was calculated using the standard formula: $\text{Size} = (\text{Length} \times (\text{Width})^2) / 2$. The animals were sacrificed once control tumors reached >800 mm³. Abraxane was resuspended in sterile saline and dosed intravenously, based on its paclitaxel content at 1, 5, or 15 mg/kg on a qod schedule. Nanogels containing taxanes were injected intravenously, qod. For targeting studies, BODIPY 630/650-labeled nanogels (1% BODIPY 630/650-DSPE fluorescent lipid incorporated in the lipid formulation) were injected iv on day 21 and the tumors were resected at 5 h post-injection. At 30 min prior to tumor harvesting, mice were injected iv with 20 µg rhodamine-labeled *Griffonia Simplicifolia* lectin to label mouse endothelial cells (GSL I – BSL I, Vector Labs). Tumors were imaged via whole mount on glass slides by confocal microscopy (Nikon C1si, Nikon Instruments) using the z-stack and channel series features in the software.

Orthotopic pancreatic cancer model

The orthotopic pancreatic carcinoma model has been previously described (19, 23, 24). Briefly, 6–8 week old athymic nu/nu mice were injected with 250,000 murine R40P cells in the tail of the pancreas. Nanogels containing taxanes or Abraxane were injected intravenously, qod, starting on day 7 post-surgical implantation of the cells. On day 21, the primary tumor as well as the hepatic hilar lymph node were resected and weighed. Dosing was identical to the breast cancer model. Immunofluorescence is described in the Supplementary Methods.

Statistical analysis

Error bars represent mean values $\pm$ s.e.m. The statistical significance of the experiments was determined using a two-tailed Student's *t*-test; *p* values < 0.05 were considered significant.

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 crosslinked core. The lipid bilayer serves a dual purpose for 1) introducing targeting ligands and polymer coatings and 2) 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 cRGDFK-PEO₄-DSPE (6:6:6:1:1 molar ratio). The desired monomers (HEMA-PEG, albumin, α 1-acid glycoprotein, etc...) containing drug and a photoinitiator (Irgacure 2959 (25)) are added to this lipid film and sonicated to form multilamellar vesicles (Fig. 1A). Extrusion, purification, and photo-crosslinking of the encapsulated monomers create a targeted lipid-coated nanogel, which enables stable loading of a wide array of chemotypes (see schematic in Fig. 1A). Nanogel core compositions include proteins such as (1) human serum albumin or α 1-acid glycoprotein, and polymers such as (2) HEMA-PEG or (3) 4-arm PEG acrylate, which demonstrate the ability to form

gels of defined size, capable of loading hydrophobic drugs, proteins, peptides, nucleic acids, and/or (4) imaging agents (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 polyethyleneglycol on the lipid bilayer that surrounds the drug loaded nanogel core. Encapsulation of albumin bound docetaxel forms homogenous nanogels with a ~ 90 nm average hydrodynamic diameter following cross-linking as observed using 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 demonstrate that photo-crosslinking slows the drug release rate of nanogels loaded with albumin bound docetaxel when compared to the non-crosslinked version (Fig. 1E). Crosslinking of the core improves docetaxel retention in mouse plasma with 50% and 30% improvement over the non-crosslinked particles at 2 h and 4 h, respectively (Fig. 1E). This finding is important for efficacy studies since most nanoparticles reach the tumor target within this time frame.

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

To demonstrate 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 $\alpha\beta3$ that is widely accepted as a targeting moiety for the tumor neovasculature (26–29). The integrin $\alpha\beta3$ targeted nanogels (RGD) were loaded with paclitaxel (β -tubulin), docetaxel (β -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 $\alpha\beta3$ (Fig. 2A and 2B). A summary of the encapsulation efficiency for each of these drugs in the human serum albumin-based nanogel core is presented in Supplementary Table S2. The EC_{50} s for cell viability with these various drug-loaded nanogels ranged from 3 nM – 8 μ M (Fig. 2A, B and Table 2). The cellular EC_{50} s of the drug-loaded nanogels compared to their respective free drugs are summarized in Table 2. In fact, drug loaded nanogels demonstrate enhanced potency when compared to exposing the cells to the free drug (Table 2). For example, the EC_{50} s of the cells exposed for 20 min. with the nanogels are comparable to cells exposed to the free drug for 72 h. It is important to note that the UV photo-crosslinking step may cause potential drug degradation, but we did not observe large potency losses in the cell viability assays, suggesting that the crosslinking was not detrimental to drug activity. These findings represent a clear advantage for nanogel drug delivery that may be relevant for predicting efficacy *in vivo*.

The contribution of targeting integrin $\alpha\beta3$ was investigated with the highly potent docetaxel-loaded nanogels. Here, we compared the cell viability of cells exposed to targeted, docetaxel-loaded nanogels (RGD-Doc-NG), untargeted, docetaxel-loaded nanogels (RAD-Doc-NG), and empty targeted nanogels (RGD-empty-NG). The $\alpha\beta3$ targeting resulted in a 13-fold enhancement in inhibiting cell viability with EC_{50} s of 0.018 and 0.238 μ M for the RGD-Doc-NG vs. RAD-Doc-NG (Fig. 2C). As expected, the empty nanogels (RGD-empty-NG) showed no effect on cell viability up to concentrations > 3 logs higher than the EC_{50} of the RGD-Doc-NGs. These findings establish that targeted nanogel drug delivery produces maximal anti-tumor cell activity.

Comparison of targeted Nanogels containing taxanes to Abraxane in Breast Cancer

Based on the approval of the taxanes and Abraxane (paclitaxel bound albumin nanoparticles) 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) (EC_{50} 0.348 and 0.231 μ M, respectively) demonstrated a > 10-fold enhancement in reducing cell viability when compared to Abraxane alone (3.374 μ M) (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 utilized an MDA-MB-231/LM2-4 orthotopic breast cancer model. Fluorescent RGD-Doc-NGs (vascular targeted) or RAD-Doc-NGs (non-targeted) were intravenously administered at a 1 mg/kg dose of docetaxel for 5 h, and the tumors were resected and imaged with confocal microscopy to view accumulation of the nanogels (green) in the tumor vasculature (red) (Fig. 3B, **upper panel**). The vascular targeted RGD-Doc-NGs targeted the vascular beds within the tumor and bright, punctate nanogel fluorescence was co-localized with the tumor vessels throughout the tumor area (Fig. 3B). As expected, the RAD-Doc-NGs did not demonstrate 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 either targeted, docetaxel-loaded nanogels (RGD-Doc-NG), untargeted, docetaxel-loaded nanogels (RAD-Doc-NG), or Abraxane. At 1 mg/kg effective taxane dose, Abraxane showed no effect when compared to saline vehicle on day 30 with tumor volumes of ~ 830 mm³ (Fig. 3B, **lower panel**). The untargeted RAD-Doc-NG showed intermediate activity with a tumor volume of 520 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 to the untargeted nanogel (RAD-Doc-NG) demonstrates the benefit of active targeting over passive tumor uptake.

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 mg/kg to 15 mg/kg total paclitaxel administration (Fig. 3C). In this study, we observed a > 5-fold enhancement in tumor growth suppression with 1 mg/kg of RGD-Pac-NG since 5 mg/kg of paclitaxel in Abraxane did not suppress tumor growth to the same tumor volume (430 vs. 710 mm³) (Fig. 3C). The 1 mg/kg docetaxel in RGD-Doc-NG produced the same result as 15 mg/kg of Abraxane treatment, demonstrating 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. Representative images demonstrate 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 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 μ M, respectively) greatly improved the cellular EC₅₀ relative to Abraxane (2.913 μ M) 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 α v β 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 demonstrated 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 mg/kg, 5 mg/kg, 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 demonstrated minor amounts of apoptosis as expected based on 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 pancreas 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 based on 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-crosslinking to form the nanogel, which enables precise control of nanogel formation. Photo-crosslinking of the nanogel core enhances drug retention (Fig. 1E), thereby improving a major limitation associated with liposomes, micelles, and co-block polymeric systems.

In our studies, we focused on utilizing proteins such as human serum albumin or α 1-acid glycoprotein since these plasma proteins are known to bind cancer therapeutics in the blood stream (32). Using human serum albumin 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 demonstrate the versatility of the nanogel platform (Fig. 2A and 2B, 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-crosslinking 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 human serum albumin as a host for hydrophobic compounds and then utilized a lipid bilayer to encapsulate this solution to form nanoparticles of desired size and provide a template for photo-crosslinking. We also demonstrated 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 (33). Additionally, other groups have used novel co-block 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 utilized poly(ethylene oxide)-b-poly(methacrylic acid) and divalent cations to form micelles, and the doxorubicin-loaded ionic cores were chemically-crosslinked with cystamine to improve drug retention within the core (34). Additionally, alginate cores have been coated with an anionic surfactant, Aerosol OTTM, for improved release kinetics of weakly basic drugs such as verapamil and doxorubicin (35). Another integrated approach termed “nanocells” utilized 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 anti-angiogenic agent and chemotherapeutic simultaneously (36). Recently, a microfluidic approach was reported for 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 to traditional emulsion-solvent evaporation techniques. Altogether, both the studies described above and the nanogel system described in this report represent a current movement towards integrated nanoparticle platforms that utilize 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 two 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 α v β 3) remains controversial. All of the major nanotherapeutics that are FDA approved or in advanced clinical trials for cancer therapy accumulate at the tumor site via the enhanced permeability and retention (EPR) mechanism. Two notable nanoparticles, Doxil®, liposomal doxorubicin (1–3), and Abraxane®, albumin bound paclitaxel nanoparticles (5, 6), demonstrate 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 utilized a cyclic RGD peptide serving as an integrin $\alpha\beta3$ 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) demonstrated intermediate tumor suppression in the orthotopic breast cancer model, which we assume is mainly from passive targeting through the EPR mechanism since 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 throughout the stromal compartment (42). To improve access to the tumor tissue, Ruoslahti and colleagues have identified a new tumor penetrating peptide system based on the use of a C-terminal arginine (CendR) that improves access of both antibodies and nanoparticles to the tumor compartment (29, 43). Although the targeted paclitaxel nanogels (RGD-Doc-NG) reduced primary tumor burden by ~40% in an orthotopic model of pancreas cancer (Fig. 4B), we envision that the CendR system can be used to further improve tumor penetration since it can be easily added to the surface of the lipid-coated nanogel. From our studies, dosing with the targeted nanogels containing 1 mg/kg total paclitaxel is equally efficacious to Abraxane carrying a 15 mg/kg total paclitaxel concentration (Fig. 4B). The targeted nanogel similarly demonstrates a 15-fold improvement over Abraxane in reducing metastasis to the hepatic hilar node, which directly correlates with suppression of primary tumor growth (Fig. 4B and 4C). For complete regression of the primary tumor in this pancreatic cancer model, the use of tumor penetrating peptides, such as the iRGD molecule, should enable greater access to the primary tumor.

When evaluating the efficacy of nanoparticle-based therapies it is important to consider the tumor models that are selected. For instance, passive uptake via the EPR mechanism is likely very different when comparing subcutaneous vs. orthotopic tumors, since the microenvironment plays a major role in the tumor properties. Additionally, the location of the tumor in the body leads to major differences in tumor properties including variation in interstitial fluid pressure, the amount of angiogenesis at the primary tumor site, and the host response, which can produce a major stromal compartment that secretes extracellular matrix proteins leading to reduced access of drugs to the tumor cells. If a tumor compartment is highly restricted, utilizing a tumor targeting ligand will likely add no benefit compared to using an untargeted nanoparticle. Since the nanoparticles in the blood are directly exposed to the endothelial cells, targeting the tumor neovasculature represents a general strategy that may be less impacted by the tumor physiology. In our efficacy studies, we utilized orthotopic tumor models of breast and pancreas cancer (Fig. 3 and Fig. 4) and found that targeting the vasculature enhances efficacy as we previously reported (19). As targeted nanotherapies continue to progress towards clinical testing in cancer, it will be important to select the appropriate preclinical tumor models to evaluate the impact of active targeting on efficacy.

In this study, we describe a new lipid-coated nanogel platform, which is capable of encapsulating a wide array of monomers that can be photo-crosslinked to improve drug retention. The nanogel platform is capable of delivering a wide range of drug chemotypes and incorporating imaging agents for theranostic applications. Using human serum albumin and taxanes as a proof of principle, we show that the nanogels improve efficacy beyond a

clinically approved nanoparticle formulation - Abraxane. In summary, the nanogel platform allows for flexibility in choosing drug cargoes and targeting ligands, and provides a unique opportunity to improve the efficacy of drugs that have poor pharmacokinetics or dose-limiting toxicities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

(DOPE)	Dioleoylphosphatidylethanolamine
(DSPC)	distearoylphosphatidylcholine
(DSPE)	distearoylphosphatidylethanolamine
HEMA-PEG	(hydroxyethylmethacrylate-polyethylene glycol)
Doc	(docetaxel)
Pac	(paclitaxel)

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Figure 1. Formation of Lipid-Coated Nanogels

A, The method for controlled photo-crosslinking 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. Proteins such as (1.) human serum albumin, α 1-acid-glycoprotein, and others serve as hosts for drug binding. Other inputs include polymers such as (2.) 2-hydroxyethylmethacrylate (HEMA)-polyethylene glycol (PEG) or (3.) a 4-arm PEG acrylate. To enable tracking with imaging agents, (4.) iron oxide nanoparticles (NPs), gold NPs, 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-crosslinked human serum albumin bound docetaxel core. **E**, Nanogels with RGD peptide on the surface and docetaxel loaded human serum albumin in the crosslinked 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 using HPLC.

Figure 2. Targeted-lipid-coated nanogels demonstrate efficacy with a wide range of chemotypes in vitro

A,B,C M21 melanomas (integrin $\alpha\text{v}\beta 3$ positive) were incubated with cyclic RGDfK nanogels (RGD-NGs) loaded with various small molecules bound to human serum albumin. After a 20 min exposure at 4°C, the nanogels were removed and the cells were incubated at 37°C for an additional 72 h before XTT measurements for cell viability. **A** and **B** compared the RGD-NGs with a wide range of chemotypes, while **C** measured the effect of targeting integrin $\alpha\text{v}\beta 3$ on cell viability of nanogels loaded with albumin bound docetaxel. RGD is the integrin $\alpha\text{v}\beta 3$ targeting peptide and RAD is the untargeted control peptide ligand. All experiments were performed in triplicate and are represented as the mean $\pm$ s.e.m.

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 performed in triplicate with data represented as the mean $\pm$ s.e.m. **B –D**, Human MDA-MB-231/LM2-4 cells were injected into the mammary fat pad # 4 of athymic nu/nu mice and primary tumors were established (average size 70–80 mm³) before initiating dosing. **B**, Upper images: 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 iv injected (1 mg/kg docetaxel concentration) and the tumors were imaged with confocal microscopy at 5 h post-injection. Red-represents rhodamine-labeled *G. simplicifolia* lectin for staining the endothelium and green represents nanogel binding. Scale bar = 100 μ m. Lower graph: A taxane dose of 1 mg/kg was compared for the nanogels and Abraxane (Abx). Dosing was via iv administration on a qod schedule until the end of the experiment. Tumors were measured every two days. Targeted nanogels carrying human serum albumin bound docetaxel (RGD-Doc-NG) were compared to either untargeted nanogels (RAD-Doc-NG) or Abraxane. (n = 8/group), $p < 0.01$ for RGD-Doc-NG or RAD-Doc-NG vs. PBS control and for RAD-Doc-NG vs. RGD-Doc-NG. **C**, At this time, nanogels containing 1 mg/kg docetaxel or paclitaxel were compared to 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 vs. Abx1. All data points are represented as the mean $\pm$ s.e.m. **D**, Immunofluorescence of sections from the tumors treated in panel C demonstrate 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 μ m.

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 performed in triplicate with data represented as the mean $\pm$ s.e.m. **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 iv administration on a qod dosing schedule. Similar to Fig. 3C, nanogels containing either 1 mg/kg of docetaxel or paclitaxel were compared to 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 **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 vs. Abx1 for both **B** and **C**. All data points are represented as the mean $\pm$ s.e.m. **D**, Immunofluorescence of sections from the primary tumors treated in panel B demonstrate 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 μ m.

Table 1

Mean hydrodynamic diameter, zeta potential, and polydispersity index measurements of nanogels prepared with various core compositions. All nanogels were prepared with the same lipid formulation as described in the Methods.

Core Composition	Diameter (nm)	Zeta Potential (mV)	PDI
HEMA-PEG	112.7	−3.09	0.264
4-arm PEG acrylate	109.3	−1.22	0.323
Albumin + Iron Oxide	102.3	−2.55	0.189
Albumin + Gold NP	116.4	6.04	0.126
Albumin + Quantum Dots	104.1	7.41	0.217
α1-acid glycoprotein	119.1	−4.36	0.410

Table 2
Summary of Cell Viability EC₅₀s in M21 cells from the curves in Figure 2A and 2B.

Drug	Target	Class	Nanogel (20min) EC ₅₀ (μM)	Free drug (20min) EC ₅₀ (μM)	Free drug (72 hr) EC ₅₀ (μM)
Docetaxel	β-tubulin	Taxane	0.0045	0.075	0.00045
Paclitaxel	β-tubulin	Taxane	0.0050	0.2354	0.020
Bortezomib	26S-proteasome	Peptide mimetic	0.1212	>1	0.017
17-AAG	Hsp90	Antitumor antibiotic	0.927	>1	0.025
Sorafenib	VEGFR2/RAF	Kinase inhibitor	5.5	>40	2.31
Sunitinib	Broad RTK	Kinase inhibitor	7.1	>40	0.94
Bosutinib	Src family	Kinase inhibitor	5.4	>40	0.56
Dasatinib	Abl/Src family	Kinase inhibitor	7.8	>40	6.8