



**Oseltamivir-Conjugated Polymeric Micelles Prepared by
RAFT Living Radical Polymerization as a New Active Tumor
Targeting Drug Delivery Platform**

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ARTICLE

Oseltamivir-Conjugated Polymeric Micelles Prepared by RAFT Living Radical Polymerization as a New Active Tumor Targeting Drug Delivery Platform

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Targeted drug delivery using polymeric nanostructures has been at the forefront of cancer research, engineered for safer, more efficient and effective use of chemotherapy. Here, we designed a new polymeric micelle delivery system for active tumor targeting followed by micelle-drug internalization via receptor-induced endocytosis. We recently reported that oseltamivir phosphate targets and inhibits Neu1 sialidase activity associated with receptor tyrosine kinases such as epidermal growth factor receptors (EGFRs) which are overexpressed in cancer cells. By decorating micelles with oseltamivir, we investigated whether they actively targeted human pancreatic PANC1 cancer cells. Amphiphilic block copolymers with oseltamivir conjugated at the hydrophilic end, oseltamivir-pPEGMEMA-*b*-pMMA (oseltamivir-poly(polyethylene glycol methyl ether methacrylate)-*block*-poly(methyl methacrylate)), were synthesized using reversible addition-fragmentation chain transfer (RAFT) living radical polymerization. Oseltamivir-conjugated micelles have self-assembling properties to give worm-like micellar structures with molecular weight of 80,000 g/mol. Oseltamivir-conjugated water soluble pPEGMEMA, dose dependently, both inhibited sialidase activity associated with Neu1, and reduced viability of PANC1 cells. In addition, oseltamivir-conjugated micelles, labelled with a hydrophobic fluorescent dye within the micelle core, were subsequently internalized by PANC1 cells. Blocking surface Neu1 with anti-Neu1 antibody, reduced internalization of oseltamivir-conjugated micelles, demonstrating that Neu1 binding linked to sialidase inhibition were prerequisite steps for subsequent internalization of the micelles. The mechanism of internalization is likely that of receptor induced endocytosis demonstrating potential as a new nanocarrier system for not only targeting a tumor cell, but also for directly reducing viability through Neu1 inhibition, followed by intracellular delivery of hydrophobic cytotoxic chemotherapeutics.

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1. Introduction

Nanoscale polymeric drug delivery systems such as polymeric micelles and liposomes have been developed in the past two decades for targeted and sustained release of chemotherapeutics. Traditional chemotherapy agents, such as doxorubicin and paclitaxel, are typically highly hydrophobic small molecules designed to work intracellularly, targeting rapidly dividing cells. Administration of these drugs alone, complicated by insolubility in biological fluids and tissues, and an incapacity to effectively localize in metastasized tumors, results in adverse toxic side effects and prevents potent

selective targeting.¹ The enhanced permeability and retention effect is the property by which functionalized drug loaded nanocarriers tend to accumulate in tumor tissue much more so than they do in healthy tissues.²

The development of amphiphilic polymeric vesicles capable of encapsulating large quantities of a chemotherapeutic agent and improving its targeting efficiency and bioavailability are emerging new delivery platforms for nanomedicine in cancer research.¹⁻³ More recently, active drug delivery platforms have been engineered for effective tumor targeting. Polymeric micelles have been modified by attaching ligand molecules to the outer shell of the micelle with specificity for unique proteins that are overly expressed on tumor cells. Such ligands such as peptides, antibodies, sugars and aptamers have enabled significant improvement in tumor selectivity and overall therapeutic efficiency of cancer treatments.³⁻⁴ The limitations of these specific “smart” delivery vesicles however, are low overtime stability especially with antibodies, immunogenic adverse effects and high preparation costs.

Amphiphilic copolymers prepared by living radical polymerization (LRP) techniques have been gaining interest in the field of polymeric micelle based delivery systems for cancer therapeutics and diagnostic tools. The ability to prepare complex polymeric structures with highly controlled molecular weights and defined architectures enables multiple functionalities such as hydrophobic/hydrophilic blocks needed for self-assembly, stimuli-responsive regions (responding to CO₂, pH and temperature) for triggered drug release, and reactive groups for drug conjugation, cross-linking and ‘click’ chemistry.⁵ In the present study, we describe the design of an amphiphilic block polymer-drug conjugate using reversible addition-fragmentation chain transfer (RAFT) polymerization which is a subclass of LRP. The mechanism of RAFT involves a degenerative chain transfer reaction facilitated by a radical-reactive chain transfer agent (CTA), known as the RAFT agent. It produces an equilibrium between the propagating chain and a deactivated or dormant chain capped by the RAFT agent (CTA).^{5b, 5c} With an equilibrium shifted heavily to the dormant polymer-CTA, the RAFT polymerization has minimum termination reactions, producing a polymer with a very narrow molecular weight distribution. The final polymer-CTA exists in a ‘living’ state, meaning it can be redissolved, and the chain can be extended with different monomers to produce complex polymeric architectures.^{5d}

Reports on the preparation of amphiphilic block copolymers for drug delivery via RAFT have dramatically increased over the last 10 years due to the ability to synthesize highly complex water soluble structures with biologically important architectures, and a wide range of acrylic monomers that can be polymerized to form a multi-functional stimuli-responsive polymer backbone.^{5d, 5e} Notably, Pascual et al⁶ have prepared a number of nanostructures by RAFT polymerization potentially useful for biomedical and drug delivery applications, including polystyrene-*b*-poly(butyl acrylate) block copolymer using xanthate as a RAFT-CTA which served as a hydrophilic end-group to stabilize the forming particles during microemulsion polymerization in water and later used to conjugate the

particle surface with functional macromolecules. Another relevant design by Jia et al⁷ was the synthesis of multifunctional nano-worms/rods via RAFT-mediated emulsion polymerization. Preparation of polymer-drug conjugates as a prodrug formulation using RAFT polymerization has been reported previously by Wang et al.⁸ The report described the conjugation of the chemotherapeutic drug gemcitabine to a RAFT agent which was then polymerized using methyl methacrylate (MMA) to produce gemcitabine-pMMA polymeric micelles. Although the gemcitabine loaded micelles were well characterized, they lack the specific “smart” chemotherapeutic delivery platform for active tumor targeting.

We recently reported that oseltamivir phosphate (OP) can impede tumor neovascularization, growth and metastasis of human ovarian⁹, triple-negative breast¹⁰ and pancreatic¹¹ cancer cells in heterotopic xenografts of these tumors in RAGxCy double mutant mice. The findings also disclosed a novel signaling paradigm that regulates epidermal growth factor receptors (EGFR).¹¹⁻¹² Mutations of the EGFR, resulting in hyperactivity of the receptor, is reported in over 90% of pancreatic cancer cases causing rapid, uncontrolled cellular growth, which contributes to the aggressive nature of pancreatic tumors.¹³ Chemotherapy designed to target EGFR hasn't been successful in the clinic since no single molecule was able to target the full range of EGFR mutations which limits the application of highly targeted chemotherapies.¹³⁻¹⁴ The role of Neu1 within the EGFR complex is that it is critical for EGF-induced receptor activation, the essential signalling platform on the cell surface. Neu1 involvement in this novel receptor signaling platform has been previously reported for a number of other receptors, all of which are known to play major roles in cancer. Oseltamivir, a synthetic analog of α -2,3-sialic acid, was shown to inhibit the Neu1 sialidase activity in complex with these receptors, and impede tumor neovascularization, growth and metastasis in a number of cancers.^{9-11, 15}

Based on these findings, an entirely new polymeric drug delivery system was designed for active tumor targeting. Micelles with oseltamivir grafted to the outer shell should provide three functions; (a) tumor cell targeting via Neu1 receptor binding, (b) anti-tumor effect induced through Neu1 inhibition, and (c) localized delivery of a chemotherapeutic drug carried by the polymeric micelle. As shown in Figure 1, oseltamivir was first linked with the RAFT agent to facilitate polymerization of polyethylene glycol methyl ether methacrylate (PEGMEMA) producing a water soluble oseltamivir conjugated micellar shell. Methyl methacrylate was polymerized from the polymer living end to produce oseltamivir-p(PEGMEMA)-*b*-p(MMA) amphiphilic block copolymer which self-assembled during polymerization into a micelle with oseltamivir on the surface of the micellar shell.

Our findings indicate that oseltamivir-micelles demonstrated targeting to PANC1 cells, a reduction in sialidase activity associated with Neu1, reduction in tumor cell viability, and subsequent internalization of the micelles carrying a fluorescent label as a model drug compound. Neu1 binding

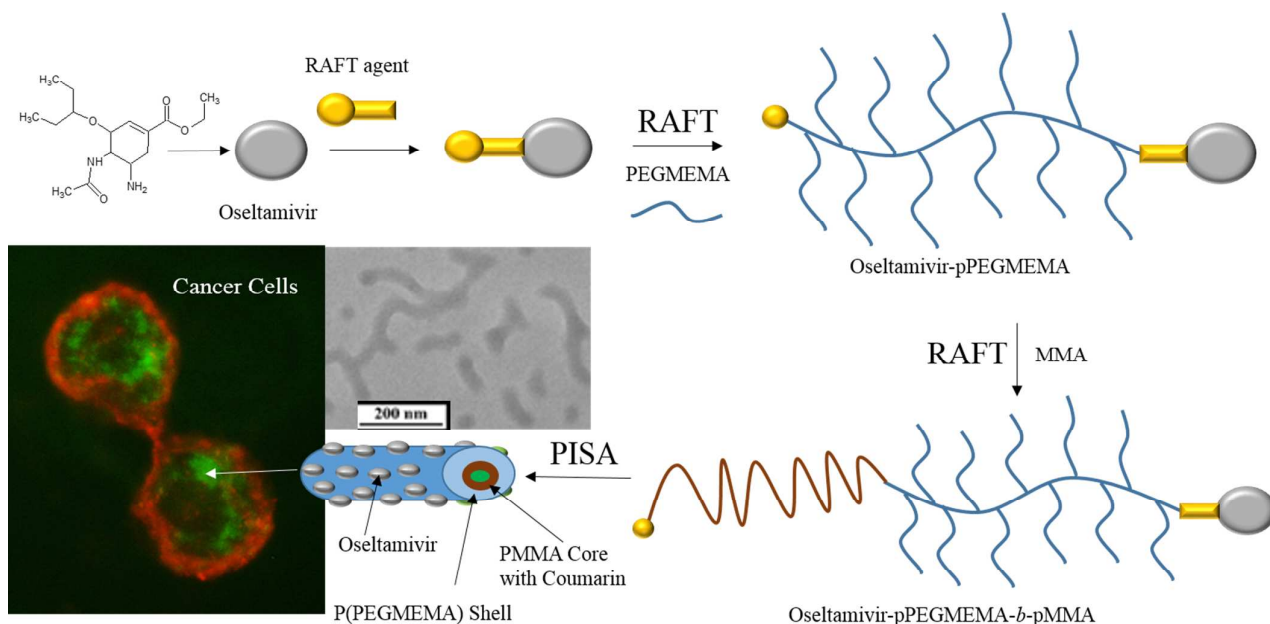


Figure 1 Schematic representation of the synthetic steps and subsequent preparation of oseltamivir-conjugated micelle capable of triggering receptor induced endocytosis in cancer cells.

was shown to be a prerequisite step toward micelle internalization since it was blocked by anti-Neu1 antibodies. The ability to both target and halt the growth of a tumor cell using a newly designed nanocarrier system, combined with the internalization of the micelle loaded with a cytotoxic chemotherapeutic represents the novel aspect of this work.

2. Experimental Section

2.1 Materials

Oseltamivir phosphate was purchased from Hangzhou Dayang Chem Co Ltd (Hangzhou City, PRC). 4-Cyano-4-(phenylcarbonothioylthio)pentanoic acid *N*-succinimidyl ester (*N*-hydroxysuccinimide-protected RAFT agent or RAFT-NHS), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid >97%, poly(ethylene glycol) methyl ether methacrylate (Mn 300), methyl methacrylate (MMA) \leq 99%, 2,2'-azobis(2-methylpropionitrile) (AIBN) 98%, triethylamine (TEA), coumarin 153 (C153) dye content 99% were purchased from Sigma-Aldrich. *N,N*-Dimethylformamide anhydrous DrySolv[®] was purchased from EMD Millipore-AA-Corp., toluene reagent A.S.C. was purchased from ACP Chemicals Montreal, QC. Human pancreatic cancer cells PANC1 (human epithelioid carcinoma, epithelial-like, ATCC[®] CRL-1469[™]) were purchased from the American Type Culture Collection. Cell culture medium contain 1 X Dulbecco's Modified Eagle's Medium (DMEM) purchased from Gibco, 10% fetal bovine serum (FBS) purchased from HyClone and 5 μ g/mL Plasmocin[™] purchased from InvivoGen. WST-1 reagent was purchased from Roche Diagnostics. CellMask[™] Deep Red plasma membrane stain was purchased from Life Technologies Inc. Antibody to human neuraminidase 1 was purchased from Santa Cruz Biotechnology Inc.

2.2 Characterization

¹H and ¹³C NMR of all products in D₂O, CDCl₃ or DMSO-d₆ were acquired with Bruker Avance 400 MHz. Dry samples were dissolved in a deuterated solvent at 15 mg/mL for ¹H and 75 mg/mL for ¹³C NMR. Overtime polymerization samples were prepared by adding 3 drops of reaction solution to 0.5 mL of deuterated solvent. Molecular weight of the polymers was analyzed by a Viscotek 270max with dual detector, (mobile phase THF), calibrated with polymethyl methacrylate standards. GPC samples were prepared at 1–2 mg/mL or by addition of 5 drops of reaction solution to 1 mL THF. Micelle morphology was observed using Hitachi H-7000 transmission electron microscope (TEM) (75 kV). Micelle size distribution was measured by dynamic light scattering (DLS) at 25°C using a Malvern Zetasizer Nano Series (Nano ZS). Thermo Scientific Orbitrap Velos Pro mass spectrometer with heated electrostatic spray ionization source (ESI-MS) was used to confirm the synthesis of the oseltamivir-RAFT agent.

2.3 Synthesis of Oseltamivir-RAFT agent

A molar excess of oseltamivir phosphate (0.8 g, 2 mmol) was dissolved in DMF (10 mL) with addition of an equimolar amount of triethylamine (TEA). RAFT-NHS (0.46 g, 1 mmol) was dissolved in DMF (5 mL) then added to the reaction flask. The solution was allowed to react for 4h at room temperature under stirring. After the reaction was completed, DMF was removed using a rotary evaporator. The remaining solid was redissolved in toluene and washed with three aliquots of water (acidified to pH 2.5 with phosphoric acid) to remove unreacted oseltamivir. The final product was then air dried to give orange solid (0.532 g, 92.4% yield) and characterized by NMR and LC-MS. ¹H NMR (400 MHz, CD₃OD): 7.6 (d, 2H, *J* = 7.3 Hz, *o*-ArH), 7.4 (t, 1H, *p*-ArH), 7.1 (m, 2H, *m*-ArH), 6.77 (d, 1H, (-CH=C-)), 4.09 (q, 2H, (-CH₂CH₃)), 3.84 (q, 1H, (-CH-NHAc)),

3.30 (quint, 1H, (-CH(CH₂CH₃)₂)), 2.80 (m, 1H, (oseltamivir-CHNHCO-RAFT)), 2.58 (dd, 1H, (-CH₂-)), 2.2-2.6 (m, 4H RAFT-(CH₂CH₂)CONH-oseltamivir), 2.2 (s, 3H, (-NH-C(O)CH₃)), 2.11-2.18 (m, 2H, (-CH₂-)), 1.86 (s, 3H, SC(CH₃)CN), 1.41 (m, 4H, (-C(CH₂CH₃)₂)), 1.19 (t, 3H, (-CH₂CH₃)), 0.84 (t, 3H, (-C(CH₂CH₃)₂)), 0.77 (t, 3H, (-C(CH₂CH₃)₂)). ¹³C NMR (CDCl₃) (δ, ppm): 8.0, 8.5, 13.1, 20.4, 22.6, 24.6, 25.2, 29.4, 31.5, 32.9, 45.2, 47.6, 52.8, 59.8, 75.7, 81.2, 124.3, 126.0, 127.2, 128.0, 130.1, 136.8, 144.4, 164.6, 170.0, 170.4. ESI-MS: m/z calc. for C₂₉H₄₀N₃O₅S₂ (M-H⁺) 574.24; found 574.24.

2.4 Synthesis of oseltamivir-pPEGMEMA via RAFT polymerization

PEGMEMA (9.0 g, Mn 300 g/mol, 30 mmol), oseltamivir-RAFT (0.10 g, 575.78g/mol, 0.17 mmol) and AIBN (0.008 g, 0.05 mmol) (monomer/CTA/initiator 150:1:0.25) were dissolved in 25 mL of toluene and purged with nitrogen for 30 min at room temperature. The solution temperature was then raised to 70 °C to thermally initiate polymerization. Samples were taken every hour and analyzed by GPC for molecular weight and NMR for conversion. In the end, reaction solution was precipitated in cold diethyl ether, and the final polymer isolated by centrifugation and cleaned by dialysis against water. ¹H NMR (400MHz): 4.1 ppm 2H (-C(O)-O-CH₂-), 3.5-3.7 ppm 16H (-C(O)-O-CH₂-CH₂-O-(CH₂-CH₂-O)3/4-CH₃), 3.3 ppm 3H (-O-CH₃), 1.7-2.1 ppm 2H (-CH₂-), 0.7-1.1 ppm 3H (a-CH₃). GPC: 35,000 g/mol.

2.5 Preparation of oseltamivir-conjugated micelle by RAFT PISA

Methyl methacrylate (MMA) (0.94 g, 9.4 mmol), oseltamivir-p(PEGMEMA)-RAFT (0.20 g, 36,000 g/mol, 0.006 mmol), AIBN (0.001 g, 0.006 mmol) (monomer/CTA/initiator 1500:1:1) were dissolved in 6 mL of water/methanol 1:1 v/v. The polymerization was allowed to proceed until the onset of PISA which typically occurred after 6-8h, with the final micellar solution appearing as milky-white colloidal suspension. Samples were taken at the beginning and the end of reaction for molecular weight analysis by GPC. The micellar solution was then dialyzed against water, while changing the dialysis solvent 3 times every 3h. After cleaning, micelle size was measured by DLS and morphology observed by TEM. A small aliquot was lyophilized for structure characterization by NMR. Micelles without oseltamivir were prepared by precisely following the two RAFT polymerization protocols described above, but using 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid as a CTA instead of oseltamivir-RAFT.

2.6 Sialidase Activity Assay

A series of solutions (0-1 mg/mL) of oseltamivir-pPEGMEMA (4000 g/mol) were prepared in sodium acetate with pH 5. To each solution, 40 μL of 9.9 unit/mL α(2→3) neuraminidase from *Streptococcus pneumoniae* (α-2,3-Neu, 50 mU/mL) (Sigma, St. Louis, MO) was used at predetermined optimal dosage and samples were incubated for 2h at 37°C. Then 5μL of 12mM 4MU-NeuAc were added to each sample and further incubated for 1h at 37 °C.

Reaction was then terminated by addition of 0.25M glycine-NaOH at pH10 and fluorescence intensity measured at 448 nm emission and 365 nm excitation.

2.7 Cell Line

PANC1 (human pancreatic carcinoma, epithelial-like, ATCC® CRL-1469™) cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in a 5% CO₂ incubator at 37°C in culture containing 1xDMEM (Gibco, Rockville, MD, USA) supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA) and 5μg/mL Plasmocin™ (InvivoGen, San Diego, CA, USA). When the cells reached ~80% confluence, they were passaged at least five times for use in the experiments.

2.8 WST-1 Cell Viability Assay

PANC1 cells were plated in 96-multiwell plates at a density of 5000 cells/well and incubated at 37°C overnight. The following day, cells were exposed to 30 mg/mL pPEGMEMA, 7.5, 15, or 30 mg/mL oseltamivir-pPEGMEMA, or left untreated as a control for 0, 24, 48, and 72h. pPEGMEMA and oseltamivir-pPEGMEMA were diluted in culture medium. 100 μL of WST-1 reagent diluted 1:10 in culture medium was added to wells for 2h prior to reading of absorbance at 420 nm at each time point. Cell viability was presented as a percentage of control using GraphPad Prism software. The following formula was used to determine cell viability as a percent of control for each time point and treatment:

$$\frac{(\text{Absorbance of cells in a given concentration of drug}) - (\text{Media absorbance})}{(\text{Absorbance of cells alone}) - (\text{Media absorbance})} \times 100\%$$

2.9 Fluorescent Microscopy with Coumarin-Loaded Micelles

PANC1 cells were plated on 12 mm glass coverslips in 24 multiwell plates at a density of 15000 cells/well and incubated overnight at 37°C. Cells were washed once with phosphate-buffered saline pH 7.4 (PBS), incubated in CellMask™ Deep Red plasma membrane stain diluted 1:1000 in PBS at 37°C for 7 min, then washed 3 times with PBS. Cells were treated with oseltamivir-pPEGMEMA or pPEGMEMA, or untreated as a control for 1h at 37°C. Cells were washed 3 times with PBS, then mounted on glass slides and visualized using a Zeiss M2 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) at 200 and 400 X magnification. The experiment was repeated, but PANC1 were treated with 25μg/mL anti-Neu1 antibody for 1h at 37°C prior to membrane staining

3. Results and Discussion

3.1 Synthesis of Oseltamivir-RAFT Agent

The first step in preparation of oseltamivir conjugated polymeric micelles was to attach the drug to the chain transfer agent (CTA) to

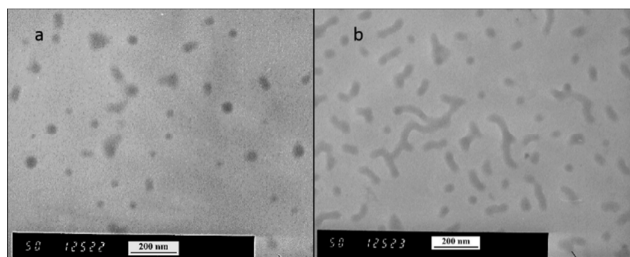


Figure 8 TEM images of pPEGMEMA-b-pMMA micelles (a) and oseltamivir-pPEGMEMA-b-pMMA micelles (b).

3.4 Oseltamivir-pPEGMEMA Inhibits Sialidase Activity

The effectiveness of oseltamivir in a polymer-conjugated form was assayed for Neu1 sialidase enzyme inhibition, simulated using a microbial $\alpha(2\rightarrow3)$ neuraminidase from *Streptococcus pneumoniae*. This enzyme, like the human Neu1, cleaves sialic acid and is inhibited competitively by free oseltamivir which is an $\alpha(2\rightarrow3)$ sialic acid analog. The inhibitory activity of oseltamivir-pPEGMEMA was measured using 4MU-NeuAc as substrate which gives a fluorescent product after being cleaved with $\alpha(2\rightarrow3)$ neuraminidase. Because of the hydrophobicity of the fluorescent molecule produced from cleavage of 4MU-NeuAc by $\alpha(2\rightarrow3)$ neuraminidase, it was decided not to conduct free sialidase activity assay with oseltamivir-conjugated micelles, due to potential diffusion of the fluorescent product into the hydrophobic core of the micelle, interfering with the fluorescent readings. Table 1 shows fluorescence intensity following reaction in the presence of three different concentrations of oseltamivir-pPEGMEMA. A concentration dependent decrease in fluorescence was observed and converted to concentration-dependent inhibition of α -2,3-neuraminidase by the oseltamivir-conjugated micelles, as depicted in Figure 9. The conversion from fluorescence intensity to inhibition was done by taking the sample with only enzyme and substrate as representing 100% activity and 0% inhibition, and calculating the percent inhibition relative to that sample. Therefore, it was shown that oseltamivir exerts Neu1 sialidase inhibitory activity in a micelle-conjugated form. Inhibition suggests a competitive binding of oseltamivir-conjugate to the neuraminidase active site, and thus shows potential for binding to cell surface Neu1 within the EGFR complex.

3.5 Oseltamivir-pPEGMEMA Reduces PANC1 Cell Viability

Since neuraminidase inhibition requiring oseltamivir-conjugate association was demonstrated using free enzyme, the potential for cell Neu1 binding was determined by the effect of conjugate on viability of PANC1 cells, which had previously been shown susceptible to free oseltamivir phosphate. Cell viability was determined using the WST-1 cell proliferation assay.¹⁶ The cell viability assay could not be conducted with oseltamivir-conjugated micelles due to the turbidity of the micellar solution interfering with the absorbance reading of the metabolite being quantified. Therefore, this assay was limited to water-soluble oseltamivir-pPEGMEMA conjugates. The data shown in Figure 10 indicate that treatment of PANC1 cells with oseltamivir-conjugated polymer reproducibly and dose-dependently decreased cell viability

expressed as a percent of the untreated control, with up to 45% viability reduction after 24, 48, and 72h of incubation. The control polymer without oseltamivir did not impede growth of PANC1 cells when used at same polymer concentration (30 mg/mL).

A direct comparison may be made between oseltamivir phosphate in the free form (1 mg/mL) to the same amount of oseltamivir, but in the conjugate form, which is the case for 30 mg/mL oseltamivir-pPEGMEMA polymer. It is noteworthy that the pure oseltamivir phosphate showed a stronger reduction in cell viability than the polymer conjugated form which was likely due to increased steric hindrance around oseltamivir reducing access to the Neu1 active site. However, overall the results indicate that oseltamivir-conjugated polymer shows anti-PANC1 cell activity and oseltamivir therefore can potentially exert anti-tumor effect in micelle-conjugated form.

Table 1 Fluorescence intensity measurements from free α -2,3-sialidase activity assay.

| [Oseltamivir-pPEGMEMA] (mg/mL) | Estimated [Oseltamivir] (μ g/mL) | Fluorescence Intensity |
|--------------------------------|---------------------------------------|------------------------|
| 0 | 0 μ g/mL | 18490 |
| 3.75 | 250 μ g/mL | 17770 |
| 7.5 | 500 μ g/mL | 17330 |
| 15 | 1000 μ g/mL | 16470 |
| Buffer | | 0.515 |

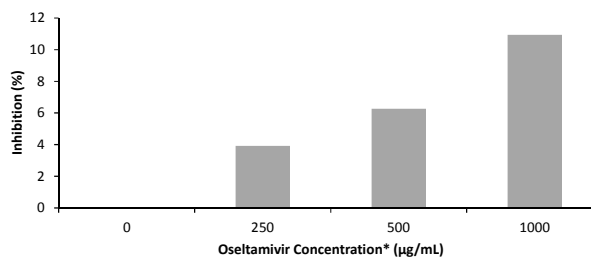


Figure 9 Inhibitory effect of oseltamivir-pPEGMEMA on $\alpha(2\rightarrow3)$ neuraminidase from *Streptococcus pneumoniae* measured by fluorescence. *- oseltamivir concentration is calculated from an estimated oseltamivir content in micelle-drug conjugate determined by ¹H NMR.

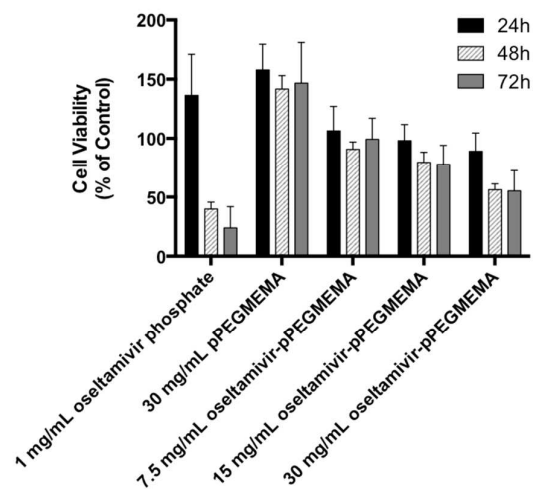


Figure 10 Cell viability as a percent relative to untreated PANC1 cells (control), showing effect of free oseltamivir phosphate, oseltamivir-free polymer, and oseltamivir-polymer conjugate (~3% w/w oseltamivir). $n=3$, with error bars - mean \pm standard error.

3.6 Interaction of oseltamivir-conjugated micelles with PANC1 cells

Oseltamivir-conjugate shows neuraminidase (Neu1) inhibition, and negatively affects viability of PANC1 cells, in a manner similar to free oseltamivir phosphate. Thus both Neu1 binding and cell inhibition can be anticipated with the new drug polymer conjugate. The potential then for not only the targeting of tumor cells, but the concomitant delivery of an oseltamivir-micelle filled chemotherapeutic was examined on PANC1 cells pre-stained with plasma membrane stain to potentially visualize surface Neu1-bound oseltamivir-micelles. To simulate the delivery of a hydrophobic chemotherapeutic, oseltamivir-micelles were loaded with the fluorescent probe coumarin-153 (C153), to serve as a hydrophobic drug model. The images depicted in Figure 11 show red fluorescence for plasma membrane stain and green fluorescence for micelle loaded C153. Figure 11a shows internalization of C153 loaded oseltamivir conjugated micelles within the plasma membrane of PANC1 cells. In contrast, C153 loaded micelles without oseltamivir did not show internalized C153, as seen in Figure 11c, comparable to the micelle-free cell control seen in Figure 11d. Thus it appears that oseltamivir conjugated to the outer surface of the micelles is necessary both for Neu1-EGFR binding, but also for cellular internalization of the micelles. To verify whether oseltamivir-conjugated micelles target Neu1, PANC1 cells were pre-treated with anti-Neu1 blocking antibodies followed by treatment with C153 loaded oseltamivir-conjugated micelles. Figure 11b shows that anti-Neu1 blocking antibody resulted in a reduction of visible label within the cell cytoplasm. Fluorescent intensity was measured from a number of images for each condition and plotted as relative C153 staining density as shown in Figure 12. Oseltamivir-conjugated micelles produced a 3.3-fold increased internalization compared to oseltamivir-free micelles, while the blocking action of anti-Neu1 antibody resulted in a 1.4-fold decrease in the

internalization of C153 loaded oseltamivir-conjugated micelles. The extent of the effect of addition of a competitive inhibitor anti-Neu1 antibody will greatly depend on the total population of Neu1 membrane protein in a given cell culture. Neu1 is also found in other membrane receptors which could in turn interfere with the output from this particular experiment. In our case, an fixed amount of antibody was added to determine whether or not there is any reduction in cellular uptake of micelles. We suggest that increasing the amount of antibody should further decrease the uptake of micelles.

Oseltamivir-conjugated micelles are thus shown to be binding to Neu1 in complex with EGFR. A mechanism that can explain micelle internalization carrying C153 as a drug model is receptor-induced endocytosis (RIE). Endocytic mechanisms have been previously explored for targeted drug delivery by attaching high affinity ligands on vesicles to bind with clathrin, transferrin and folate receptors.¹⁷ Endocytosis of EGFR is a part of the receptor recycling process triggered by extracellular signal molecules followed by internalization of receptor bound molecule complex into lysosomes for degradation.¹⁸ It has been reported that binding of EGF to EGFR induces the internalization of the receptor into lysosomes for intracellular degradation.¹⁹ Although it was found that Neu1 inhibition by free oseltamivir did not result in receptor internalization¹¹, it is possible that the oseltamivir decorated micelle exerts additional steric and/or conformational changes to the receptor that now triggers endocytosis, as illustrated in Figure 13. Thus oseltamivir conjugated micelles could induce endocytosis of EGFR via binding to the receptor's Neu1 sialidase located at the ectodomain of EGFR and through interaction with EGFR, triggers internalization. The exact mechanism of EGFR activation by the oseltamivir-micelle and subsequent internalization at the molecular level is presently the subject of further investigated. Nevertheless, this active targeting approach has potential for delivery of hydrophobic anti-cancer agents such as doxorubicin into the target cell cytoplasm.

Oseltamivir-conjugated micelles designed to target Neu1 in complex with EGFR offer a new and effective way of targeting, delivering and internalizing a chemotherapeutic agent via oseltamivir-Neu1 binding followed by receptor-induced endocytosis. Compared to other targeting ligands such as growth factors and antibodies used in active tumor targeting, which are typically expensive, unstable and potentially immunogenic, oseltamivir is a small, relatively inexpensive molecule which can be easily attached to the end of polymer via reactive amine group and is expected to be biologically stable when conjugated to a micelle. A synthetic route for oseltamivir-conjugated micelles via RAFT polymerization by using the drug itself as a chain transfer agent is only one of many ways to prepare oseltamivir decorated nanocarriers, serving as a template for development of other oseltamivir-based cancer targeting platforms. Finally, as seen from sialidase enzyme activity assay, and PANC1 cell viability assays, oseltamivir, in the polymer conjugated form, shows both sialidase inhibition and anti-PANC1 cell activity of around 45% of its free form, oseltamivir phosphate. Therefore, oseltamivir-decorated nanocarriers can provide a triple-effect of targeting and exerting an

anti-tumor cell effect directly from oseltamivir, combined with a subsequent internalization and delivery of a separate chemotherapeutic as demonstrated by the C153 as a model hydrophobic drug.

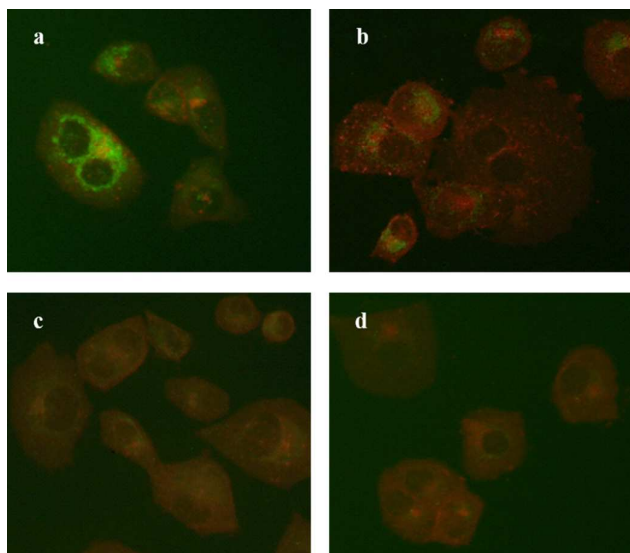


Figure 11 Fluorescent microscopy images of pancreatic tumor cells, stained with deep red cell mask, incubated with a) C153-loaded oseltamivir-conjugated micelle, b) C153-loaded oseltamivir-conjugated micelle plus anti-Neu1 antibody, c) C153-loaded micelles without oseltamivir and d) media (no micelles).

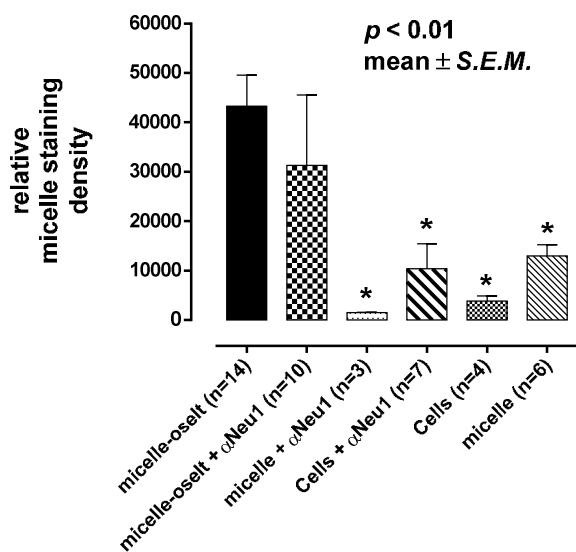


Figure 12 Quantitative analysis of cell uptake of micelle-conjugated oseltamivir (oselt) loaded with coumarin 153 by measuring relative fluorescence staining density from microscopy images. *Number of samples n = number of individual cells from one or more images from each experiment. Oselt – oseltamivir; αNeu1 – anti-Neu1 antibody.

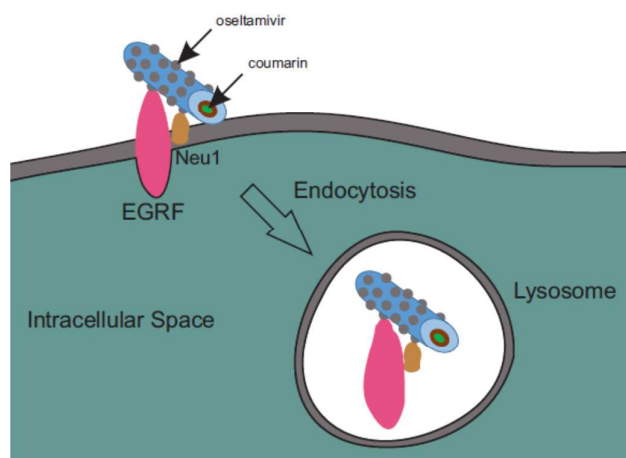


Figure 13 Graphic representation of EGFR induced endocytosis of oseltamivir micelle via binding to Neu1.

4. Conclusion

The synthesis and preparation of oseltamivir-conjugated micelles formed via RAFT polymerization was designed for active targeted delivery of a hydrophobic chemotherapeutic. This new drug delivery platform was designed to target the EGFR complex located and overexpressed on the surface of tumor cells, and more specifically bind to Neu1 within the EGFR complex. The synthetic route consisted of three major steps: conjugating oseltamivir with RAFT chain transfer agent, RAFT polymerization of a hydrophilic polymer block, and finally RAFT polymerization of a hydrophobic block. The resultant oseltamivir-conjugated block copolymer self-assembled into nanosize micelles with the oseltamivir ends orienting into the aqueous phase. Polymer-conjugated oseltamivir was shown to both inhibit neuraminidase activity representing sialidase activity of Neu1 within the EGFR, and reduce the viability of PANC1 cells, in a manner previously demonstrated for free oseltamivir phosphate. Further evidence for Neu1 cell surface binding was provided by demonstrating that C153 loaded oseltamivir conjugate was internalized by PANC1 cells but not so in the absence of the linked oseltamivir, or when cells had been pre-treated with anti-Neu1 blocking antibody. It is proposed that that oseltamivir micelle conjugate cell internalization occurs as a result of binding to cell surface Neu1 within the EGFR complex, triggering receptor-induced endocytosis, as part of the EGFR recycling process. Therefore, oseltamivir conjugated to the surface of the micelle, acts as a targeting molecule, enabling internalization of the micelles. The addition of C153 loaded within the micelles as model hydrophobic chemotherapeutic, demonstrates the potential for targeted delivery and subsequent internalization of other hydrophobic chemotherapeutics. These newly designed micelles for active tumor targeting thus have dual functionality by exerting an anti-tumor cell effect, and at the same time delivering and internalizing a hydrophobic chemotherapeutic.

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Graphical Abstract: Synthetic steps and subsequent preparation of oseltamivir-conjugated micelles capable of targeting and triggering receptor induced endocytosis in cancer cells

