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Improved Tumor Targeting of Polymer-based Nanovesicles Using Polymer-Lipid Blends

Zhiliang Cheng¹, Drew R. Elias¹, Neha P. Kamat¹, Eric D. Johnston¹, Andrei Poloukhine², Vladimir Popik², Daniel A. Hammer¹, and Andrew Tsourkas^{1,*}

¹Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104

²Department of Chemistry, University of Georgia, Athens, GA 30602

Abstract

Block copolymer-based vesicles have recently garnered a great deal of interest as nanoplateforms for drug delivery and molecular imaging applications due to their unique structural properties. These nanovesicles have been shown to direct their cargo to disease sites either through enhanced permeability and retention or even more efficiently via active targeting. Here we show that the efficacy of nanovesicle targeting can be significantly improved when prepared from polymer-lipid blends compared with block copolymer alone. Polymer-lipid hybrid nanovesicles were produced from the aqueous co-assembly of the diblock copolymer, poly(ethylene oxide)-*block*-polybutadiene (PEO-PBD), and the phospholipid, hydrogenated soy phosphatidylcholine (HSPC). The PEG-based vesicles, 117 nm in diameter, were functionalized with either folic acid or anti-HER2/neu affibodies as targeting ligands to confer specificity for cancer cells. Our results revealed that nanovesicles prepared from polymer-lipid blends led to significant improvement in cell binding compared to nanovesicles prepared from block copolymer alone in both *in vitro* cell studies and murine tumor models. Therefore, it is envisioned that nanovesicles composed of polymer-lipid blends may constitute a preferred embodiment for targeted drug delivery and molecular imaging applications.

Keywords

Diblock copolymer; phospholipids; PEG; tumor targeting; polymersome

INTRODUCTION

For traditional drug delivery systems, only a small fraction of the therapeutic agents reach the disease site. Targeted drug delivery seeks to improve the accumulation of these agents in the tissues of interest while reducing their relative concentration in off-target sites. If successful, this would lead to a significant improvement in treatment efficacy, while reducing drug toxicity, and/or dose. Accordingly, the use of nanoparticulate systems to facilitate the targeted delivery of therapeutic agents has been extensively explored in recent years.^{1–10} Drug delivery nanoplateforms have included dendrimers, polymers, liposomes, micelles, emulsions, and silica nanoparticles. Beyond their enhanced targeting capabilities and ability to carry high drug payloads, nanoparticle-based drug delivery systems have also

*CORRESPONDING AUTHOR FOOTNOTE. Phone: (215) 898-8167. Fax: (215) 573-2071, atsourk@seas.upenn.edu.

SUPPORTING INFORMATION AVAILABLE. Synthesis of ADIBO-PEG₄-NHS Ester, Cloning and expression of HER2-Affibody, Expressed protein ligation, Copper free click conjugation, Image of giant vesicle prepared from 100 % PEO-PBD (Figure S1), Flow cytometric analysis of KB cells following incubation with folate receptor-targeted vesicles with varying lipid-polymer ratios, but equal number of folate per vesicle (Figure S2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

been shown to provide protection of some drugs against degradation and have enabled the efficient delivery of substances with poor inherent solubility or low membrane permeability.²

Among the many nanoparticulate systems that have been reported, hollow nanometer-sized vesicles are particularly attractive due to the increased functionality imparted by their amphiphilic structure. Specifically, hydrophilic compounds can be loaded into the aqueous lumen of the nanovesicles, the hydrophobic domain serves as a natural carrier environment for hydrophobic drugs, and the exterior surface can be functionalized with molecularly specific targeting ligands.

Polymer-based nanovesicles (i.e. polymersomes), prepared from high molecular weight diblock copolymers, have a thick hydrophobic domain, typically ~6–10 nm.¹¹ This is significantly larger than the hydrophobic domain of most liposomes, which are typically ~3 nm in thickness. Compared to liposomes, polymersomes possess several beneficial properties, including increased mechanical robustness and the ability to carry large quantities of hydrophobic and hydrophilic molecules.^{11, 12} The vast majority of polymersomes are formed from diblock copolymers with poly(ethylene glycol) (PEG) as the hydrophilic block. This creates a relatively inert, brush-like outer shell, which imparts “stealth” like characteristics to the nanovesicles and allows them to effectively avoid the reticuloendothelial system, resulting in longer circulation times. Further, polymersomes can be finely tuned through polymer selection to yield vesicles with diverse functionality, i.e. biodegradability, biocompatibility, permeability, elasticity, etc.^{13–15} As a result of all these characteristics, polymersomes have garnered a great deal of interest as nanoplatfroms for a range of biomedical applications, including drug delivery, in vivo imaging, and cell mimicry.^{13, 16–18} For example, it has recently been shown that both hydrophobic paclitaxel and hydrophilic doxorubicin can be co-loaded into polymersomes to effectively treat tumors,¹³ porphyrins have been loaded into the hydrophobic domain of polymersomes creating highly fluorescent imaging agents,^{19, 20} and gadolinium-labeled dendrimers have been encapsulated within the lumen of polymersomes to create highly efficient magnetic resonance imaging (MRI) contrast agents.^{16, 17} When administered intravenously, polymeric vesicles generally maintain a long circulation time within the body and will non-specifically accumulate within tumors due to enhanced permeability and retention (EPR). Although EPR can allow for a sufficient quantity of polymersomes to accumulate within tumors for effective treatment and/or imaging, it is generally desirable to develop actively targeted nanoplatfroms to minimize nonspecific toxicity and/or enhance the efficiency of therapy.²¹ Targeting, however, can often be compromised by the brush-like PEG coatings on the vesicle surface. Specifically, despite the benefits of pegylation in reducing polymer uptake by the reticuloendothelial system, it has been shown the presence of PEG on vesicle surfaces can inhibit ligand-mediated targeting of the vesicle, due to steric effects.^{22, 23} Therefore, we hypothesized that the efficiency of polymersome targeting could be improved by diluting the surface density of PEG by integrating phospholipids into the membrane bilayer.

Recently, phospholipid–block copolymer hybrid nanoparticles have been reported and have drawn much attention.^{17, 24, 25} The main components of these hybrid nanoparticles are the hollow polymeric scaffold with the incorporated phospholipid. Such hollow vesicles possess the structural stability and mechanical strength of polymer membranes and the biocompatibility and biofunctionality of phospholipid membranes. Owing to their unique structural properties, lipid-polymer hybrid vesicle may constitute a preferred nanoplatfrom for targeted drug delivery and molecular imaging applications. For example, Kanger and co-workers have prepared biofunctionalized lipid-polymer hybrid nanocontainers with controlled permeability for triggered delivery of drugs or imaging agents.^{13, 26}

In this study, we show that polymer-based nanovesicles prepared from polymer-lipid blends, as opposed to block copolymer alone, exhibit a significant improvement in tumor cancer cell binding. Specifically, lipid-polymer hybrid vesicles were produced from the aqueous co-assembly of the diblock copolymer, Poly(ethylene oxide)-*block*-polybutadiene, and the phospholipid, hydrogenated soy phosphatidylcholine (HSPC). The vesicles were functionalized with either folic acid or HER2/neu-targeted affibodies as targeting ligands to confer specificity for cancer cells. HER2-Affibodies comprise a new class of high affinity ligands based on a protein scaffold derived from the IgG-binding domains of staphylococcal protein A.²⁷ These small (6.5 kDa), robust molecules have been shown to exhibit remarkable specificity and affinity (pm range) for the HER2/neu receptor. The key features of the developed nanomaterials include nanometer-sized vesicles formed from self-assembly of amphiphilic diblock copolymers and phospholipids, brush-like PEG outer shell and tumor-targeting ligands conjugated onto hybrid nanovesicles, which serve to localize the therapeutic agent to the site of interest. Here, the design, assembly, characterization, and tumor-cell targeting of the polymer-lipid hybrid vesicles are discussed.

EXPERIMENTAL PROCEDURES

Materials

Poly(ethylene oxide)-*block*-polybutadiene copolymer (denoted PEO-PBD) was purchased from Polymer Source (Dorval, Quebec, Canada). Average molecular weights of the poly(ethylene-oxide) and poly(butadiene) block were 600 and 1,200, respectively. Hydrogenated soy phosphatidylcholine (HSPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-PE), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)2000-N'-carboxyfluorescein] (DSPE-PEG2K-CF), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000-Amine), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (DSPE-PEG2000-Folate) were obtained from Avanti Polar Lipids (Alabaster, AL). ADIBO-dPEG4-NHS was synthesized in the lab (see supporting information). All other chemical were used as received. All buffer solutions were prepared with ultrapure grade water.

Preparation of giant vesicles

Giant vesicles were prepared by hydration of dry polymer/lipid films. Stock solutions of PEO-PBD and HSPC in chloroform were mixed in the following molar ratios: PEO-PBD/HSPC (100:0), PEO-PBD/HSPC (90:10), and PEO-PBD/HSPC (75:25). The total amount of PEO-PBD for each of the vesicle compositions was 1 mg. In all cases, a small amount of the fluorescent label, Rhod-PE, was also added for vesicle visualization. The solvent was removed using a direct stream of nitrogen prior to vacuum desiccation for a minimum of 4 h. Giant (micron-sized) vesicles were formed by adding 2 mL sucrose (285 mM) solution to dried film and incubating in a 65 °C water bath for more than 24 h.

Preparation of nanometer-sized vesicles

Stock solutions of PEO-PBD and HSPC in chloroform were mixed in the following molar ratios: PEO-PBD/HSPC (100:0), PEO-PBD/HSPC (90:10), and PEO-PBD/HSPC (75:25). The total amount of PEO-PBD for each of the vesicle compositions was 1 mg. For folate-receptor targeting, DSPE-PEG2000-Folate (10 μ L, 1 mg/mL in chloroform) was added to the PEO-PBD/HSPC mixture. Rhod-PE (2.5 μ L, 1 mg/mL in chloroform) was also added as a fluorescent indicator for fluorescence microscopy images. For flow cytometric study, DSPE-PEG2K-CF (2.5 μ L, 1 mg/mL in chloroform) instead of Rhod-PE was used for vesicle preparation. For Her2/neu-receptor targeting, DSPE-PEG2000-Amine (10 μ L, 1 mg/mL in chloroform) was added to the PEO-PBD/HSPC mixture. The solvent was removed

using a direct stream of nitrogen prior to vacuum desiccation for a minimum of 4 h. Vesicles were formed by adding an aqueous solution (0.1 M PBS, pH 7.4) to the dried film and incubating in a 65 °C water bath for 0.5 h and then sonicating for another 1 h at the same temperature. Samples were subjected to 10 freeze–thaw–vortex cycles in liquid nitrogen and warm H₂O (65 °C), followed by extrusion 21 times through two stacked 100 nm Nuclepore polycarbonate filters using a stainless steel extruder (Avanti Polar Lipids).

Micropipette aspiration

The micropipette aspiration experiment was performed as described previously.^{20, 28} Briefly, micropipettes made of borosilicate glass tubing (Friedrich and Dimmock, Milville, NJ) were prepared using a needle/pipette puller (Model 720, David Kopf Instruments, Tujunga, CA) and microforged using a glass bead to give the tip a smooth and flat edge. Pipettes were filled with 290 mOsm PBS and mounted on a micromanipulator, and the pipet was connected via tubing to a manometer. A negative pressure, produced through the manometer using a syringe, was created in the pipet to pick up a giant vesicle. The pressure applied to the vesicle was increased in stepwise increments (4 cm H₂O) and from this suction pressure (ΔP), the membrane tension (T) for a fluid membrane can be calculated from LaPlace's Law.²⁹ The length of the vesicle extension into the pipette in response to suction pressure was used to calculate the resulting vesicle area strain, $\alpha \equiv \Delta A/A_0$. The area elastic modulus was then measured by plotting α vs T in the high tension regime ($T > 0.5$ dyne/cm) and calculating the slope of this tension-strain curve. Experiments were imaged using DIC optics with a 40 $\times$ objective and a Cohu black-and-white CCD camera (Cohu, Inc., San Diego, CA). ImageJ software was used to measure membrane extensions and vesicle diameters.

Cloning and Expression of HER2-Affibody and copper free click conjugation

See Supporting Information.

Lipid contents in lipid-polymer hybrid nanovesicles

Lipid-polymer hybrid nanovesicles, i.e. 10 mol % HSPC/90 mol % PEO-PBD and 25 mol % HSPC/75 mol % PEO-PBD, were lyophilized either before or after extrusion and purification. In these studies, samples were prepared in pure water. The residual solid was weighed and re-suspended in pure water. Phospholipid content was determined by measuring the total content of phosphorus in the sample.³⁰ The polymer weight was calculated as the dry weight minus the lipid weight. The molar ratio of lipid to polymer was then calculated based on their respective molecular weights.

Quantification the targeting ligand number on vesicle surface

The content of folate in the samples was determined by quantitative UV spectrophotometric analysis using the molar extinction coefficient value of 15,760 M⁻¹ cm⁻¹ at $\lambda = 358$ nm. Background absorbance measurements were determined using analogous samples with no folate. To calculate the number of folate per vesicle, the number of vesicles in the purified sample was calculated based on the amount of polymer and phospholipid in each vesicle. For this calculation, the average diameter of each vesicle formulation was measured via dynamic light scattering. Further, the average area occupied by single polymer molecules in the bilayer was previously determined to be ~1 nm.¹⁴ The average area occupied by single phospholipid molecules in the bilayer was previously determined to be ~0.65 nm,³¹ and the thickness of the polymersome bilayer was considered to be ~6 nm based on previous study.¹² A similar approach was used to quantify the content of Rhod-PE in each vesicle. The molar extinction coefficient value used for Rhod was 93,000 M⁻¹ cm⁻¹ at $\lambda = 568$ nm.

Once the Rhod content per vesicle was established it was possible to quantify the number of conjugated HER2-affibody per vesicle based on the ratio of absorbance of conjugated fluorescent-HER2 affibody relative to incorporated Rhod-PE. Notably, each Her2-affibody is labeled with a single near infrared dye, Hilyte Fluor 750. The content of Hilyte Fluor 750 was determined by spectrophotometric analysis using the molar extinction coefficient value of $275,000 \text{ M}^{-1} \text{ cm}^{-1}$ at $\lambda = 753 \text{ nm}$.

Cell culture

KB cells (human nasopharyngeal epidermoid carcinoma cells) were purchased from the America Type Tissue Collection (ATCC) and cultured in RPMI 1640 folic acid free media with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 . T6-17 cells that were engineered to stably express the Her2/neu receptor (T6-17) were kindly provided by Mark Greene, MD/PhD (University of Pennsylvania). T6-17 cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO_2 .

Cell viability via MTT assay

NIH 3T3 cells were seeded in 96-well plates at a density of 10,000 cells per well. After incubation overnight (37°C , 5% CO_2), the medium in each well was aspirated off and loaded with 100 μL of fresh medium containing nanovesicles with four different formulations including 100 mol % PEO-PBD, 10 mol % HSPC/90 mol % PEO-PBD, 25 mol % HSPC/75 mol % PEO-PBD and 100 mol % HSPC. For PEO-based vesicles, the final polymer (PEO-PBD) concentration in the cultured media was 10 μM . For pure HSPC vesicles, the final HSPC concentration in the cultured media was also 10 μM . After incubation for 24 h, the nanoparticle containing medium in each well was aspirated off and replaced with 100 μL of medium and 10 μL of MTT reagent. The cells were incubated for 2 to 4 hours, then 100 μL detergent reagent was added and left at room temperature in the dark for 2 hours. The absorbance at 570 nm was measured using a microplate reader. Analogous viability studies were also conducted with the nanoparticle formulation consisting of 25 mol % HSPC/75 mol % PEO-PBD at PEO-PBD concentrations of 0, 5, 10, 50, 100, and 200 μM .

Fluorescence microscopy

Cells plated in 8-glass well plates were washed once with PBS and then incubated in 200 μL of RPMI (for KB cells) or DMEM (for T6-17) containing vesicles for 2 hours. For competitive inhibition experiments, cells were treated with the same vesicles but in the presence of 1 mM free targeting ligands in the media. The final PEO-PBD concentration in the cultured media was 5 μM for all imaging experiments. Prior to acquisition of fluorescence images, cells were washed with PBS three times. All microscopy images were acquired with an Olympus IX81 motorized inverted fluorescence microscope equipped with a back-illuminated EMCCD camera (Andor), an X-cite 120 excitation source (EXFO), and Sutter excitation and emission filter wheels. Images of the Rhod-PE were acquired using the filter set HQ545/30, HQ610/75, Q570LP. Images of FITC fluorescence were acquired using the filter set HQ480/40, HQ535/50, Q505LP. All filter sets were purchased from Chroma.

Flow cytometric analysis

Cells were dissociated from culture flasks using PBS-based enzyme free dissociation buffer and transferred to sterile 96-well plates at a final concentration of 50,000 cells per well. Targeted vesicles were added to the wells for 2 hours at 37°C . The final polymer (PEO-PBD) concentration in the cultured media was 5 μM for all flow cytometric experiments. For competitive inhibition experiments, cells were treated with the same vesicles but in the

presence of 1 mM free targeting ligands in the media. Cells were transferred to 1.5 mL centrifuge tubes and washed in triplicate by pelleting cells at 1000 RCF for 3 minutes and then resuspending in PBS. Cells were resuspended in 250 μ L of PBS and seeded in a 96-well plate (50,000 cells per well) and analyzed using a Guava Easycyte Plus system (Guava Technologies, Hayward, CA). Flow cytometry data were analyzed using FlowJo software (TreeStar Inc., San Francisco, CA).

In vivo imaging

Approximately 6-week old female Fox Chase SCID mice (Charles River Laboratory, Charles River, MS) were maintained in accordance with the Institutional Animal Care and Use Committee of the University of Pennsylvania. Mice were anesthetized via isoflurane and T6–17 cells were injected subcutaneously into the back right flank (2×10^6 cells in 0.2 mL PBS). Tumors were grown to an approximate size of 100 mm³. Near infrared (NIR) HER2-targeted nanovesicles were injected retro-orbitally (100 μ M PEO-PBD in 0.2 mL PBS). Post-contrast images were collected 24 h post-injection. Fluorescent images were acquired using a Pearl NIR imaging system. Region of interest (ROI) analysis was performed using Pearl imaging software. Mean fluorescence of the tumor was measured after background subtraction.

Instrumentation

Dynamic light scattering (DLS) measurements were performed on a Zetasizer Nano from Malvern Instruments. The scattering angle was held constant at 90°. Fluorescence spectra measurements were acquired on a SPEX FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon).

RESULTS AND DISCUSSION

Evidence of forming polymer-lipid blends in giant vesicles

PEO-PBD are a class of synthetic amphiphilic diblock copolymers in which hydrophobic PBD is linked to hydrophilic PEG. When dispersed in aqueous medium, PEO-PBD with appropriate hydrophilic volume fractions can self-assemble to form polymeric vesicles.¹¹ As shown in Figure S1, the PEO-PBD selected for this study, PEO(600)-*b*-PBD(1200) (PEO-PBD), was capable of forming giant vesicles utilizing a classical swelling technique. Similarly, lipid-polymer hybrid nanovesicles could be formed from the aqueous co-assembly of PEO-PBD and the phospholipid, hydrogenated soy phosphatidylcholine (HSPC). A schematic of a targeted lipid-polymer hybrid vesicle functionalized with either folic acid or HER2/neu-targeted affibodies as targeting ligands to confer specificity for cancer cells is shown in Figure 1. The ability to integrate phospholipids into the membrane of polymer-based vesicles was confirmed by preparing micron-sized lipid-polymer hybrid vesicles with a low molar percentage (0.5%) of fluorescent phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhod-PE). As shown in Figure 2, incorporation of Rhod-PE into the vesicle membrane was evident at the resolution of the optical microscope, for vesicles doped with either 0, 10 or 25 mol % HSPC. The phospholipid-based fluorescent probe within polymer-lipid hybrid giant vesicles did not exhibit any obvious amphiphile phase segregation on optically resolvable length scales. However, the possibility of forming nanoscale lipid domains below the length scale of optical resolution cannot be ruled out.^{12, 25, 32}

Membrane Elasticity

To provide evidence that HSPC was successfully incorporated into the vesicle bilayer, the elastic modulus (K_a) for three types of vesicles, 100 mol % PEO-PBD, 25 mol % HSPC/75

mol % PEO-PBD and 100 mol % HSPC, were obtained via micropipette aspiration. The K_a is a common mechanical parameter of vesicle membranes that measures the in-plane mechanical elasticity of the membrane, and reflects the interfacial tension of the membrane interface.¹¹ Because the interfacial tension is a function of the chemical composition of the membrane, subtle changes in membrane lipid:polymer composition can be detected through changes in the membrane area elastic modulus.^{33, 34} As shown in Figure 3, polymersomes made from pure PEO-PBD were more elastic, as illustrated by their significantly lower elastic modulus ($K_a = 72$ dyne/cm) than pure HSPC liposomes ($K_a = 206$ dyne/cm). When PEO-PBD and HSPC were blended at a molar ratio of 75 to 25, the resulting vesicles exhibited an intermediate elastic modulus ($K_a = 112$ dyne/cm) between the K_a values of the pure lipid and pure polymer vesicles. This intermediate elastic modulus provides further support that the vesicles were indeed a blend of the polymer and lipid.

Surface chemistry of hybrid polymer-lipid vesicles

To provide evidence that the outer surface of pure polymer and polymer-lipid hybrid nanovesicles (10% and 25% HSPC) could be modified with various functional molecules, a small percentage of amine-terminated phospholipid, DSPE-PEG2K-NH₂, was first doped into membrane during fabrication. The surface amines were then labeled with the amine reactive dye ATTO740 (ATTO740-NHS ester) and visualized by fluorescence microscopy. Figure 4 shows the fluorescence images obtained for giant vesicles prepared from 100 mol % PEO-PBD, 10 mol % HSPC/90 mol % PEO-PBD and 25 mol % HSPC/75 mol % PEO-PBD after conjugation of ATTO740. In all cases, conjugation of the ATTO740 into the vesicles membrane was evident. To ensure that the ATTO dye was specifically bound to the vesicle surface, the nanovesicles were also prepared in the absence of DSPE-PEG2K-NH₂, and then incubated with ATTO740-NHS ester. In contrast to the aminated nanovesicles, these control vesicles did not exhibit a fluorescent signal, suggesting that the bound ATTO dyes were conjugated through a surface chemical reaction and not from non-specific absorption. This result indicates that biologically-active molecules could be conjugated through the reactive groups on the hybrid polymer-lipid vesicle surface.

Nanometer-sized vesicles using polymer-lipid blends

Following the formation of micron-sized vesicles, the vesicle size was reduced to the ~100 nanometer size-range by subjecting the sample to multiple freeze-thaw cycles and extrusion through a 100 nm polycarbonate filter. Dynamic light scattering (DLS) presented in Figure 5 revealed that hybrid vesicles with 10 and 25 mol % HSPC had mean diameters of 112 and 117 nm, respectively, while pure PEO-PBD vesicles had a mean diameter of 105 nm. To confirm that the lipid-to-polymer molar ratio was unaffected by the extrusion and purification process, the lipid content in samples containing nano- or giant hybrid vesicles was determined by measuring the phosphorous content. The polymer content was calculated by subtracting the measured lipid weight from the total dry weight of the sample (see Experiment section for details). These measurements indicated that HSPC was incorporated into the hybrid membrane at the predetermined molar ratios and that there was no significant loss of HSPC during processing. To evaluate the ability to store lipid-polymer hybrid nanovesicles, the hydrodynamic diameter of the vesicles was measured by DLS for 10 days following suspension in PBS buffer (0.1 mM phosphate, pH 7.4). It was found that vesicles prepared with 10 and 25 mol% HSPC did not exhibit any significant change in hydrodynamic diameter over this time frame.

Viability

Prior to evaluating hybrid nanovesicles for targeting in living cells or tissues, their cytotoxicity was examined in a MTT cell proliferation assay (MTT=3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide). Specifically, four different vesicle

samples, 100% HSPC, 100% PEO-PBD, 90% PEO-PBD/10% HSPC and 75% PEO-PBD/25% HSPC, were incubated with NIH 3T3 fibroblasts for 24 h. Each vesicle was tested in triplicate. Cell viabilities were normalized to a control cell sample that was not incubated with any vesicles. As shown in Figure 6A, none of the vesicles formulations had any significant effect on the proliferation of NIH 3T3 cells at a PEO-PBD concentration of 10 μ M. The average cell viability of NIH 3T3 remained unaffected for PEO-PBD concentrations up to 100 μ M for the 75% PEO-PBD/25% HSPC vesicles and only a very slight loss in viability (<8%) was observed at 200 μ M PEO-PBD (Figure 6B).

Targeting efficiency of lipid-polymer hybrid vesicles in vitro

To demonstrate the feasibility of utilizing hybrid lipid-polymer vesicles to target tumor cells, we developed two different types of polymer-lipid vesicles, one targeting the folate receptor and one targeting the HER2/neu-receptor. In the first approach, the small molecule folic acid (folate) was used for tumor targeting since folate specifically binds to folate receptors, which are frequently overexpressed on tumor cancer cells. KB cells were used in this study as a model cell line to evaluate the targeting capabilities of folic acid-labeled nanovesicles since they are known to overexpress the folate receptor. Vesicles were prepared with 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (PEG2000-DSPE-Folate) and Rhod-PE at a fixed molar ratio of folic acid/Rhod per polymer. A summary of the number of targeting ligands on each vesicle is provided in Table 1. After a 2-hour incubation with KB cells, fluorescent images were acquired. A bright fluorescent signal could be observed for KB cells incubated with polymer-lipid hybrid vesicles, especially PEO-PBD vesicles doped with 25% HSPC, as seen in Figure 7A. In contrast, very little fluorescence was observed from cells incubated with vesicles that did not contain HSPC. To verify that uptake of the vesicles was mediated through the folate-receptor, competitive inhibition studies were performed by incubating the folate-receptor-targeted vesicles with KB cells in the presence of excess free folic acid. Under these conditions, fluorescence was significantly reduced; confirming that cellular binding of the polymer-lipid hybrid vesicles was specifically mediated by the folate receptor. Analogous findings were also obtained via flow cytometry (Figure 7B). A clear shift in the mean cellular fluorescence was observed between cells incubated with folate-targeted lipid-polymer hybrid vesicles compared with cells incubated with folate-targeted vesicles that did not contain HSPC.

To confirm that the difference in targeting efficiency between pure polymer vesicles and lipid-polymer hybrid vesicles did not result from the differing number of targeting ligands on each vesicle, we further prepared 100 mol % PEO-PBD, 10 mol % HSPC/90 mol % PEO-PBD and 25 mol % HSPC/75 mol % PEO-PBD vesicles with an equal number ($n = 310$) of folate ligands and fluorescent lipids on their surface. As can be seen in Figure S2, even at equal ligand densities, the lipid-doped nanovesicles exhibited a significantly higher degree of cell labeling than non lipid-doped nanovesicles.

To evaluate whether polymer-lipid hybrid vesicles that had targeting agents conjugated to their outer surface also exhibited improved cellular binding, pre-formed vesicles were coupled to HER2/neu targeted affibodies via click conjugation.³⁵ After incubating the HER2-targeted nanovesicles with HER2/neu-positive T6-17 cells for 2 hours, fluorescent images were acquired. As shown in Figure 8A, the HER2/neu-targeted nanovesicles composed of 25% HSPC/75% PEO-PBD exhibited a significantly higher degree of cell labeling than nanovesicles composed of 100% PEO-PBD. This was further supported by flow cytometric analysis (Figure 8B). Competitive inhibition studies using a molar excess of free HER2/neu affibody confirmed that the binding was specific for the HER2/neu receptor.

Targeting efficiency of lipid-polymer hybrid vesicles in vivo

To examine whether the incorporation of phospholipids into polymeric vesicles could be used to improve the targeting of HER2/neu-positive tumors in living subjects, near-infrared (NIR) fluorescent images of mice with T6-17 cell xenografts were acquired 24 hours after retro-orbital injection of HER2-targeted lipid-polymer hybrid vesicles or HER2-targeted pure polymeric vesicles. As shown in Figure 9A, the HER2/neu-targeted nanovesicles composed of 25%HSPC/75%PEO-PBD exhibited a significantly higher signal in the tumor compared with nanovesicles composed of 100%PEO-PBD. Notably, some fluorescent signal was also observed in the kidney for both samples. Quantitative analysis of the NIR images revealed that HER2/neu-targeted nanovesicles composed of lipid-polymer blends exhibited more than a 1.5-fold improvement in tumor uptake compared with images from nanovesicles composed of 100%PEO-PBD (Figure 9B).

CONCLUSION

In summary, we have demonstrated that incorporation of HSPC into PEO-PBD-based nanovesicles can lead to a significant improvement in the efficiency of cell surface receptor targeting. It is hypothesized that incorporation of phospholipids into the vesicle bilayer diluted the density of the PEG-brush on the vesicle surface and as a result reduced the steric effect that PEG exerted on the targeting ligand. This is consistent with previous reports, which have also indicated that PEG-coatings can inhibit ligand-mediated targeting of the vesicle.^{22, 23} It is envisioned that the use of phospholipids can be extended to any polymer-based vesicles as a means to improve binding. Therefore, we believe that polymer-lipid hybrid vesicles constitute a promising nanopatform for a broad variety of research areas, ranging from targeted drug delivery to molecular imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Alexis F, Basto P, Levy-Nissenbaum E, Radovic-Moreno AF, Zhang L, Pridgen E, Wang AZ, Marein SL, Westerhof K, Molnar LK, Farokhzad OC. HER-2-targeted nanoparticle-affibody bioconjugates for cancer therapy. *Chem Med Chem*. 2008; 3:1839–1843. [PubMed: 19012296]
2. Febvay S, Marini DM, Belcher AM, Clapham DE. Targeted cytosolic delivery of cell-impermeable compounds by nanoparticle-mediated, light-triggered endosome disruption. *Nano Lett*. 2010; 10:2211–2219. [PubMed: 20446663]
3. Han HD, Mangala LS, Lee JW, Shahzad MMK, Kim HS, Shen DY, Nam EJ, Mora EM, Stone RL, Lu CH, Lee SJ, Roh JW, Nick AM, Lopez-Berestein G, Sood AK. Targeted gene silencing using RGD-labeled chitosan nanoparticles. *Clin Cancer Res*. 2010; 16:3910–3922. [PubMed: 20538762]
4. Liu Y, Sun J, Han JH, He ZG. Long-circulating targeted nanoparticles for cancer therapy. *Curr Nanosci*. 2010; 6:347–354.
5. Mei H, Shi W, Pang ZQ, Wang HF, Lu WY, Jiang XG, Deng J, Guo T, Hu Y. EGFP-EGF1 protein-conjugated PEG-PLA nanoparticles for tissue factor targeted drug delivery. *Biomaterials*. 2010; 31:5619–5626. [PubMed: 20413154]

6. Murphy EA, Majeti BK, Barnes LA, Makale M, Weis SM, Lutu-Fuga K, Wrasidlo W, Cheresch DA. Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis. *Proc Natl Acad Sci U S A*. 2008; 105:9343–9348. [PubMed: 18607000]
7. Patil Y, Sadhukha T, Ma LN, Panyam J. Nanoparticle-mediated simultaneous and targeted delivery of paclitaxel and tariquidar overcomes tumor drug resistance. *J Control Release*. 2009; 136:21–29. [PubMed: 19331851]
8. Petros RA, DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. *Nat Rev Drug Discov*. 2010; 9:615–627. [PubMed: 20616808]
9. Rivkin I, Cohen K, Koffler J, Melikhov D, Peer D, Margalit R. Paclitaxel-clusters coated with hyaluronan as selective tumor-targeted nanovectors. *Biomaterials*. 2010; 31:7106–7114. [PubMed: 20619792]
10. Veisheh O, Gunn JW, Zhang MQ. Design and fabrication of magnetic nanoparticles for targeted drug delivery and imaging. *Adv Drug Deliv Rev*. 2010; 62:284–304. [PubMed: 19909778]
11. Discher BM, Won YY, Ege DS, Lee JCM, Bates FS, Discher DE, Hammer DA. Polymersomes: Tough vesicles made from diblock copolymers. *Science*. 1999; 284:1143–1146. [PubMed: 10325219]
12. Photos PJ, Bacakova L, Discher B, Bates FS, Discher DE. Polymer vesicles in vivo: correlations with PEG molecular weight. *J Control Release*. 2003; 90:323–334. [PubMed: 12880699]
13. Ahmed F, Pakunlu RI, Brannan A, Bates F, Minko T, Discher DE. Biodegradable polymersomes loaded with both paclitaxel and doxorubicin permeate and shrink tumors, inducing apoptosis in proportion to accumulated drug. *J Control Release*. 2006; 116:150–158. [PubMed: 16942814]
14. Discher BM, Bermudez H, Hammer DA, Discher DE, Won YY, Bates FS. Cross-linked polymersome membranes: Vesicles with broadly adjustable properties. *J Phys Chem B*. 2002; 106:2848–2854.
15. Ghoroghchian PP, Frail PR, Susumu K, Park TH, Wu SP, Uyeda HT, Hammer DA, Therien MJ. Broad spectral domain fluorescence wavelength modulation of visible and near-infrared emissive polymersomes. *J Am Chem Soc*. 2005; 127:15388–15390. [PubMed: 16262400]
16. Cheng ZL, Thorek DLJ, Tsourkas A. Porous polymersomes with encapsulated Gd-labeled dendrimers as highly efficient MRI contrast agents. *Adv Funct Mater*. 2009; 19:3753–3759.
17. Cheng ZL, Tsourkas A. Paramagnetic porous polymersomes. *Langmuir*. 2008; 24:8169–8173. [PubMed: 18570445]
18. Ghoroghchian PP, Frail PR, Susumu K, Blessington D, Brannan AK, Bates FS, Chance B, Hammer DA, Therien MJ. Near-infrared-emissive polymersomes: Self-assembled soft matter for in vivo optical imaging. *Proc Natl Acad Sci U S A*. 2005; 102:2922–2927. [PubMed: 15708979]
19. Christian NA, Milone MC, Ranka SS, Li GZ, Frail PR, Davis KP, Bates FS, Therien MJ, Ghoroghchian PP, June CH, Hammer DA. Tat-functionalized near-infrared emissive polymersomes for dendritic cell labeling. *Bioconjugate Chem*. 2007; 18:31–40.
20. Robbins GP, Jimbo M, Swift J, Therien MJ, Hammer DA, Dmochowski IJ. Photoinitiated destruction of composite porphyrin-protein polymersomes. *J Am Chem Soc*. 2009; 131:3872–3873. [PubMed: 19249827]
21. Discher DE, Ahmed F. Polymersomes. *Annu Rev Biomed Eng*. 2006; 8:323–341. [PubMed: 16834559]
22. Gabizon A, Horowitz AT, Goren D, Tzemach D, Mandelbaum-Shavit F, Qazen MM, Zalipsky S. Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: In vitro studies. *Bioconjugate Chem*. 1999; 10:289–298.
23. Yamada A, Taniguchi Y, Kawano K, Honda T, Hattori Y, Maitani Y. Design of folate-linked liposomal doxorubicin to its antitumor effect in mice. *Clin Cancer Res*. 2008; 14:8161–8168. [PubMed: 19088031]
24. Nam J, Beales PA, Vanderlick TK. Giant phospholipid/block copolymer hybrid vesicles: mixing behavior and domain formation. *Langmuir*. 2011; 27:1–6. [PubMed: 21133340]
25. Ruysschaert T, Sonnen AFP, Haefele T, Meier W, Winterhalter M, Fournier D. Hybrid nanocapsules: Interactions of ABA block copolymers with liposomes. *J Am Chem Soc*. 2005; 127:6242–6247. [PubMed: 15853329]

26. Dudia A, Kocer A, Subramaniam V, Kanger JS. Biofunctionalized lipid-polymer hybrid nanocontainers with controlled permeability. *Nano Letters*. 2008; 8:1105–1110. [PubMed: 18311934]
27. Nilsson FY, Tolmachev V. Affibody (R) molecules: New protein domains for molecular imaging and targeted tumor therapy. *Curr Opin Drug Discov Dev*. 2007; 10:167–175.
28. Pangu G, Johnston E, Petkov J, Parry N, Leach M, Hammer DA. Targeted particulate adhesion to cellulose surfaces mediated by bifunctional fusion proteins. *Langmuir*. 2007; 23:10682–10693. [PubMed: 17845062]
29. Evans EA, Waugh R, Melnik L. Elastic area compressibility modulus of red-cell membrane. *Biophys J*. 1976; 16:585–595. [PubMed: 1276386]
30. Zhou X, Arthug G. Improved procedures for the determination of lipid phosphorus by malachite green. *J Lipid Res*. 1992; 33:1233–1236. [PubMed: 1431602]
31. Heerklotz H, Binder H, Lantzsch G, Klose G. Membrane water partition of oligo(ethylene oxide) dodecyl ethers and its relevance for solubilization. *Biochim Biophys Acta-Biomembr*. 1994; 1196:114–122.
32. Le Meins JF, Sandre O, Lecommandoux S. Recent trends in the tuning of polymersomes' membrane properties. *Eur Phys J E*. 2011; 34:1–17. [PubMed: 21337017]
33. Bermudez H, Brannan AK, Hammer DA, Bates FS, Discher DE. Molecular weight dependence of polymersome membrane structure, elasticity, and stability. *Macromolecules*. 2002; 35:8203–8208.
34. Mabrouk E, Cuvelier D, Pontani LL, Xu B, Levy D, Keller P, Brochard-Wyart F, Nassoy P, Li MH. Formation and material properties of giant liquid crystal polymersomes. *Soft Matter*. 2009; 5:1870–1878.
35. Elias DR, Cheng ZL, Tsourkas A. An intein-mediated site-specific click conjugation strategy for improved tumor targeting of nanoparticle systems. *Small*. 2010; 6:2460–2468. [PubMed: 20925038]

Figure 1.

Schematic diagram of folate receptor or Her2/neu receptor-targeted, polymer-lipid hybrid vesicles. Nanovesicles were formed through the co-assembly of the diblock copolymer PEO-PBD and the phospholipid HSPC. Folate receptor targeting was achieved by doping the vesicles with low percentage of PEG2000-DSPE-Folic acid. Her2/neu receptor targeting was achieved by conjugation of HER2/neu targeted affibodies to the vesicle surface via copper free click conjugation.

Figure 2.

Fluorescent images of giant vesicles composed of PEO-PBD and HSPC at three different molar ratios. Vesicles composed of 100% PEO-PBD, 90% PEO-PBD/10% HSPC, or 75% PEO-PBD/25% HSPC were doped with a low amount (< 1 mol %) of the fluorescent phospholipid Rhod-PE. Notably, it was observed that Rhod-PE was incorporated into all of the vesicles in each sample.

Figure 3. Elastic moduli of vesicles composed of PEO-PBD and HSPC at three different molar ratios. Micropipette aspiration was used to measure area elastic modulus (Ka) of giant vesicle formulations with different compositions of PEO-PBD and HSPC. Inset: Image of a giant polymer-lipid hybrid vesicle (75 mol% PEO-PBD/25 mol% HSPC) during aspiration procedure.

Figure 4.

Fluorescent images of giant lipid-polymer vesicles following surface modification with a fluorescent dye. Vesicles prepared with (A) 100% PEO-PBD, (B) 90% PEO-PBD/10% HSPC, or (C) 75% PEO-PBD/25% HSPC were doped with low amount (<1%) of DSPE-PEG2K-NH₂ and were subsequently labeled with NHS-ATTO740. (D) For comparison, 100% PEO-PBD vesicles were also prepared in the absence DSPE-PEG2K-NH₂ for NHS-ATTO740 surface conjugation.

Figure 5. Intensity-weighted size distribution of three different vesicle formulations as measured by dynamic light scattering (DLS).

Figure 6.

(A) The cell viability of NIH 3T3 cells incubated with nanovesicles with different lipid-polymer compositions. (B) The cell viability of NIH 3T3 cells incubated with 75 % PEO-PBD/25 % HSPC nanovesicles at different polymer concentrations. Viability was measured and normalized to cells grown in the absence of any particles based on an MTT assay.

Figure 7.

In cellulo analysis of folate receptor-targeted nanovesicles prepared with different lipid-polymer compositions. (A) Microscopy images of KB cells that have been incubated with various folate receptor-targeted vesicles prepared with PEO-PBD/HSPC at three different molar ratios. The top two rows show phase contrast and fluorescence images, respectively, collected in the absence of free folic acid in the media. The bottom two rows show images collected in the presence of 1 mM free folic acid in the media. All vesicles were incubated with KB cells for 2 hours before images were acquired. Folate receptor targeting was achieved by doping the vesicles with low percentage of PEG2000-DSPE-Folic acid. All vesicles were also doped with a small amount of Rhod-PE. (B) Flow cytometric analysis of KB cells following incubation with folate receptor-targeted vesicles prepared with PEO-PBD/HSPC at three different molar ratios. Normalized mean fluorescent intensities are shown $\pm$ S.D (n=3). To confirm that cell binding was mediated through the folate receptor,

competitive inhibition studies were performed by incubating KB cells with folate-targeted vesicles in the presence of excess free folic acid.

Figure 8.

In cellulo analysis of Her2/neu receptor-targeted nanovesicles prepared with different lipid-polymer compositions. (A) Microscopy images of T6-17 cells that have been incubated with HER2/neu receptor-targeted vesicles prepared with PEO-PBD/HSPC at three different molar ratios. The top row shows phase contrast images and the bottom row shows fluorescent images of the same cells. (B) Flow cytometric analysis of T6-17 cells following incubation with HER2/neu receptor-targeted vesicles prepared with PEO-PBD/HSPC at three different molar ratios. Normalized mean fluorescent intensities are shown $\pm$ S.D (n=3). To confirm that cell binding was mediated through the HER2/neu receptor, competitive inhibition studies were performed by incubating T6-17 cells with HER2/neu-targeted vesicles in the presence of excess free HER2/neu affibody.

Figure 9.

In vivo analysis of Her2/neu receptor-targeted nanovesicles prepared with different lipid-polymer compositions. (A) Fluorescence images of T6-17 tumor-bearing mice 24 h post-injection of NIR HER2/neu receptor-targeted vesicles prepared with PEO-PBD/HSPC at two different molar ratios. Tumors are indicated by white arrows. (B) Quantitative analysis of fluorescent images.

Table 1

Vesicle diameters and number of targeting agents per vesicle

Vesicle composition	Diameter (nm)	Folate/vesicle	HER2/vesicle
100 mol % PEO-PBD	105	310	24
90 mol %PEO-PBD/10 mol % HSPC	112	261	22
75 mol %PEO-PBD/25 mol % HSPC	117	193	21