Published in final edited form as:

Polym Chem. 2014 March 7; 5(5): 1791–1799. doi:10.1039/C3PY01404E.

Synthesis and characterization of transferrin-targeted chemotherapeutic delivery systems prepared via RAFT copolymerization of high molecular weight PEG macromonomers

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Abstract

Reversible addition-fragmentation chain transfer (RAFT) polymerization was employed to prepare a nanoparticulate drug delivery system for chemotherapeutics. The nanoparticles contain a PEG “stealth” corona as well as reactive anhydride functionality designed for conjugating targeting proteins. The multifunctional carrier functionality was achieved by controlling the copolymerization of the hydrophobic monomer lauryl methacrylate (LMA), with a reactive anhydride functional methacrylate (TMA), and a large polyethyleneglycol methacrylate monomer ($M_n \sim 950$ Da) (O950). RAFT polymerization kinetics of O950 were evaluated as a function of target degrees of polymerization (DP), initial chain transfer agent to initiator ratio ($[CTA]_0/[I]_0$), and solvent concentration. Excellent control over the polymerization was observed for target DPs of 25 and 50 at $[CTA]_0/[I]_0$ ratio of 10 as evidenced by narrow and symmetric molecular weight distributions and the ability to prepare block copolymers. The TMA-functional copolymers were conjugated to the tumor targeting protein transferrin (Tf). The targeted copolymer was shown to encapsulate docetaxel at concentrations comparable to the commercial single vial formulation of docetaxel (Taxotere). In vitro cytotoxicity studies conducted in HeLa cells show that the Tf targeting enhances the cancer killing properties relative to the polymer encapsulated docetaxel formulation.

Introduction

Chemotherapy remains a frontline approach to managing cancer but are associated with a range of serious dose-limiting toxicities including cardiomyopathy, febrile neutropenia, anemia, and thrombocytopenia.^{1,2} The use of nanoparticle-based therapies to deliver cytotoxic agents has the potential to significantly improve the activity and side-effect profiles. Chemotherapeutic nanoparticle formulations such as Doxil (liposomal encapsulated doxorubicin) exhibit enhanced circulation half-lives (up to 100 times greater than the unencapsulated drug) yet cause substantially lower deleterious side effects.¹ In the case of Doxil, the risk of cardiotoxicity is 7-fold lower than the free drug despite the large

difference in circulation half-lives.^{2,3} The application of controlled radical polymerization (CRP) technology to prepare chemotherapeutic drug delivery systems has opened new material systems. For example, Zhongfan et al. described the development of block copolymers of N-(2-Hydroxypropyl)methacrylamide (HPMA) with a bioconjugatable monomer 2-(2-pyridyldisulfide)ethylmethacrylate (PDSMA) via the RAFT process.⁴ The resultant diblock copolymer was then simultaneously conjugated to doxorubicin and crosslinked via hydrazone linkages to form micellar assemblies that released free drug upon a decrease in pH. The sequestration of hydrophilic platinum-based therapeutics has also been achieved via a combination of RAFT, thiol-ene, and thiol-yne chemistry and yielded materials with pendent Pt drugs.⁵ These authors have also described the RAFT synthesis of Pt delivery systems based on block copolymer micelles consisting of a hydrophilic biocompatible polyethylene glycol methacrylate (PEGMA) corona and a hydrophobic styrene core containing reactive isocyanate groups for conjugation of cisplatin prodrug.⁶

Polyethylene glycol is a common functional component connected to the enhanced circulation properties. Poly(ethylene glycol) methyl ether methacrylate (PEGMA) is a polymerizable PEG containing macromonomer that has been widely employed as a precursor for the preparation of therapeutic nanoparticles.⁷⁻⁹ The wide variety of bioapplications of these polymers stem from their stealth properties. Previously, various polymerization techniques such as anionic, cationic, ring opening metathesis, and free radical polymerization have been employed to polymerize PEG macromonomers.¹⁰⁻¹¹ The advent of controlled radical polymerization (CRP) methods such as atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer (RAFT) polymerization have further broadened the scope of available PEG-based macromolecular architectures. Armes et al. first reported the controlled ATRP polymerization of PEGMA with 7/8 ethylene oxide (EO) units in aqueous medium.¹² Lutz et al. demonstrated the well controlled ATRP polymerization of PEGMA with 8/9 ethylene oxide units ($M_n = 475$ g/mol) in organic solvents.¹³ RAFT polymerization technique has also been used to synthesize well controlled poly(PEGMA) with 8/9 ethylene oxide in aqueous environment^{9,14} or with 4/5 in organic solvents.⁷ The solution and stealth properties of PEGMA depend on its fine balance between hydrophilic and hydrophobic groups.⁹ While the ether oxygen atoms on the polyethylene oxide segment form stabilizing hydrogen bonds with water,¹⁵ the apolar carbon-carbon backbone increases hydrophobicity.¹⁶ As a result, poly(PEGMA) with short PEG side chains are only mildly hydrophilic, whereas poly(PEGMA)s with 10 or more ethylene oxide units exhibit enhanced hydrophobicity.⁹ While the controlled radical polymerization of PEGMA has been thoroughly investigated for shorter PEG side chains (EO units between 4/5 to 8/9), the CRP of PEGMA with longer PEG side chains (EO units > 10) are sparse.¹⁶ The purification of relatively large PEGMA monomers from the corresponding poly(PEGMA) has been shown to be problematic.^{16,11}

Targeted nanotherapies have the potential to further enhance the effectiveness of nanoparticle therapies. Active targeting of tumor surface receptors via antibodies, peptides, aptamers, and vitamins has been shown to significantly enhance the specificity of these therapies compared to passive targeting alone.^{17,18} Chemotherapeutic delivery systems may be coupled to targeting agents through the use of activated ester-containing compounds

resulting in conjugates linked via stable amide bonds. Typical activated esters include succinimidyl^{19,20} and pentafluorophenyl esters^{21,22} as well as mercaptothiazoline.^{23,24} Herein we report the successful RAFT polymerization of PEGMA with high molar mass (M_n = 950 g/mol, EO units 17/18) and its integration into a targeted drug delivery platform that can also incorporate protein targeting ligands and potent chemotherapeutic drugs.

Experimental details

Materials

Chemicals and all materials were supplied by Sigma-Aldrich unless otherwise specified. Docetaxel, was obtained from LC Laboratories. 4-Methacryloxyethyl trimellitic anhydride (TMA) was obtained from Polysciences and used as received. Cell mask Far Red, Alexafluor 488 NHS ester, and Hoechst stains were obtained from invitrogen. Spectra/por regenerated cellulose dialysis membranes (6-8 kDa cutoff) were obtained from Fischer. Lauryl methacrylate was passed through a short column of basic alumina. PD10 columns were obtained from GE life sciences. MTS cytotoxicity kits were obtained Promega. HeLa cells, human cervical carcinoma cells (ATTC), were maintained in minimum essential media (MEM) containing L-glutamine (Gibco), 1% penicillin-streptomycin (Gibco), and 10% fetal bovine serum (FBS, Invitrogen) at 37 °C and 5% CO₂. O950 (Aldrich) (30 g) was dissolved in 70 g of tetrahydrofuran (THF) and then passed through a 6-inch plug activated basic alumina. The monomer was collected slowly due to height of packed Al₂O₃ and the viscosity of the concentrated monomer solution. After collection the THF was removed under reduced pressure using a rotary evaporator followed by a high vacuum line. The PEGMA 950 solution, which became a waxy solid under reduced pressure, was gently melted by immersion of the flask in a warm water bath under high vacuum. NMR analysis confirmed the presence of 3 % (by mass) residual THF which was accounted for in subsequent polymerization calculations.

RAFT polymerization kinetics for poly(ethylene glycol) methyl ether methacrylate (O950)

Kinetic evaluation of the RAFT polymerization of O950 was conducted with 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid (CTP) and 4,4'-Azobis(4-cyanovaleric acid) (ABCVA) as the RAFT chain transfer agent and initiator respectively in inhibitor free dioxane at 70 °C. The initial monomer ($[M]_0$): $[CTA]_0$: $[I]_0$ ratio were 25:1:0.05 and 50:1:(0.10/0.05) respectively. Individual polymerization solutions were transferred to a septa-sealed vial and purged with nitrogen for 30 minutes. After this time, the polymerization vials were transferred to a preheated oil bath at 70 °C and allowed to polymerize for the prescribed time period. A representative procedure for the synthesis of a poly(O950) targeting a DP of 25 is as follows. To a 50 mL round bottom flask was added O950 (6.00 g, 6.315 mmol), CTP (70.6 mg, 252 μ mol), ABCVA (7.08 mg, 25.2 μ mol), and inhibitor free dioxane (24 g). The solution was then purge with nitrogen and allowed to heat for 24 h at 70 °C. Following polymerization, the individual vials were quenched by immersion in liquid nitrogen and evaluated via ¹H NMR in CDCl₃ and GPC (DMF eluent). Isolation of PEGMA 950 homopolymers was achieved by precipitation of 5 mL aliquots of the polymerization solution into 45 mL of diethyl ether in 50 mL tubes. The combined mixture was then centrifuged for 10 minutes at 4200 rpm (note: it is important not to cool the precipitation

solution as the O950 macromonomer will coprecipitate at low temperatures). The nonsolvent was then decanted and the pink oil was diluted with 2 mL of acetone. This solution was then precipitated as described above (x6). Following precipitation the viscous oil was dried overnight under vacuum to yield the pure polymer as a waxy solid.

RAFT copolymerization of lauryl methacrylate (LMA), O950, and methacryloxyethyl trimellitic anhydride (TMA)

The RAFT copolymerization of LMA, TMA, and PEGMA 950 was conducted in inhibitor free dioxane at an initial total comonomer concentration of 10 wt. % with an CTA to initiator ratio ($[CTA]_0/[I]_0$) of 5 to 1 with an monomer to CTA ratio ($[M]_0/[CTA]_0$) of 50 to 1. Polymerization were purged with nitrogen for 1 hour and then heated at 70 °C for 24 hours. After this time the polymerization solutions were transferred to spectrapor regenerated cellulose dialysis membranes (preequilibrated in deionized water) and then dialyzed against acetone at 5 °C for 1 week. Following dialysis the polymer solution in acetone was filtered through a plug of cotton after which time the acetone was removed via rotary evaporation. The resultant viscous solid was then dried under high vacuum for 48 hours. A representative procedure is as follows: To a 50 mL round bottom flask was added O950 (3.12 g, 3.28 mmol), LMA (2.50 g, 9.83 mmol), TMA (1.00g, 3.28 mmol), CTP (92 mg, 0.033 mmol), azobiscyanovaleric acid (ABCVA) (18.4 mg, 0.066 mmol), and Dioxane (27 g). The polymerization solution was then purged with nitrogen for 60 minutes and then transferred to a preheated oil bath at 70 °C and allowed to react for 24 hours. Copolymer composition was determined by integration of the aromatic resonances (7.70 – 8.80 ppm) and the methoxy resonance (3.37 ppm) associated with 3 protons from TMA and O950 respectively allowed algebraic determination of the LMA content via subtraction of the corresponding methylene and methyl resonance for each of these comonomers from the backbone region (0.5 – 3.0 ppm).

Conjugation of Tf to poly[(LMA_{co}TMA_{co}O950)]

Tf was conjugated to the diblock copolymer via reaction of pendant anhydride residues incorporated throughout the copolymer via the methacrylate comonomer (TMA) with lysine residues on the proteins. The copolymer and protein stocks were prepared at 75 and 21 mg/mL in ethanol and buffer respectively. The protein solution was diluted with additional buffer such that the final protein concentration following addition of the ethanolic polymer stock was 0.3 mg/mL. To this solution the copolymer in ethanol was added to produce the desired polymer/protein molar ratios over a range of 1:1 to 128:1 The degree of protein conjugation to the polymer was verified using polyacrylamide gel electrophoresis (PAGE) using Mini-PROTEAN TGC precast gels (4-20 %) (BIORAD) with tris-glycine-SDS (10x stock = 0.25 M tris, 1.92 glycine, 1 % SDS) (national diagnostics). The gel was run for 1 hour at 150 Volts at room temperature and subsequently stained for 18 hours in GelCode blue and destained overnight with deionized water.

Micelle encapsulation of docetaxel

Doxetaxel was codissolved with the poly(LMA_{co}TMA_{co}PEGMA 950) according to the formulation parameters outlined in Table 1. The concentrated docetaxeethanol-polymer was then diluted with 5 % dextrose solution containing 0.2 M HEPES buffered to pH 7.4 to a

final drug concentration of 0.5 mg/mL. The solutions were then sterile filtered through a 0.2 μ m filter and then stored at 5 °C. For in vitro toxicity experiments, where the desired drug-loading is significantly lower than the corresponding clinical formulations, the polymer concentration was fixed at 0.2 mg/mL. To a solution of copolymer (400 μ g) in dextrose HEPES buffer 20 mM pH 7.4 (93 μ L) was added 5 μ L of docetaxel as stock solutions in ethanol. This solution was then incubated at 5 °C overnight at which time 1900 μ L of media was added. The solution was then sterile filtered with a 0.2 μ m filter and 200 μ L was added to 96 well plates such that the final DTX concentration was between 100 and 0.5 nM.

In vitro cytotoxicity measurements

The cytotoxicity of drug-loaded micelles as well as the drug free diblock copolymer were evaluated in HeLa cells using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) (Promega Corp., Madison, WI). HeLa cells were seeded at a density of 5000 cells/well in 96-well plates and allowed to adhere for 24 h. The media was then replaced with 200 μ L of fresh media containing the unloaded and drug loaded micelles at the appropriate concentrations. After 72 hours the media was replaced with new media and the cells were evaluated using the CellTiter MTS assay according to the manufacturer's instructions. The absorbance at 490 nm was evaluated using Tecan Safire 2 microplate reader. Untreated cells in media were used as a negative control. All experiments were carried out at a series of four replicates for at least two experiments.

Gel permeation chromatography (GPC)

Absolute molecular weights and dispersity indices were determined using Tosoh SEC TSK-GEL α -3000 and α -4000 columns (Tosoh Bioscience, Montgomeryville, PA) connected in series to an Agilent 1200 Series Liquid Chromatography System (Santa Clara, CA) and Wyatt Technology miniDAWN TREOS, 3 angle MALS light scattering instrument and Optilab rEX, refractive index detector (Santa Barbara, CA). HPLC-grade DMF containing 0.1 wt.% LiBr at 60 °C was used as the mobile phase at a flow rate of 1 ml/min.

Critical micelle concentration (CMC) via fluorescence

The critical micelle concentration (CMC) for the copolymer micelles were determined using Rhodamine 6G as a fluorescence probe. The concentration of copolymer was varied between 1 and 1000 μ g/mL, with a fixed concentration of Rhodamine 6G of 10 μ M. The fluorescence spectra were recorded using a Tecan Safire 2 microplate reader with an excitation and emission wavelength of 480 and 550 nm respectively. The CMC was estimated as the cross-point when extrapolating the intensity at 550 nm between low and high concentration regions.

Transmission Electron Microscopy (TEM)

A 1.0 mg/mL solution of the polymeric nanoparticles in PBS was applied to a carbon-coated copper grid for 30 min, fixed in Karnovsky's solution, and washed in cacodylate buffer and water. The grid was stained with a 6% solution of uranyl acetate for 15 min and then dried until analysis. Transmission electron microscopy was carried out on a Tecnai G2 F20, 200

kV scanning transmission electron microscope (S/TEM). Average particle size and circularity were determined using ImageJ by evaluating 100 separate nanoparticles.

Fluorescence microscopy: acquisition and analysis

The intracellular distribution of fluorescently labeled Tf-polymer conjugates was evaluated in cells using live-cell fluorescence microscopy. Cells were seeded in chamber slides at a concentration of 5 000 cells/well. After allowing the cells adhere for 18 hours the media was replaced with fresh media containing the appropriate concentration of the copolymer-Tf conjugates. Thirty minutes prior to imaging Hoechst (5 µg/mL) was added to the media and allowed to incubate at 37 °C. After this time, the media was carefully aspirated and replaced with PBS. Immediately prior to imaging individual wells were aspirated and then treated with 2 µL of a 10 mg/mL cell mask stock in 200 µL of ice cold PBS. After 3 minutes the PBS was removed and washed three times with additional ice cold PBS. The mediChamber slides were placed on a Live-Cell Fluorescence Microscope (Nikon Ti-E) equipped with an environmental control chamber. Cells were imaged with a mercury lamp and a 100X objective using the following filter sets: 480/40, (EX) and 535/50 nm (EM) for AF488, Hoechst and Cell Mask Deep red. (Chroma 49000 Series, Rockingham, VT). For each image stack, 24 z-sections with a 0.5 µm step size were collected using a 1/8 neutral density filter and 400 millisecond exposure. After image acquisition, image stacks were deconvolved using object-based measurement software, Velocity (Perkin Elmer), to remove out-of focus fluorescence for the identification of conjugate containing compartments. To deconvolved image stacks, point spread functions were calculated for the green, blue, and deep red channel and applied using 25 iterations to reach a near 100% confidence interval.

Results and discussion

Synthetic design for the preparation of protein-reactive drug carriers

Shown in Fig. 1 is the synthetic strategy for the preparation of polyethylene glycol (PEG) containing copolymers capable of efficiently encapsulating hydrophobic drugs such as the antineoplastic agent docetaxel. Under aqueous conditions these materials are designed to self-assemble to form micelles with phase separated LMA residues stabilized in solution by hydrophilic O950 residues. This combination of monomers closely mimics the physiochemical properties of the clinically established surfactant-based drug stabilizer Polysorbate 80 (PS80). PS80 stabilized formulations of docetaxel show high intrinsic tumor killing activity but are associated with severe dose-limiting toxicity.²⁵ In order to minimize these toxic side affects and enhance the effectiveness of current taxane-based therapies the drug delivery system outlined in Fig. 1 is designed employ tumor specific targeting ligands. These ligands are conjugated to the hydrophilic micelle corona via the reaction of amine residues (present on many large proteins and antibodies) with polymeric anhydride residues. These conjugation reactions can be conducted directly in physiological saline without the need for protein modification reactions (Fig. 1). Anhydride functionality is easily integrated directly into the copolymers through the addition of 4-Methacryloxyethyl trimellitic anhydride (TMA) to the polymerization.

RAFT polymerization kinetics for O950

In order to effectively copolymerize the monomers outlined in Fig. 2 we first examined the polymerization behavior of O950 alone by conducting polymerization kinetics for the large sterically bulky macromonomer in dioxane. Dioxane was selected as the polymerization medium based on the ability of this solvent to dissolve both hydrophilic and hydrophobic monomers as well as the resultant copolymers. Additionally the relatively high boiling point and lack of reactivity of this solvent allowed polymerization to be conducted at 70 °C without degrading the anhydride functionality present on TMA. Shown in Fig. 2(a-d) are the kinetic results for the homopolymerization of O950 targeting DPs of 25 and 50.

Polymerizations targeting a DP of 25 were conducted at a $[CTA]_0/[I]_0$ of 10 to 1 at an initial monomer concentration of 20 wt. % while those targeting a DP of 50 were evaluated at $[CTA]_0/[I]_0$ of 5 and 10 to 1 at initial monomer concentrations of 10 and 20 weight % respectively. As can be seen from Fig. 2a the pseudo first order rate plots for the RAFT polymerizations of O950 conducted at 20 weight % monomer are linear over the entire polymerization period up to high monomer conversion. For both polymerizations approximately 80 % monomer conversion is reached at 24 hours even at an initial CTA to initiator ratio of 10 to 1. In contrast polymerizations targeting a DP of 50 at an initial monomer concentration of 10 weight % and a $[CTA]_0/[I]_0$ of 5 to 1 plateau above 34 % monomer conversion after an initial linear period. These results highlight the strong monomer concentration dependence for the polymerization of O950. Although polymerization rates and monomer conversions were increased at higher initial monomer concentrations a high molecular weight shoulder was observed at higher monomer conversion for polymerizations conducted above an initial monomer concentration of 20 wt. % (not shown). Evaluation of the M_w/M_n and M_n vs conversion plots (Fig. 2b, c, d) illustrate the controlled nature of PEGMA 950 under these conditions. For all three polymerizations M_w/M_n remains low through out the course of the polymerization. While an initial overshoot of the theoretical molecular weight is observed at low monomer conversions good agreement between the theoretical and experimentally determined molecular weights is observed at intermediate and high monomer conversions. In all cases the molecular weight distributions remain unimodal, symmetric, and remain narrow (Fig. 3a). A slight increase in M_w/M_n from 1.05 at 26.5 % monomer conversion to 1.27 at 81 % monomer conversion was observed for polymerizations targeting a DP of 50 ($[CTA]_0/[I]_0 = 10$ to 1). This increase in M_w/M_n is not observed for polymerizations targeting a DP of 25 with sub 1.1 values observed at low (20 %) and high (80%) monomer conversions.

Copolymerization of O950 with a hydrophobic LMA comonomer display narrow molecular weight distributions over a range of target DPS between 12.5 and 50 yield copolymer molecular weights of between 7 100 and 23 500 Da (Fig. 3b). Excellent chain end retention of the thiocarbonyl thio group is also observed polymerization of O950 conducted under these conditions (Fig. 3c). Chain extension of poly(O950) (19 kDa, PD = 1.06) with a copolymer of LMA and TMA (50:50 mol/mol feed) shows a clear shift in the MWD to lower elution volumes yielding a final diblock copolymer with a molecular weight and M_w/M_n of 39 800 Da, and 1.19 respectively. Clear shifts in the molecular weight distributions and narrow M_w/M_n values were also observed for the chain extension of O950 macroCTAs containing reactive comonomers. As shown in Fig. 3d the Block copolymerization of O950 and LMA (50:50 mol %) from a poly[O950-coTMA] macroCTA (30 900 Da, M_w/M_n of 1.13) to yield a

poly[(O950_{co}TMA)_b(O950_{co}LMA)] diblock copolymer with a molecular weight of 5200 and 1.18 respectively.

Nanoparticle synthesis and characterization

The polymerization conditions established for homopolymerization of O950 were subsequently employed to prepare a series of protein-reactive drug nanocarriers targeting a range of hydrophobic LMA to hydrophilic O950 ratios at a fixed molar feed ratio of 25 % for the reactive TMA comonomer as outlined in Fig. 1. The molecular weight, M_n , and composition data for these copolymers are outlined in Table 1. Shown Fig. 4 a, and b, are the MWD and ¹H NMR spectrum for polymer 3, which was conducted with an initial molar percentage of TMA:O950:LMA of 25:25:50 %. The molecular weight distribution for the copolymerization is quite narrow and symmetric and is representative of the other copolymer compositions outlined in Table 1. The ¹H NMR spectrum for polymer 3 conducted in CDCl₃, which is a good solvent for all three comonomer residues in the copolymer, show resonances associated with each of the individual comonomer units. For example the large aliphatic resonance associated with the LMA sidechain (v) is visualized as an intense resonance at 1.28 ppm. Also present are the intense resonances associated with the O950 ethylene oxide repeat (iii) units and –OCH₃ (iv) at 3.66 and 3.39 ppm as well as the aromatic TMA resonances (i) between 8 and 9 ppm. Dissolution of this copolymer in D₂O shows a strong attenuation of the hydrophobic resonances while the O950 resonances remain prominent. This result is consistent with the formation of the desired micellar morphology with the hydrophobic PEGMA 950 residues stabilizing the hydrophobic LMA residues in aqueous solution. Dynamic light scattering results suggest that these micelles are small with sizes around 5 nm which is consistent with intramolecular or unimolecular micelles.

Critical micelle concentration (CMC) determination

The critical micelle concentration for the copolymers was evaluated as a function of hydrophobic lauryl methacrylate content for copolymers containing between 59 and 77 mol % LMA over a concentration range of 1 and 1000 µg/mL in phosphate buffer (pH = 7.4) using rhodamine 6G as a polarity sensitive fluorophore (Fig. 4c). Rhodamine 6G is strongly fluorescent in water but upon localization in a less polar environment this fluorescence is usually quenched. While all three copolymers showed similar fluorescence trends higher LMA containing copolymers showing slightly lower CMCs (Table 1). These values were found to be between 52 µg/mL for copolymer 3 containing 59 % LMA and 26 µg/mL for copolymer 1 containing 68 % LMA. Copolymers containing higher mole fractions of LMA (e.g. n_f LMA = 80 %) were not readily dispersible in aqueous solution but could be dispersed in water to form 60 nm particles following the formation of films cast from chloroform (not shown). The morphology of these particles as well as their potential application as polymersome-based drug delivery vehicles is currently under investigation.

Targeting protein conjugation to polymers

Conjugation of the copolymer to large proteins was investigated using Tf under a variety of pH and analyte conditions. Tf receptors are highly overexpressed in a number of cancers

including cervical cancers.²⁶ Protein conjugation studies were conducted by first dissolving the polymer in ethanol at a concentration of between 50-500 mg/mL. The concentrated ethanolic stock was then induced to form micelles by dilution directly into the appropriate buffer. These conditions were designed to closely match formulation of docetaxel (Taxotere) which consists of a concentrated stock of the drug plus ethanol in polysorbate 80 and a second aqueous diluent. The effect of pH on the conjugation of polymer 1 to Tf was evaluated as a function of solution pH. As can be seen in Fig. 5 the free Tf band remains approximately constant from lanes 1 to 9 at both pH 9.6 and 8.3 with only slight attenuation at the highest polymer to protein ratio. Under the staining conditions employed in these studies the free polymer band (lane 9) does not stain. In striking contrast the conjugation reactions conducted in pH 7.4 HEPES buffer containing 5 % glucose show significant reductions in the free Tf band at even the lowest polymer to protein ratios with complete conjugation at a ratio of approximately four. This significant difference in conjugation efficiency could either be related to the solution pH or the presence of 5 wt % glucose in the buffer. Given the significant differences in conjugation efficiency and the clinical relevance of physiologically buffered glucose solutions these conditions were employed in all subsequent conjugation reactions.

Encapsulation of docetaxel

The ability of the protein-reactive copolymers to encapsulate docetaxel under clinically relevant conditions was evaluated using formulation conditions similar to the single vial formulation of Taxotere 20 mg vials. This formulation consists of 20 mg of Taxotere in 1 mL of 50:50 (v/v) polysorbate 80 to ethanol which is then diluted into an IV of 5 % dextrose or 0.9 % NaCl to yield a final docetaxel concentration of between 0.3 and 0.74 mg/mL. This solution is reported to be stable for 4 hours if stored at ambient temperature and lighting conditions. Based on these formulation parameters a range of polymer to docetaxel ratios between 5 and 11 % (w/w) were evaluated as a preconcentrate in 50 % ethanol to polymer (v/v) (Table 1). Following complete dissolution of the docetaxel in the polymer ethanol mixture the resultant preconcentrate stocks were then diluted with 5 % dextrose containing 20 mM HEPES buffer pH 7.4 to a final docetaxel concentration of 0.5 mg/mL. These buffer conditions were selected because they are already commonly employed in clinical IV formulations and were shown in the previous section to provide excellent protein conjugation to the reactive polymer scaffold. Under all formulation conditions evaluated in Table 1 the preconcentrate stock completely dissolved in the aqueous buffer without any observable precipitate. The stability of the encapsulated DTX formulations was evaluated over a period of 24 hours at 37 °C in the presence of human serum albumin (50 mg/mL) at pH 7.4. HPLC analysis of the dialysis solution shows that approximately 87 % of the DTX remains encapsulated at 8 hours with approximately 70 % DTX retention at 24 hours. Dynamic light scattering of the resultant aqueous solutions showed that the nanoparticles had diameters of approximately 5 nm and remained stable for extended periods of time without any evidence of precipitation over a period of two weeks (See Supporting Information). Nanoparticle size and morphology was also evaluated via Transmission electron microscopy (TEM) as shown in Fig. 6. These studies show spherical particles (circularity ratio = 0.995 ± 0.007) with mean diameters of $12.34 \text{ nm} \pm 1.6 \text{ nm}$.

Live cell fluorescence imaging

Live cell imaging experiments conducted with fluorescently (ALEXA 488) labeled Tf confirm that the polymer bound protein retains the ability to access intracellular compartments via receptor-mediated endocytosis. As can be seen Fig. 7 the green fluorescence from Tf is strongly localized within endosomes/lysosomes upon incubation with HeLa cells for 1 hour at 37 °C. Hoechst (blue) and CellMask (red) were also employed in order to visualize the nucleus and cell membrane respectively.

In vitro cytotoxicity in HeLa cells

The cytotoxicity of the copolymer-encapsulated docetaxel was evaluated in HeLa (human cervical cancer) cell lines that overexpress Tf receptors. Formulations were prepared over a range of DTX concentrations between 1 and 100 nM. Control experiments conducted with the free polymer without encapsulated DTX were employed to establish the biocompatibility of the polymeric delivery system. At polymer concentrations of up to 1000 µg/mL cell viability levels (not shown) were similar within error to untreated controls for HeLa cells incubated with the free polymer for 72 hours. The ability of the Tf-nanoparticle conjugates to enhance the cytotoxicity of encapsulated docetaxel was evaluated as shown in Fig. 8. Tf receptors continually cycle from the cell surface to intracellular compartments with an average period of 7 minutes required to turnover the total population of surface receptors.²⁷ Once internalized these receptors have an intracellular resonance time of approximately 21 minutes suggesting that approximately three-quarters of the Tf receptor are present inside the cells at any moment.²⁷ Evaluation of the LD_{50} values in HeLa cells following a 72 h incubation period with both the polymer encapsulated docetaxel and the corresponding Tf conjugates showed values of 19 and 9.7 nM respectively. The increase in toxicity observed for the Tf targeted micelles is consistent with studies by Sahoo and Labhasetwar in which the authors found that the incorporation of Tf-targeting resulted in enhanced antiproliferative activity for paclitaxel-loaded poly(lactic-co-glycolide).²⁸ These studies demonstrated the greater and sustained antiproliferative activity of the Tf targeted nanoparticles in dose- and time dependent studies in MCF-7 and MCF-7/Adr cells. The authors attributed the greater antiproliferative properties of the Tf-targeted system to higher levels cellular uptake and reduced exocytosis of the targeted nanoparticles. Also shown in Fig. 8 are cell viability results for HeLa cells incubated with free unencapsulated DTX. These studies suggest that the free drug shows slightly greater concentration-dependent HeLa cell toxicity than the polymer encapsulated DTX but less than the DTX encapsulated with the TF-polymer conjugates.

Conclusion

RAFT polymerization kinetics of PEGMA 950 targeting a DP of 25 and 50 result in symmetric unimodal molecular weight distributions that move to lower elution volumes with increasing monomer conversion. SEC analyses of the molecular weight distributions confirm the close agreement between theoretical and experimentally determined molecular weights and low molar mass distribution. High monomer conversion where obtained for polymerizations conducted at an initial monomer concentration and $[CTA]_0/[I]_0$ of 20 wt. % and 10 respectively but where limited to approximately 30 % conversion for

polymerizations conducted at lower initial monomer concentration even at a $[CTA]_0/[I]_0$ of 1. Copolymerization of the PEGMA 950 with LMA, and TMA successfully yielded well-defined copolymers with controllable compositions. These copolymers were shown to efficiently encapsulate the potent chemotherapeutic agent docetaxel with formulation parameters comparable to the clinically established Taxotere. Aqueous formulations of the copolymer were shown to spontaneously form covalent bonds with Tf under clinically relevant buffer conditions. The presence of Tf targeting was shown significant improvements in docetaxel for the polymer-encapsulated docetaxel are comparable to the parent compound while fluorescent microscopy show substantial levels of internalized Tf-protein conjugates. These results taken together suggest that the targeted drug delivery technology outlined in this report could enhance the therapeutic index of clinically well-established chemotherapeutic agents by directing their accumulation in tumor tissue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by the National Institutes of Health (Grant R01EB002991 and Grant 1R21EB014572- 01A1) as well as The Life Science Discovery Fund (Grant 2496490).

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Fig. 1. Synthetic scheme for the preparation of protein-reactive hydrophobic drug carriers via RAFT copolymerization. Schematic representation of the copolymer drug delivery system integrating tumor specific transferrin targeting groups, a hydrophilic polyethylene glycol (PEG) stealth corona, and a hydrophobic drug sequestering core.

Fig. 2.

Kinetic evaluation of the RAFT polymerization of O950. a). Pseudo first order rate plot, M_n and vs. conversion for $[M]_0:[CTA]_0:[I]_0$ equal to (b) 50:1:0.1 (c) 25:1:0.1, and (d) 50:1:0.2 for the RAFT polymerization of O950 at 70 °C with CTP and ABCVA as the chain transfer agent and initiator respectively. Polymerizations were conducted in dioxane at an initial monomer concentration of 10 wt % (d) and 20 wt % (b,c). Absolute molecular weight values were determined by SEC equipped with inline laser light scattering detectors. Monomer conversion was determined by ^1H NMR by comparison of the vinyl resonances normalized to the total ester region relative prepolymerization values.

Fig. 3.

SEC analysis of O950 and copolymers prepared by RAFT. (a) Molecular weight distributions RAFT polymerization of O950 conducted at different initial target DPs, $[CTA]_0/[I]_0$ ratios, and solvent concentrations. (b) Molecular weight distributions for the copolymerization of O950 and LMA at equimolar feed ratios. All (Co)polymerizations shown were conducted in dioxane ($[M]_0 = 20$ wt% monomer) at 70°C with $[M]_0:[CTA]_0:[I] = 50:1:0.1$ unless otherwise indicated. (c) Block copolymerization from a p(O950) macroCTA ($M_n = 19\,000$ Da; $\text{PDI} = 1.06$) with an equimolar ratio of TMA and O950 resulting a diblock copolymer with an M_n of $39\,800$ Da and PDI of 1.19 respectively. (d) Block copolymerization of O950 and LMA ($50:50$ mol %) from a poly[O950_{co}TMA] macroCTA ($30\,900$ Da; PDI of 1.13) to yield a poly[(O950_{co}TMA)_b(O950_{co}LMA)] diblock copolymer with a molecular weight and PDI of $52\,500$ and 1.18 respectively.

Fig. 4. Molecular weight, composition, and critical micelle concentration values for poly(LMA_{co}O950_{co}TMA) prepared by RAFT. (a) Representative SEC chromatogram (refractive index channel) showing the narrow and symmetric molecular weight distribution for a copolymer of PEGMA 950, LMA, and TMA (25:50:25) feed ratio and (b) ^1H NMR spectrum of the copolymer in CDCl_3 with assignment of the resonances associated with the respective comonomer residues. (c) Rhodamine 6G fluorescence as a function of copolymer composition and concentration in phosphate buffered saline (20 mM sodium phosphate, 150 mM NaCl; pH 7.4) with an excitation and emission of 480 and 550 nm respectively.

Fig. 5.

Evaluation of Transferrin conjugation via gel electrophoresis following overnight incubation with poly(LMA_{co}O950_{co}TMA). Polyacrylamide gel electrophoresis for (left) transferrin and (right) Trastuzumab monoclonal antibody conjugation reactions conducted at various polymer to protein ratios. The degree of protein conjugation to the polymer was verified using polyacrylamide gel electrophoresis (PAGE) using Mini-PROTEAN TGC precast gels (4-20 %) (BIORAD) with tris-glycine-SDS (10× stock = 0.25 M tris, 1.92 glycine, 1 % SDS) (national diagnostics). The gel was run for 1 hour at 150 Volts at room temperature and subsequently stained for 18 hours in GelCode blue and destained overnight with deionized water.

Fig. 6. Transmission Electron Microscopy (TEM). A 1.0 mg/mL solution of the polymeric nanoparticles in PBS was applied to a carbon-coated copper grid for 30 min, fixed in Karnovsky's solution, and washed in cacodylate buffer and water. The grid was stained with a 6% solution of uranyl acetate for 15 min and then dried until analysis. Transmission electron microscopy was carried out on a Tecnai G2 F20, 200 kV scanning transmission electron microscope (S/TEM).

Fig. 7.

Live cell deconvoluted fluorescence microscopy images of HeLa cells treated with fluorescently labelled Transferrin-polymer conjugates. (a) (extended view) showing green fluorescence from fluorescently labeled AlexaFluor 488-labeled (green) transferrin-polymer conjugates and (b-c) 3D view for HeLa cells treated with. Nuclear stain (Hoechst) (blue) and plasma membrane (Cellmask deep red) (red) administered 30 minutes and 2 minutes prior to analysis, respectively.

Fig. 8. Cytotoxicity of the copolymer-encapsulated doctaxel evaluated in HeLa (human cervical cancer) cells. Formulations were prepared over a range of docetaxel concentrations between 1 and 100 nM. Dose response curves are for cells at 72 hours post-treatment with polymer-encapsulated drug with and without Transferrin targeting. Cell viability numbers are relative to untreated controls as determined by MTS. Data represent the average of 2 experiments conducted in quadruplicate.

Table 1

Theoretical and experimentally determined copolymer composition, number average molecular weights (Mn), and molar mass distributions (D) for a series of PEGMA 950, LMA, and TMA copolymers prepared by RAFT.

Poly. #	TMA (Feed)	O950 (Feed)	LMA (Feed)	TMA ^a (Exp.)	O950 (Exp.)	LMA ^a (Exp.)	Mn ^b (kDa)	b	CMC (μg/mL)	D _H Poly (nm) ^a	D _H DTX (nm) ^a
1	25	15	60	21	11	68	26.6	1.10	26	6.2	4.9
2	25	20	55	23	14	63	31.6	1.12	34	6.7	4.3
3	25	25	50	23	18	59	37.8	1.09	52	5.1	5.6

^a As determined by 500 mhz ¹H NMR spectroscopy in CDCl₃ by evaluation of PEGMA 950 Methoxy resonance [A] at 3.39 ppm, the TMA aromatic resonances [B] between 8 and 9 ppm, and the combined ester region [C] at 4.1 and 5.0 ppm using the formulas: ¹H PEGMA 950 = [A]/3, ¹H TMA = [B]/3, and ¹H LMA = ([C]-2/3 × [A] -2/3 × [B])/2.

^b As determined by size exclusion chromatography using Tosoh SEC TSK-GEL α-3000 and α-4000 columns (Tosoh Bioscience, Montgomeryville, PA) connected in series to an Agilent 1200 Series Liquid Chromatography System (Santa Clara, CA) and Wyatt Technology miniDAWN TREOS, 3 angle MALS light scattering instrument and Optilab TREF, refractive index detector (Santa Barbara, CA). HPLC-grade DMF containing 0.1 wt.% LiBr at 60 °C was used as the mobile phase at a flow rate of 1 ml/min.