Dual Drug Delivery of Tamoxifen and Quercetin in Polyester Nanosponges: Enhanced Bioavailability and Regulated Metabolism for Anticancer Treatment

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ABSTRACT: We report the synthesis and encapsulation of polyester nanosponge particles (NPs) co-loaded with tamoxifen (TAM) and quercetin (QT) to investigate the loading, release and *in vitro* metabolism of a dual drug formulation. The NPs are made in two variations, 4% and 8% crosslinking densities, to evaluate the effects on metabolism and release kinetics. The NP-4% formulation with a particle size of 89.3 ± 14.8 nm was found to have loading percentages of 6.91 ± 0.13 % TAM and 7.72 ± 0.15 % QT after targeting 10 % (w/w) each. The NP-8% formulation with a particle size of 91.5 ± 9.8 nm was found to have loading percentages of 7.26 ± 0.10 % TAM and 7.80 ± 0.12 % QT. The stability of the formulation was established in simulated gastrointestinal fluids, and the metabolism of TAM was shown to be reduced 2-fold and 3-fold for NP-4% and NP-8%, respectively, while QT metabolism was reduced 3 and 4-fold. The implications for improved bioavailability of the NP formulations were supported by cytotoxicity results that showed a similar efficacy to free dual drug formulations and even enhanced anti-cancer effects in the recovery condition. This work demonstrates the suitability of the nanosponges not only as a dual release drug delivery system but also enabling a regulated metabolism through the capacity of the nanonetwork. The variation in crosslinking enables a dual release with tailored release kinetics and suggests improved bioavailability aided by a reduced metabolism.

INTRODUCTION. Substantial therapeutic benefits are observed from a combination of drugs, in which one drug affects the activity of another drug when administered together, also known as the synergistic effect.¹ Dual and multiple drug delivery have to be carefully studied because overdosing or other side effects can occur due to the enhanced activity. Many of these drug combinations are described and studied for cancer chemotherapy² but are not limited to these disease profiles.³ In fact, it can be witnessed that nanoscopic delivery systems are ideal not only to solubilize BCS class IV drugs but also provide opportunities to co-deliver a synergistic drug combination. Several nano-sized platforms including micelles,^{4, 4b} squalene based micelles, traditional^{5,6} poly (ethylene glycol)-block-poly(lactide) (PEG-PLA) particles,⁷ and poly (lactic-co-glycolic acid) (PLGA) particles⁸ have been investigated. Other examples of nanoscale dual delivery vehicles are vitamin D₃ nanoparticles,⁹ nanocells,¹⁰ minicells,¹¹ drug-nano-particles,¹² micelleplexes,¹³ layer-by-layer nanoparticles,¹⁴ carbon nanotubes,¹⁵ graphene-oxide,¹⁶ geliposome¹⁷ and mesoporous silica nanospheres¹⁸ which have been explored for the treatment of a multitude of cancers. It is noted that the delivery platforms specialized to deliver two hydrophobic drugs are mainly guided by the degradation of the carrier and the location of these

drugs within the nanoscale structure. When a more water-soluble drug is in the mix, in these cases a prodrug is formed to avoid a rapid escape from the carrier. Recently, the response of a polymeric delivery carrier towards a stimulus such as pH or temperature has received a lot of attention to achieve a control over the time point and speed of delivery. These developments can be seen as a response to the often limited control in degradation-guided delivery systems in which a fast release in the first 72 hrs is observed. Degradation-guided delivery systems are traditionally prepared via solvent-change methods or salting out, leading to a self-assembled nanoscale particle containing the drug. Therefore the degradation is guided by physiochemical properties only and limited control is observed. With the introduction of a chemical crosslinking that can be varied, we have developed the nanosponges to enhance the control over the degradation. In numerous examples we have shown that the crosslinking density plays a main role in the delivery kinetics of these crosslinked nanonetworks. While we have conducted targeted drug delivery with one drug to be delivered per nanoparticle and also demonstrated the use of a sequential approach, we sought in this work to investigate the dual delivery of a synergistic drug combination to be combined into the same particle. Furthermore, the effect of the na-

nanoparticle architecture to influence or even regulate the metabolism has not been studied extensively. We hypothesized that the nanonetwork density in nanosponges might lead to an effect that is typically observed in pro-drug approaches. The incorporation of a drug combination which is protected by two different nanonetworks of varied composition is thought to lead to a different exposure to the metabolizing factors together with the aspects leading to degradation and release of the dual drug combination. In this work, we chose to study the effect on dual drug release and metabolism with tamoxifen and quercetin as drug combination known in synergistic anti-breast cancer treatments. Tamoxifen (TAM) has been the preeminent treatment option for women with advanced breast cancer¹⁹ but TAM is challenging to administer because of solubility issues and undergoes extensive first-pass metabolism by CYP3A4 in the liver.²⁰ This causes a low intestine which reduces its bioavailability drastically.²¹ In bioavailability and necessitates repetitive high doses.^{22,23,24,25,26 27,28 29,30} Quercetin (QT) on the other hand is a potent antioxidant and dietary flavanoid,³¹ and has been shown to enhance the antitumor efficacy of TAM by promoting intestinal absorption³² and reducing first-pass metabolism.^{25, 33 34 35} However, it is also not easily soluble in water and can be rapidly glucuronidated in the last few years, combination treatment of TAM with QT has been an effective way to improve the uptake of TAM and alleviate the hepatotoxicity generated throughout course of treatment.^{25,36} To study this drug combination we sought to synthesize two types of nanosponge particles (NP) with two crosslinking densities of 4% and 8%, and postload them with TAM and QT (1:1) to evaluate the release kinetics, metabolism, gastric stability and cytotoxicity. To test the metabolism activity, the specific P450 enzymes CYP3A4 and UGT1A9 were selected due to their high contributions in metabolizing TAM and QT, respectively. In here, with increasing the crosslinking density we show that NP-TAM-QTs exhibit a tuned metabolism towards a slower rate, enhanced potential bioavailability, together with release kinetics that indicate that a dual loading does not alter the release kinetics of the individual drugs when released from a single carrier.³⁰

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MATERIALS AND METHODS

Materials. TAM (Free Base), QT (anhydrous), Tween-80, 2,2'-(ethylenedioxy) diethylamine crosslinker, tin triflate, D- α -tocopherol polyethylene glycol 1000 succinate (Vit-E Peg), uridine diphosphoglucuronic acid (UDP-GluA) 1,1,1 tris-hydroxymethyl ethane, glucose-6-phosphate, and β -glucuronidase and were purchased from Sigma, USA. γ -valerolactone and meta-chloroperoxybenzoic acid was also purchased from Sigma and purified via Kugelrohr distillation before use. The γ -allyl- γ -valerolactone was synthesized in-house according to a published protocol.³⁸ Tin scavenger SiliaMetS[®]Cysteine was purchased from Silicycle, USA. Phosphate buffered saline (PBS, 1X) was obtained from Gibco by Life Technologies, pH adjusted to 7.4, and supplemented with Tween-80 (0.1% v/v) for *in vitro* drug release studies. Dialysis tubing SpectraPor[®] (10k

MWCO) was purchased from Spectrum Laboratories, Inc. Human CYP3A4 BACULOSOMES[®], MTT reagent, and Human UGT1A9 microsomes (20mg/mL) were purchased from Life Technologies, NADPH from Santa Cruz biotech, and Glucose-6-phosphate Dehydrogenase (G6P) was purchased from Millipore. All other solvents or reagents were purchased from Sigma Aldrich and used as received.

Characterization. ¹H NMR spectra were obtained from a Bruker AV400 Fourier transform spectrometer with CDCl₃ as the solvent. High performance liquid chromatography (HPLC) was carried out using a Waters system equipped with a Waters 2996 variable wavelength photodiode array detector, a Waters 1525 binary HPLC pump, and a Thermo Synchronis C18 (100 x 4.6mm, 5 μ m) column. Runs were carried out using an isocratic gradient of methanol and water (70:30) with 0.1% TFA at a flow rate of 1.2 mL/min. Polyester nanoparticle samples for transmission electron microscopy (TEM) were prepared by dissolving 0.5 mg of nanoparticles in a 1 mL of cell culture water (GIBCO). Samples were sonicated for 5 min and then stained with 6 drops of 3% phosphotungstic acid. Carbon grids were prepared by dipping an Ultrathin Carbon Type-A 400 Mesh Copper Grid (Ted Pella, Inc., Redding, CA) into the particle solution three times, followed by drying overnight at room temperature. TEM images were obtained using a FEI Technai Osiris transmission electron microscope operated at 200 kV in bright field mode. Cell cytotoxicity was measured by a Synergy HT Microtiter Plate reader (BIO-TEK) at 540nm.

Nanoparticle Synthesis and Optimization.

Linear polymer precursor synthesis poly(vl-avl) 4%. A (α -allyl- γ -valerolactone, γ -valerolactone) (AVL-VL, 4% AVL) polymer was synthesized in a N₂-purged flame-dried 25-mL round bottom flask containing a catalytic amount (2.63mg, 1.97x10⁻² mol, 3.2x10⁻⁴ eq.) of tin triflate in the bottom of the round bottom flask, and then the flask was again purged with nitrogen. The 3-methyl-1-butanol initiator (73 μ L, 0.25 mmol, 0.37 eq) was added to the flask, and the mixture was stirred for 30 minutes to allow the catalyst to coordinate to the alcohol group on the initiator. The α -valerolactone (1.75 mL, 9.03 mmol, 13.3 eq) and the α -allyl- γ -valerolactone (0.103 mL, 0.68 mmol, 1.0 eq) were added to the reaction mixture along with the DCM (1.08 mL, 6.6 M), and the reaction was allowed to stir for 16-18 hours. After the reaction a tin scavenger, SiliaMetS[®]Cysteine (Silicycle, 8 mol eq/mol tin) was added to the copolymer, stirred for 1 h, and removed by gravity filtration in DCM. The resulting polymer was purified by precipitation in methanol at -80°C and dried overnight. 1.6g yield (80%). ¹H NMR (400 MHz, CDCl₃) δ : 0.92 (6H, d, CH₃), 1.47-1.78 (8H, m, CH₂), 1.86 (1H, m, CH), 2.14-2.52 (5H, m, CH₂, CH), 3.65 (2H, t, CH₂), 4.08 (4H, m, CH₂), 5.04 (2H, m, CH₂) 5.73 (1H, m, CH). A similar preparation was followed to synthesize the 8% AVL/VL polymer with VL (3.21 mL, 0.03456 mol), AVL (0.50mL, 0.003832 mol), 3-methyl-1-butanol (145 μ L, 0.1172 mol), and 1.97mL DCM (6.6 M). 3.3 g yield (83%). M_w(NMR): 3245 g/mol. In a 6 dram vial, the VL/AVL polymer (0.500 g, 0.301 mmol, 1.0

eq) was added along with m-CPBA (62.4 mg, 0.362 mmol, 1.2 eq/allyl). CDCl_3 was added to a final concentration of 6.5×10^{-2} M. The vial was capped allowed to stir at room temperature for 48 h. Oxidized polymer was purified by washing with saturated sodium bicarbonate (3 times) and brine (1 time), 65% yield. ^1H NMR (400MHz, CDCl_3): In all cases, 100% conversion of allyl to epoxide was achieved as indicated by the disappearance of alkene proton shifts at 5.0 and 5.7 ppm and the presence of small broad peaks at 2.4, 2.7 and 2.9 ppm from the epoxide ring (see supplemental materials). M_w (NMR): 3343 g/mol

Nanoparticle Synthesis: 4% crosslinking with 1.5 of amine per epoxide. 340 mg of the oxidized poly (vl/evl) linear polymer with a M_w (NMR) of 3400 g/mol was added to a 50-mL round bottom flask and dissolved in 35.8 ml DCM to reach a 3.24×10^{-3} mol concentration of the epoxide unit. 2,2'-(ethylenedioxy) diethylamine crosslinker, 12.7 μL (calculated for 0.75 eq of crosslinker molecules per epoxide, or 1.5 eq of amine per epoxide) was added via syringe to the polymer solution and refluxed at 46°C for 12 hours. The resulting particles were purified by dialysis using Snakeskin Dialysis tubing (MWCO = 10,000) against DCM for 72 hours, and product was dried by rotovap and put overnight under vacuum. Particle size and uniformity were determined by transmission electron microscopy at 200kV. Yield: 240 mg, 71% isolated nanoparticles as an off-white solid.

Nanoparticle Synthesis 8% crosslinking with 1.5 amine per epoxide. 120 mg of the oxidized poly (vl/evl) linear polymer with 8 wt % of epoxide and a M_w (NMR) of 3400 g/mol was added to a 50mL round bottom flask and dissolved in 28.9 ml DCM to reach a 3.24×10^{-3} mol concentration of the epoxide unit. 2,2'-(ethylenedioxy) diethylamine crosslinker, 10.3 μL (calculated for 0.75 eq of crosslinker molecules per epoxide, or 1.5 eq of amine per epoxide) was added via syringe to the polymer solution and refluxed at 46°C for 12 hours. The resulting particles were purified by dialysis using Snakeskin Dialysis tubing (MWCO = 10,000) against DCM for 72 hours, and product was dried by rotovap and put overnight under vacuum. Particle size and uniformity were determined by transmission electron microscopy at 200kV. Yield: 98 mg, 82%, of isolated nanoparticles as an off-white solid.

Post-Loading TAM and QT into NPs. A solution of 0.1% D- α -tocopherol polyethylene glycol 1000 succinate (0.40 g) was dissolved in distilled water (40.0 mL). To a separate tube, poly(vl-evl)nanoparticles (80.0 mg), TAM (10 mg) and QT (10 mg) were added and dissolved in minimal dimethyl sulfoxide (100 μL). The polyethylene glycol solution was stirred vigorously, and the particle-drug solution was added dropwise. The dual drug loaded particles were centrifuged for two cycles at 7830 rpm for 20 minutes/cycle. The resulting pellet was re-suspended in fresh distilled water and lyophilized to aid in the removal of any residual dimethyl sulfoxide. Freeze-dried NP-TAM-QTs were stored at 4°C until use. The weight percent of post-loaded tamoxifen and quercetin was determined by HPLC by comparing the areas under the curves at 265nm to previously constructed calibration curves.

Dual Drug Release of NP-TAM-QTs. Polyester nanoparticles (4% and 8% NPs) containing encapsulated QT and TAM were suspended in PBS (1X, pH 7.4, 0.1% Tween-80) to a final drug concentration of 0.3 mM TAM and QT (1:1). The mixture was stirred at 37°C , and buffer was collected and exchanged every 48 hours for 21 days. At each time point, the suspension was centrifuged at 7830 rpm for 15 minutes and the supernatant was collected and replaced with fresh buffer. Injections of 30 μL of supernatants were made on the HPLC as described in the characterization section. The amount of drug released at each time point was calculated from a calibration curve, and all experiments were performed in triplicate. Tween-80 was used as surfactant to solvate the released TAM and prevent it from binding to the walls of the incubation container.³⁰

Stability in Simulated gastro-intestinal (GI) Fluid. Simulated GI fluid was prepared by dissolving 2.0 grams NaCl in 7.0 mL concentrated HCl, diluting to 1.0 L of water, adjusted to pH 1.2 and supplemented with Tween-80 (0.1% v/v). Freeze-dried NP-TAM-QTs were suspended in GI media to a final drug concentration of 0.3 mM TAM and QT (1:1), and allowed to incubate at 37°C . At 30 min, 1h, and 2 h time points, the mixture was pelleted at 7830 rpm, and the supernatant was transferred, neutralized with sodium bicarbonate to pH 7 and extracted three times with 5 mL CH_2Cl_2 . The CH_2Cl_2 was evaporated and the resulting solid was reconstituted in methanol/water (1:1 v/v). Drug content was quantified by HPLC, and all experiments were performed in triplicate.

Metabolism of NP-TAMs. Procedure adapted from Kim, et al.³⁹ Tamoxifen was incorporated into 4% and 8% cross-linked NPs using a loading procedure as previously described.³⁹ Free TAM (0.5 μM , 0.2mg/mL) and NP-TAM were incubated separately at 37°C for 2 h in 1X PBS, pH 7.4 (GIBCO), with 50 pmol of CYP3A4 BACULOSOMES® (Life Technologies), 1.3mM NADPH, 3.3mM glucose-6-phosphate, 3.3mM MgCl_2 , and glucose-6-phosphate dehydrogenase (0.4U/mL) in a 1.5-mL Eppendorf tubes with a total reaction volume of 0.5mL. The reaction mixture was pre-warmed at 37°C , and metabolism was started by the addition of enzyme (kept on ice until use). A control reaction with free TAM was run alongside with no enzyme added, and each reaction (NP-TAM, TAM, TAM control) was performed in triplicate. After 2h, the reaction was stopped by the addition of 500 μL of cold methanol. The samples were mixed by vortex and then centrifuged at 7830 rpm for 15 min. 30 μL of supernatant was injected directly onto the HPLC and peak areas were compared to a standard curve 6.25-100 $\mu\text{g/mL}$ in MeOH:Water (1:1) to quantify the amount of free TAM remaining after 2h.

Metabolism of NP-QTs. QT metabolism procedure was adapted from Oliveira et al.^{21a} In a 1.5-mL Eppendorf tube, a 0.5mL reaction mixture contained 1 mg/mL UGT-1A9 microsomes, 4.5mM UDP-GluA, 100mM Tris Buffer (pH 7.5), 10mM MgCl_2 , and 300 μM quercetin (0.1mg/mL). A separate reaction mixture contained 300 μM quercetin encapsulated in 4% crosslinked nanoparticle, and another reaction with 8% crosslinked nanoparticles with querce-

tin entrapped. The reactions started upon addition of cold UGT-1A9 microsomes to the pre-warmed (37°C) reaction mixtures. The mixtures were vortexed briefly before incubation. A control reaction with free drug and without enzyme was run in parallel, and each reaction was performed in triplicate. After 2h of incubation, the reactions were stopped by the addition of 0.5mL of cold methanol, centrifuged at 4000 rpm for 20 min, and 300µL of supernatant was injected onto the HPLC. A standard curve of 6.25-100 µg/mL in MeOH:Water (1:1) was constructed to determine the concentration of free drug remaining after 2 h.

4T1 Cell Culture Experiments. Mammalian breast cancer cells (4T1; ATCC® CRL-2539™, Manassas, VA, USA) were grown in tissue culture flasks (75 cm²) and maintained under 5% CO₂ atmosphere at 37 °C in complete growth media, which was made with RPMI-1640 (Corning, with glutamate, without phenol red) supplemented with 10% FBS (Gibco, USA). Growth media was changed every 2-3 days, and once 80% confluent, cultured cells were trypsinized and split (1:5 to 1:10 ratio) with 0.25% trypsin-0.53mM EDTA solution (Gibco, USA). For the MTT assay, 4T1 cells were seeded at a density of 10,000 cells/well in 96-well cell culture plates (BD Falcon, NJ, USA).

Cell Cytotoxicity Assay. Standard MTT (Life Technologies, USA) assay was used to determine the cell cytotoxicity of NP-TAM-QTs in mouse breast cancer cells (4T1, ATCC). The 4T1 cells were harvested from confluent cultures by trypsinization and adjusted to 600,000 cells/ml incomplete media. The suspension was added (0.2 ml/well) and incubated for 3 h for cell attachment. Following attachment, the media was replaced with fresh complete media (0.2 ml/well) containing free TAM, free QT, a mixture of free TAM with QT (1:1 w/w), or NP-TAM-QTs in different wells to achieve net concentrations of 0.01, 0.1, 1, and 10 µg/mL (equivalent to free TAM) and incubated for 24, 48, and 72 hours. After incubation was finished, the media containing drug formulation was aspirated, and cells were washed with PBS (1X, pH 7.4). Then, 150 µL of MTT solution (500 µg/mL in complete media) was added to each well and incubated for 3-4 h in order for the live cells to produce formazan crystals. Following incubation, all but 25 µL were aspirated carefully. The formazan crystals were dissolved by addition of 50 µL of DMSO with gentle mixing, and the absorbance (A) of each well was measured at 540nm using a Synergy HT Microtiter Plater reader (BIO-TEK). Cell viability was assessed using the following equation:

$$\% \text{ cell viability} = \frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

Recovery Condition. In a separate 96-well plate, free drugs or NP-drugs were incubated to estimate cell cytotoxicity in the recovery condition. In this experiment, 4T1 cells were dosed with free TAM, free QT, a mixture of free TAM and QT (1:1 wt/wt), or NP-TAM-QTs for 24 h at the

same concentrations as other experiments, followed by washing with PBS (1X) and reincubation in fresh complete media up to 72 h. The cell cytotoxicity was measured by MTT under same protocol as above.

Statistical analysis. All results from experiments are expressed as mean ± standard deviation (SD). Statistical analysis was performed in Microsoft Excel. Mean particle size and standard deviations were calculated using ImageJ software (Mac OS X).

RESULTS AND DISCUSSION

We first synthesized the nanoparticles via the intermolecular crosslinking reaction from linear poly(avl-evl) with 4% or 8% weight percent epoxide stemming from a complete oxidation of the allyl functionality in the corresponding poly(vl-avl). It has been observed that Sn(OTf)₂ with its high reactivity at room temperature needs to be carefully removed to not affect the subsequent oxidation step by an unwanted ring opening of the epoxide^{40,41,42}. This can be done by efficient precipitation as reported before³⁰ or in a more efficient way with SiliaMetS®Cysteine that is added at the end of the polymerization and filtered off before a final precipitation of the polymer into cold methanol. With this, we found that the subsequent epoxidation reaction is more robust. The nanosponges synthesis was adapted from the procedure in Stevens, et al.³⁰ and characterization data including ¹H-NMR and TEM can be found in the supplementary materials (see Figures S1-S3). After successful nanoparticle synthesis the particles were loaded with QT and TAM via a developed nanoprecipitation method. As shown in Table 1, the entrapment efficiency improved from 69.1 to 72.7% for TAM and 77.2 to 78% for QT when comparing the post-loading for NP-4% particles to NP-8% particles. We attribute the higher loading efficiency to the denser network in the 8% crosslinked nanoparticles. The overall higher loading efficiency of QT in contrast to TAM might be the result of a higher a higher loading of hydrogen bonding between the hydroxyl groups of the QT molecule with the secondary amines in the crosslinker of the nanoparticle network (see Figure S4). The particle size was very similar between the NP-8% and NP-4% as determined by TEM and around 100 nm.

Table 1. Particle sizes and entrapment efficiencies for NP-4% and NP-8% particles.

Formulation		NP-4%	NP-8%
Particle Size (nm)		89.3 ± 14.8	91.5 ± 9.8
Theoretical loading (%)		20	20
Total Practical Loading (%)		14.63	15.06
Practical Loading (%)	TAM	6.91 ± 0.13	7.26 ± 0.10
	QT	7.72 ± 0.15	7.80 ± 0.12
Loading Efficiency (%)	TAM	69.11 ± 1.31	72.66 ± 1.02
	QT	77.24 ± 1.54	78.02 ± 1.16

*Values are in mean ± SD (n=3)

Figure 1. Cumulative free drug release of QT and TAM from NP-8% and NP-4% particles in PBS over the course of 21 days.

Figure 2. Cumulative TAM and QT release from NP-4% particles in simulated gastrointestinal fluid (SGF) media at 37°C.

can provide a faster release where 50% drug is released in 9 days (NP-4%), or slow release where 50% drug is released in 21 days (NP-8%) as observed in the Paclitaxel release in comparable particle densities. Therefore, the dual release can be tailored for the desired application.

Free TAM and QT were monitored for its stability in the NP-4% formulation under conditions that mimic a gastrointestinal environment and the stability test was run for 2 h which are standard conditions.¹⁹ The resulting plot (Figure 2) shows that after 2 h, only 20% of TAM and 12% QT is released, which indicates a high stability of the NP-TAM and NP-QT formulations in SGF media.³⁰ Interestingly, more TAM than QT was released into the SGF media, indicating that TAM may be more likely to release at low pH conditions. Nonetheless, the nanoparticle formulation is stable in SGF and presents a promising platform application for combination oral drug delivery of TAM and QT.

The bioavailability of tamoxifen and quercetin is limited because of fast metabolism in the liver.⁴³ We wanted to

After determining the drug loading of around 14%, we sought to investigate whether the dual loading would influence the release of the individual drugs and a drug-drug interaction is occurring that results in an unexpected release profile. As shown in Figure 1, there was an expected initial release of 22 and 25% from the NP-4% for a nanoparticle in the first day for TAM and QT, respectively, followed by a slow, linear release ending at 89 and 97% after 21 days. The TAM release kinetics from our NP-4% and NP-8% were more consistent with previous reports of drug release by the anti-cancer drug Paclitaxel, which had an initial 1 day burst of 25% from the NP-4% and 12% release in the NP-10%.³⁰ Moreover, the release plot shows QT and TAM exhibited similar linear release kinetics with QT releasing slightly more on day 2 (38%) than TAM (30%). We attribute this effect to the higher water solubility of QT versus TAM. A similar difference in release was noticed in the NP-8%, when 30% of TAM and 52% QT was released after 21 days. It also can be said that the lower crosslinking density of the NP test our NP-4% and NP-8% dual drug formulations against enzymes under *in vitro* conditions consistent with experiments commonly cited in the literature for free TAM and QT.^{21a,39} TAM is extensively metabolized by the P450 CYP3A4 enzyme to metabolites N-desmethyldamoxifen, 4-hydroxytamoxifen and endoxifen.⁴⁴ QT is rapidly conjugated by UGT1A9 microsomes in the intestinal wall.^{45,21b} In order to detect how much free drug is metabolized in the NP formulations, we monitored the parent loss of TAM and QT in buffers under physiological temperature (37°C) and pH = 7.5. The quantity of free drug remaining after two hours for the NP-drug groups directly were compared with the free drug groups (See Table S2). Figure 3 illustrates a roughly 2-fold reduction in metabolism from 65% TAM (NP-4%) to 36% TAM remaining, and a 77% (NP-8%) to 35% TAM remaining after 2 h incubation with enzyme. The effect on QT was even more substantial with a 3-fold reduction in metabolism from 74% QT (NP-4%) to 19%, and a 4-fold reduction from 80% to

Figure 3. Metabolism of QT and TAM in NP-4% and NP-8% particles by CYP3A4.

21% (NP-8%) parent drug remaining. Each reaction set was run with a blank and control (no enzyme) to correct

from any contact loss or compound degradation. It is apparent that the polymeric nanonetwork of the particle provides a protection effect for the drug, and the data suggests using a particle composition with higher cross-linking density can reduce metabolism further and improve bioavailability of the combination drug therapy.

As the literature suggests,²⁵ the combination of QT and TAM exhibited increased anti-cancer cell activity in combination compared with either drug alone,⁴⁶ and our results indicate that free TAM+QT and NP-TAM-QT formulations imposed a similar effect on 4T1 cell viability after 24, 48 and 72 h of incubation (See Figure 5) in MTT cytotoxicity assays. A time and concentration dependent decrease in cell viability against the 4T1 cell line was observed in all formulations tested. In this study, we only tested NP-4% because we anticipate that using NP-8% would result in very similar results as the NP-4% because of the nearly identical composition. Regression analysis showed IC₅₀ values in Table 2 were similar between NP-drugs and free drugs (0.1 µg/mL at 72 h). On the other hand, recovery experiments revealed the IC₅₀ value was

significantly lower for NP-TAM-QTs (2.12 µg/mL) compared with free drug (305.85 µg/mL), which suggests the anti-cancer cell activity is preserved in the NP formulation and may even enhance the TAM and QT dual drug efficacy more than one hundred fold.

Figure 4. Metabolism of TAM and QT in NP-4% and NP-8% particles by UGT1A9.

Figure 5. Cell cytotoxicity of free TAM, free QT, combined free QT and TAM (1:1) and NP-TAM-QTs after (A) 24 h, (B) 48, (C) 72 and (D) recovery condition. Each data point represents the mean $\pm$ SD (n=3).

Table 2. IC₅₀ of free TAM, free QT, combination of TAM and QT and NP-TAM-QTs upon treatment with 4T1 cells.

Formulation	IC ₅₀ value (µg/mL)*			
	24h	48h	72h	Recovery
Free QT	65.32 $\pm$ 3.56	52.17 $\pm$ 2.23	4.90 $\pm$ 0.83	365.12 $\pm$ 14.54
Free TAM	42.39 $\pm$ 1.35	20.18 $\pm$ 1.28	0.35 $\pm$ 0.02	450.32 $\pm$ 16.23
Free TAM + QT (1:1)	10.82 $\pm$ 1.71	0.51 $\pm$ 0.03	0.088 $\pm$ 0.011	305.85 $\pm$ 10.87
NP-TAM-QTs	14.15 $\pm$ 1.19	0.27 $\pm$ 0.04	0.104 $\pm$ 0.013	2.12 $\pm$ 0.22

*Data expressed as mean $\pm$ SD (n=3).

CONCLUSION

We can summarize that this work supports a formulation strategy to synthesize and co-load a clinically relevant drug combination, TAM and QT, into a single dosage form. Previous reports have demonstrated how the targeting potential of our NPs can further enhance the therapeutic efficacy of cancer treatments.⁴⁷ The inclusion of an antioxidant helps in easing the oxidative stress induced by free radicals generated during the course of TAM therapy,⁴⁸ which is corroborated by our MTT cytotoxicity study. The NP-TAM-QT preparation posed higher cell cytotoxicity in contrast to the free drug combination. In the future, multiple dose kinetics and *in vivo* distribution studies can be performed to better understand the advantages of our combined formulation over any individual free drug regimen. NPs can be synthesized to encapsulate and protect TAM and QT and tune down the *in vitro* metabolism by up to 4-fold for QT and over 2-fold for TAM. There are many potential therapeutic applications where a slowed metabolism is beneficial.⁴⁹ Crosslinking density of our NPs can be modified to control the release and impact the metabolism of the encapsulated drug, and all particles were prepared in 100nm size range, which is ideal for both I.V. administration and oral delivery of drugs.⁵⁰ In summary, we hopefully have demonstrated the potential for tunable metabolism, improved therapeutic efficacy and controlled dual drug release functionality our NP-drug formulation can offer for breast cancer and many other potential therapeutic applications.

ASSOCIATED CONTENT

Supporting Information. Nanoparticle synthesis and characterization, HPLC methodology and calibration drug quantification, TEM image, and MTT studies. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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