Chemotherapeutics Loaded Poly(Dopamine) Core-Shell Nanoparticles for Breast Cancer Treatment

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Multifunctional nanoparticles for TNBC treatment

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Abbreviations:

4- cyano- 4- [[(ethylthio) thioxomethyl] thio] -pentanoic acid (TCT-2)
5-Carboxytetramethylrhodamine (TAMRA)

chain transfer agent (CTA)

Dynamic Light Scattering (DLS)

Fourier Transform Infrared (FTIR)

Near infra-red region (NIR)

Paclitaxel (PTX)

Poly(dopamine) (PDA)

poly(ethylene glycol) (PEG)

poly(vitamin B5 analogous methacrylamide) (poly(B5AMA))

reversible addition–fragmentation chain-transfer polymerization (RAFT)
Starch encapsulated PDA nanoparticles PDA-SNPs

Transmission Electron Microscope (TEM)

Section: Other ‘Nanotherapeutics in Cancer Research

Abstract

Chemophotothermal therapy is an emerging treatment for metastatic and drug resistant cancer anomalies. Among various photothermal agents tested, polydopamine provides an excellent biocompatible alternative that can be used to develop novel drug delivery carriers for cancer treatment. This study explores the synthesis of starch encapsulated, polydopamine coated core-shell nanoparticles, in a one pot synthesis approach and by surfactant free approach. The nanoparticles produced are embellished with polymeric stealth coatings and are tested for their physiological stability, photothermal properties, and drug delivery in metastatic triple negative breast cancer cell (TNBC) lines. Our results indicate that stealth polymer coated nanoparticles exhibit superior colloidal stability under physiological conditions, and are excellent photothermal agents, as was determined by the increase in temperature of solution in the presence of nanoparticles, upon laser irradiation. The chemotherapeutic drug loaded nanoparticles also showed concentration dependent toxicities in TNBC and in a brain metastatic cell line.

Significance Statement: This study for the first time develops biocompatible core-shell nanoparticles in template free approach that can serve as drug delivery carrier and as photothermal agents for cancer treatment.

Introduction

Chemo photothermal therapy is an emerging and promising strategy to ensure effective drug delivery and complete ablation of residual tumors, where elevated temperatures enhance the
sensitivity of cancer cells towards chemotherapeutics, along with facile drug release in the tumor environment (Zhang et al. 2019, Ambekar et al. 2019, Tian et al. 2019, Ho et al. 2013, Griffith et al. 2019, and Xiong et al. 2019). Among various organic and inorganic materials that have been explored for their potential as photothermal agents, poly(dopamine) (PDA) are advantageous due to their biocompatibility, biodegradability, adhesiveness, facile synthesis and superior drug loading capacity. (Zhu et al. 2017, Li et al. 2021, Liu, et al. 2021, & Jin et al. 2020) PDA are melanin like structures that are prepared by the simple oxidation of 3,4-dihydroxy-L-phenylalanine (DOPA) in alkaline aqueous environment, in the presence of oxygen and their size can easily be tuned as a function of pH of the solution. (Ho et al. 2013, Li et al. 2021, & Zhang et al. 2015) Owing to superior adhesive properties, PDA nanoparticles are extensively studied for various biomedical applications including drug delivery, sensors fabrication and for tissue engineering. (Ambekar et al. 2019 & Griffith et al. 2018)

PDA nanoparticles are especially exciting for chemotherapeutics delivery, as PDA absorbs near infra-red region (NIR), converting light energy into hyperthermia, hence providing a multi-pronged strategy for cancer treatment. (Griffith et al. 2018) Furthermore, the highly adhesive nature of PDA enables superior drug loading capacity via hydrophobic, and π-π interactions and provides active surface for functionalization of various biomolecules including targeting agents, imaging probes and stimuli responsive polymer chains. (Jin et al. 2020) Surface coating of PDA nanoparticles with hydrophilic, biomacromolecules including polymers, DNA and proteins by electrostatic interactions, surface adsorption and covalent bonding has yielded physiologically stable and stimuli responsive nanocarriers with superior drug delivery efficacies in various tumor models in vitro and in vivo. (Liu et al. 2022, Li et al. 2017, He et al. 2017 & Siani et al. 2022) To further improve the drug loading capacity of PDA based carriers, mesoporous nanoparticles are
synthesized by soft-template approach and are tested for their photothermal properties and drug delivery efficacies. (Chen et al. 2021 & Zhang et al. 2019) Similarly, core-shell nanoparticles, comprising of organic core and PDA shell have been developed to improve the stability and chemotherapeutic potential of nanoparticles in cancer cells. (Zhang et al. 2015) The biocompatible, FDA-approved poly(lactide-co-glycolide) (PLGA) nanoparticles coated with PDA are further modified with stealth polymers and targeting moieties as photothermally active drug loaded carriers. (He et al. 2017) Similarly, Paclitaxel (PTX) loaded, poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanoparticles prepared by emulsion solvent evaporation method were coated with PDA shell, followed by their surface functionalization with RGD peptide to yield pH-responsive spherical nanoparticles with \textit{in vitro} and \textit{in vivo} anti-tumor efficacies in liver cancer model. (Wu et al. 2021) Others have demonstrated the encapsulation of 2-diestearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] (DSPE-PEG) micelles in a PDA shell can yield potent dual drug delivery carriers for DOX and Bortezomib delivery in breast cancer cells \textit{in vitro} and in mice xenograft model. (Zhang et al. 2015) PDA coated polymeric nanoparticles are typically prepared by multi-step approach that first require the fabrication of polymeric core, followed by coating and surface functionalization with PDA shell and stealth/targeting moieties.

In this study, we report a facile one-step method for the synthesis of biodegradable, core-shell nanoparticles comprised of a starch core and PDA shell (Scheme 1). The nanoparticles synthesized are then fabricated with stealth layers of poly(ethylene glycol) (PEG) and poly(vitamin B5 analogous methacrylamide) (poly(B5AMA)) in one pot approach. Poly(B5AMA) is a hydrophilic polymer that has recently been studied for its PEG like antifouling and stealth properties. (Combita et al. 2021 & Nazeer et al. 2021) Starch
encapsulated PDA nanoparticles embellished with PEG or poly(B5AMA); namely PEG@PDA-SNPs and P(B5AMA)@PDA-SNPs respectively, were loaded with chemotherapeutic drugs and were studied for their anticancer effects in primary and metastatic breast cancer cells. Our results show that stealth polymers functionalized PDA coated starch nanoparticles prepared by one step fabrication method and in the absence of any template exhibit excellent physiological stability, photothermal properties and \textit{in vitro} anticancer activities in breast cancer cell lines.

\textbf{Materials & Methods}

Dopamine hydrochloride, water soluble starch, 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris Base), DMEM high glucose media, Fetal Bovine Serum (FBS), Penicillin-Streptomycin, (2-N-Morpholino)ethanesulfonic acid sodium salt (MES sodium salt), ethylenediaminetetraacetic acid (EDTA), sodium chloride, methanol, calcium chloride and acetic acid were purchased from Fisher Scientific. 5kDa Polyethylene Glycol (Thermofisher), Paclitaxel 99.5%, and 5-TAMRA (5-Carboxytetramethylrhodamine) were purchased from Sigma Aldrich. MDA-MB-231 cell line was purchased from ATCC and MDA-MB-231- BR was a gift from Dr. Kata Henji Japan. Synthesis of B5AMA was achieved according to previously established procedures.\textsuperscript{20-21}

\textbf{Polymerization of B5AMA}

Polymerization of B5AMA was conducted using the reversible addition–fragmentation chain-transfer polymerization (RAFT) technique at 50 °C, using deionized water/methanol solution as a solvent in a ratio of 10:1 (v/v). 4- cyano- 4- [[(ethylthio) thioxomethyl] thio]-pentanoic acid (TCT-2) was used as a chain transfer agent (CTA) and VA-044 as an initiator. In a typical procedure, a 25 mL two-neck flask was charged with B5AMA monomer (1290 mg, 5 mmol), TCT-2 (77 mg, 0.25 mmol, target DP = 20), and VA-044 (27 mg, 0.083 mmol) dissolved in 5 mL of solvent. The reagent solution was degassed by three freeze-vacuum-thaw cycles and was
then allowed to react at 50 °C. At the end of the reaction, the polymer solution was dialyzed against deionized water for 48 hours at least. Finally, 5kDa polymer was obtained by freeze-drying and was analyzed by GPC chromatography (Supplemental Figure 1).

**Synthesis of Core-Shell Nanoparticles**

A 0.05 w/v% stock solution of starch was prepared in Tris buffer of pH 8.5 and was heated at 85 °C and mixed until starch dissolves completely, then was then cooled to 19-21°C. A solution containing 2 mg/mL of dopamine and 1mg/mL PEG/P(B5AMA) was prepared in distilled water and 1 mL of this solution was mixed with 1mL of cooled starch solution, drop wise. The final concentration of dopamine, PEG/P(B5AMA) and starch was 1mg/mL, 0.5 mg/mL and 0.25 mg/mL, respectively, in the reaction solution. The solution was left stirring at room temperature covered from light for 2.5 hours. The nanoparticles formed were centrifuged at 17,000 RPM for 10 minutes and were washed 2 times with filtered distilled water at 17,000 RPM for 10 minutes.

For drug loaded nanoparticles, 60 µg Paclitaxel was added to 1 ml of starch solution, followed by the addition of dopamine solution to obtain final total volume of 2 mL. The drug loaded nanoparticles synthesis was achieved as described above.

**Synthesis of PDA Nanoparticles**

A solution containing 2 mg/mL of dopamine was prepared in 1mL of distilled water and 1mL of Tris buffer (pH 8.5) was added. The solution was left stirring at room temperature covered from light for 2.5 hours. The nanoparticles formed were centrifuged at 17,000 RPM for 10 minutes and were washed 2 times with filtered distilled water at 17,000 RPM for 10 minutes.

**Dynamic Light Scattering and Zeta Potential Analysis**

Size, net charge and polydispersity analysis of the nanoparticles were performed using Dynamic Light Scattering (DLS) (Nano Brook 90 Plus (Brookhaven, Holtsville, NY, USA). The 2.5
µg/mL nanoparticles solution was prepared in PBS buffer of pH 7.4, supplemented with 1% FBS and the nanoparticles were incubated for 24 hours. The changes in the size and polydispersity of nanoparticles were analyzed at different time points 0 hours, 1, 2, 3 and 24 hours. All the measurements were taken at room temperature at a 90° scattering angle, equilibration count of 180 seconds and count time of 300 seconds. The zeta potential of nanoparticles was analyzed at room temperature in deionized water.

**Transmission Electron Microscopy**

Transmission Electron Microscope (TEM) (Hitachi 7700) was performed at 80 kV with a Lab6 filament. Samples of 3 µL were drop casted onto the 200 mesh copper TEM grids, with a layer of formvar and thin film of carbon (Ted Pella) and were allowed to air dry. Histogram characterization was performed with image J software by counting over 100 nanoparticles for each sample and organizing by different number distribution ranges. The average median size was calculated to determine the sample size distribution.

**UV-Vis-NIR Spectroscopy**

Absorbance of nanoparticles was performed at range of wavelength (400 to 1000nm) for different concentrations (150-37.5 µg/ml) of nanoparticles, prepared by serial dilution.

**Fourier Transform Infrared (FTIR) Spectroscopy**

Samples of polydopamine and dopamine coated core-shell nanoparticles were prepared, as described above and were freeze dried. FTIR analysis was performed on a Bruker Alpha-T spectrometer operated in transmission mode.

**Starch Release and Encapsulation Efficiency**

Starch encapsulation in core-shell nanoparticles was evaluated with a starch test comprised of Iodine-KI solution. (Pesek et al 2022) The standard curve was first prepared with varying
concentrations of starch (250-1.95 µg/ml) in water. In order to address the interference of PDA-NPs with the blue color development during starch detection using Iodine-KI solution, a second standard curve was prepared by the addition of 4% volume/volume (v/v) of PDA-NP washes and was used as a control to evaluate the interference of PDA with starch assay. The starch solution mixed with PDA washes was read at 600 nm to produce a standard curve. The comparison of absorbance value of the two standard curves indicated that free starch can be detected in nanoparticles wash with a sensitivity of 15.6 µg/mL. The presence of residual nanoparticles color in reaction washes interferes with starch concentrations that are lower than 15.6 µg/mL, when starch is detected using Iodine-KI based starch assay. The amount of residual starch in supernatant of the reactions was analyzed by starch assay and starch encapsulation efficacy in dopamine core-shell nanoparticles was calculated using the standard curves generated. The encapsulation efficiency is given by

\[
Encapsulation\ Efficiency = \frac{\text{Amount Loaded} - \text{Amount in Washes}}{\text{Amount Loaded}} \cdot 100
\]

**Drug Encapsulation Efficiency**

The drug encapsulation efficiency of nanoparticles was evaluated using TAMRA fluorescent dye as a probe, and is given by

\[
Drug\ Encapsulation\ Efficiency = \frac{(\text{Amount Loaded} - \text{Amount in Wash})}{\text{Amount loaded}} \cdot 100
\]

The synthesis of nanoparticles was performed in the presence of 300 µg of TAMRA dye, as indicated above. The encapsulation efficacy of the dye was calculated by measuring the amount of residual dye post-synthesis of nanoparticles in the reaction supernatant and was quantified using the calibration curve of TAMRA dye at 552nm excitation and 578nm emission.

**Drug Release Study**

Release of TAMRA dye from nanoparticles was evaluated as follows:
Nanoparticles (300 µg/mL) were suspended in PBS (pH 7.4 and 5.5) and were irradiated with an 808 nm diode laser (Luck Laser Model 7/24, 200 mW nominal power) for 10 minutes. Samples were taken over different time periods then centrifuged for 10 minutes at 17,000 RPM to remove nanoparticles and absorbance of TAMRA dye in the supernatant was measured at excitation and emission wavelengths of 552nm and 578nm, respectively. Fresh PBS was added to the pellet and nanoparticles were added back to the samples for continued evaluation. The dye release was studied at different periods of time ranging from 0 to 120 hours at 37°C. The dye release was quantified using the calibration curve of TAMRA dye at 552nm excitation and 578nm emission.

**Laser Characterization**

The operating power of the laser was measured using a laser power meter (Nova II, OPHIR). The laser beam dimensions at the operating distance from the top of the sample were measured using a graded ruler system.

**Temperature Studies**

Nanoparticles were suspended at the concentration of 200 µg/ml in distilled water and were irradiated for 10 minutes. The temperature of the solution was evaluated at time 0 and following the 10 minutes of laser irradiation using a thermal camera (FLIR ONE Pro) positioned near the center of the sample. Deionized water was used as a control.

**Cellular Uptake Studies**

MDA-MB-231 and MDA-MB-231-BR cells were seeded in in high glucose DMEM containing 10 % FBS and 1 % antibiotic at 37 °C and 5% CO₂ in 6 well plates on glass coverslips and were treated with TAMRA loaded nanoparticles at a final concentration of 40 µg/mL. The nanoparticles were incubated with cells for 4-hours, were fixed with 3.7% formalin and were
stained with DAPI. The glass coverslips were mounted onto the glass slides and were examined by use of fluorescent microscopy with the excitation and emission of the two dyes, TAMRA of 552 nm and 578 nm respectively and for DAPI 359 nm and 457 nm respectively.

In vitro Cytotoxicity

MDA-MB-231 and MDA-MB-231-BR were seeded in 96 well plate at the density of 2x10^4 cells per well in high glucose DMEM containing 10% FBS and 1% antibiotic at 37 °C and 5% CO₂. After 24 hours, the cells were treated with 50-200 µg/mL nanoparticles in high glucose DMEM. The cells were incubated with the nanoparticles containing media for 4 hours and were then irradiated using the 808 nm laser for 10 minutes. Cells were then incubated overnight, and MTS assay was performed according to manufacturer’s protocol. Briefly, treated cells were washed with PBS and media containing MTS was added and incubated for 3 hours, the absorbance of the samples was read at 490nm. Statistical analysis was performed with use of Graph Pad Prism version 10. Unpaired two tailed t-test with welch correction was performed on cytotoxicity data. All asterisks (*) indicate p<0.05, the number of asterisks indicate the groups that are significantly different to each other. Square symbols (■) indicate p<0.1.

Results

One-pot synthesis of core-shell nanoparticles

In this study, dopamine coated starch nanoparticles were synthesized in one step by dissolving different concentrations of water-soluble starch in Tris buffer (pH = 8.5), followed by the addition of dopamine that undergoes rapid oxidation under alkaline pH. (Zhu et al. 2017) The nanoparticles synthesized were optimized as a function of concentration of reactants, pH, and reaction time and were characterized by FTIR, DLS and zeta potential equipment and by TEM, for the composition, size and surface charge of nanoparticles. The dispersion of 0.05% w/v of
starch alone in Tris buffer yielded nanoparticles of ~150 nm with a PDI 0.31. The addition of dopamine to starch solution of pH = 8.5 quickly resulted in color change from transparent to brown and dark brown nanoparticles were obtained after 24 hours of reaction time (Supplemental Figure 2). Poly(dopamine) nanoparticles strongly absorb at 600 nm and synthesis of nanoparticles, as a function of time can be monitored by UV-Vis-NIR spectroscopy. (Chen et al. 2021) The increase in the absorbance of reaction solution with time indicated successful formation of nanoparticles and no significant change in the absorbance values was observed 2.5 hours post-reaction time (A600 = 0.7 after 1 hour, 1.15 after 2 hours, 1.4 after 3 hours versus 1.7 after 24 hours(without starch) , indicating the optimal time for nanoparticles formation.

The purified nanoparticles analyzed by DLS showed discrete nanoparticles of 400-600 nm, and the size of nanoparticles was dependent on the concentration of reactants in solution. (Supplemental Table 1). The PDA-SNPs prepared at low w/w ratio of starch/dopamine (0.05 and 0.1) showed large particle sizes, possibly due to the agglomeration of starch molecules on the surface of dopamine nanoparticles and presence of insufficient starch in the reaction solution to serve as nano-template for the formation of a PDA shell. The nanoparticles obtained at dopamine : starch w/w ratio of 0.25 and 0.5, however showed discrete nanoparticles of 465 ± 53 nm and 470± 48 respectively, indicating the presence of near monodisperse PDA-SNPs. PDA-NPs prepared in the absence of starch were synthesized as a control and were 330 ± 52 nm in size (Table 1). The larger sizes of PDA-SNPs compared to PDA-NPs are attributed to the swelling capacity of starch present in the nanoparticles core under alkaline pH. (Chou et al. 2020 & Jivan et al. 2014)

The supernatant of the nanoparticles post-synthesis was analyzed for the presence of free starch in reaction solution by Iodine-KI assay (Pesek et al. 2022) and encapsulation efficacy of starch in
PDA shell was evaluated by developing a starch calibration curve prepared at 600 nm (Supplemental Figure 3). The possible interference of dopamine nanoparticles present in nanoparticle washes in Iodine-KI assay was evaluated by developing the calibration curve of known amount of starch in the presence of bare dopamine nanoparticles washes and sensitivity of the assay was confirmed by comparing the data with the calibration curve of starch prepared in deionized water. Our data indicated that free starch in dopamine nanoparticle washes can be detected accurately up to the concentration of 15.6 µg/mL (Supplemental Figure 4), without any significant interference of dopamine nanoparticles with the assay absorbance. The encapsulation efficacy of starch in dopamine core was calculated to be 75 ± 15% for PDA-SNPs.

The synthesis of PDA-SNPs was further optimized at pH 10 and 13 and at starch: dopamine w/w ratio of 0.25. PDA-SNPs prepared at various pH showed pH dependent size variations. The nanoparticles prepared in Tris-buffer of pH 8.5 yielded hydrodynamic diameter of 465 ± 53 nm. However, the increase in pH to 10.5 and 13 resulted larger aggregates (1-2 µm) and poor encapsulation of starch in PDA shell, as was apparent by the presence of white starch precipitates in the reaction tube (Supplemental Table 2).

The nanoparticles synthesized at starch: dopamine w/w ratio of 0.25 in Tris buffer (pH = 8.5) and after 2.5 hours of reaction time were further characterized by FTIR spectroscopy (Figure 1). FTIR spectra of PDA-NPs, starch and PDA-SNPs was compared to confirm the encapsulation of starch in PDA core. FTIR spectra of PDA-NPs revealed broad peak from 2500-3200 cm⁻¹ consistent with OH stretching of polydopamine. The peak at 1587 cm⁻¹ was due to stretching vibration of N-H bonds of dihydroxyindole moiety. The characteristic peaks at 1045, 1124, 1199, 1257 and 1488 cm⁻¹ are due to CH₂ bending vibrations, C-O-H bending, C-O symmetry vibration and C-C stretching mode. (Batul et al. 2020) FTIR spectra of water-soluble starch showed
pronounced peaks at 3000-3700 cm\(^{-1}\) indicating OH stretching, and a strong absorption bands in the region of 1000-1200 cm\(^{-1}\) arising from C-O-C and C-O-H stretching and C-O-H bending (Supplemental Figure 5). (Warren et al. 2016) In contrast PDA-SNPs showed an overall dampening and shifting of peaks to 1013, 1340, and 1620 cm\(^{-1}\) indicating the interactions between starch core and PDA shell.

**Stealth Layer Coated Core-Shell Nanoparticles**

PDA-SNPs were modified with stealth polymeric coatings to aid physiological stability and to reduce the aggregation of nanoparticles. PDA-SNPs were modified with the stealth layer of PEG-SH and P(B5AMA) of similar molecular weight (5k Da) and were compared for their physiological stability in situ. PEG@PDA-SNPs and P(B5AMA)@PDA-SNPs produced under identical synthesis condition (pH 8.5, starch: dopamine w/w ratio of 0.25 and 2.5 hr reaction time) were purified and redispersed in deionized water and PBS (supplemented with 1% FBS) and were analyzed for their hydrodynamic size and net charge by DLS and zeta potential. The stealth layer coated nanoparticles showed sizes ranging from 250-470 nm and net negative surface potential of -13 to -21 mV in deionized water. Interestingly, nanoparticles suspended in the presence of serum proteins showed relatively smaller sizes (240-330 nm).

The stability of nanoparticles under physiological conditions was evaluated as a function of time and aggregation propensity of PEG@PDA-SNPs, and P(B5AMA)@PDA-SNPs was compared with PDA-SNPs in the presence of serum proteins. As expected, PDA-SNPs showed time dependent aggregation and size of nanoparticles increased from 330 ± 34nm to >500nm, after 3-hours of incubation in serum containing PBS (Supplemental Figure 6). PEG@PDA-SNPs and P(B5AMA)@PDA-SNPs showed sizes of 290 ± 53 and 240 ± 41 nm respectively, under physiological solution and negligible change in size and PDI of nanoparticles was observed after
24 hours, as was measured by the DLS analysis, suggesting that both PEG and P(B5AMA) has the potential to serve as excellent stealth coating for PDA-SNPs.

Nanoparticle morphology and size was further analyzed by TEM for stealth layer coated nanoparticles (Figure 2). TEM image showed diameter of 97±27 nm and 65±13nm for PEG@PDA-SNPs and P(B5AMA)@PDA-SNPs, respectively. The size of nanoparticles was quantified by image J and the histograms demonstrated a near normal size distribution. The larger sizes of core-shell nanoparticles by DLS (~250-300 nm) when compared with TEM (60-100 nm) are attributed to the hydration capacity of nanoparticles when analyzed in aqueous solution by DLS. (Chou et al. 2020 & Jivan et al. 2014)

**Photothermal Properties and Laser Parameters**

The absorbance intensity of the nanoparticles is a major determinant of their photothermal capability. PEG@PDA-SNPs, P(B5AMA)@PDA-SNPs and PDA-SNPs were analyzed and were compared with PDA-NPs for the change in absorbance as a function of concentration (Figure 3). As seen from the UV-vis-NIR spectra in figure 3, the intensity of absorbance of core-shell nanoparticles is directly correlated with the concentration of nanoparticles and is comparable with PDA-NPs of similar concentration, indicating that encapsulation of starch in PDA core does not change absorption capacity of PDA-NPs. The surface coating of PDA-SNPs with PEG yielded absorption spectra similar to PDA-NPs and PDA-SNPs, however, P(B5AMA)@PDA-SNPs showed significant reduction in absorption spectra, when compared with the other core-shell nanoparticles.

The laser power was measured to be 162 ± 4 mW. The resulting laser power density at the surface of the samples was calculated to be 1.4 W/cm² based on the output collimated rectangular beam, 6.0 mm by 2.0 mm, as viewed on a NIR laser card (Figure 4A).
NIR absorption capacity of nanoparticles and conversion of light energy into heat in the presence of nanoparticles was then studied in situ (Figure 4b, Supplemental Figure 7). After laser irradiation, the three nanoparticle solutions synthesized, all at a concentration of 200 µg/mL, showed a temperature increase from 15-18°C by thermal camera, (Figure 4B). The temperature increase for water alone was 5 °C. Interestingly, the temperature change of 15-18 °C, as was measured by thermal camera was consistent for all the nanoparticles synthesized; PDA-NPs prepared at similar concentration (200 µg/mL) were used as a control and showed an increase of 18 °C in temperature upon irradiation with 1.4 W/cm² laser power density.

**Drug Encapsulation Efficiency and Release Studies**

The drug loading capacity of the stealth layer coated core-shell nanoparticles was studied using TAMRA fluorophore, as a cargo molecule and was compared with PDA nanoparticles. The fluorescent dye was encapsulated in starch core during nanoparticles synthesis and TAMRA loading capacity of the nanoparticles was analyzed by measuring the presence of residual dye in the supernatant of reaction by the microplate reader, using excitation and emission wavelength of 552nm and 578nm, respectively (Supplemental Figure 8). PEG@PDA-SNPs and P(B5AMA)@PDA-SNPs showed TAMRA encapsulation efficacies of 66-72%, (~45 µg of dye/300 µg of nanoparticles) and PDA-NPs used as a control showed comparable dye encapsulation efficacies (70%) (Figure 5).

The release profile of TAMRA loaded nanoparticles was then studies at various pH (5.5 and 7.4) and as a function of time at 37 °C, as shown in figure 5. PEG and P(B5AMA)-coated core-shell nanoparticles showed sustained-release of TAMRA dye over a period of 5 days at all studied pH. Interestingly, the release of TAMRA dye at physiological pH (7.4) was ~20% after 24 hours, while only ~5% release of the dye was observed at pH 5.5 for all nanoparticles studied.
Similarly, release profile of the dye from nanoparticles was ~40% at pH 7.4, while only ~20% of dye was released at pH 5.5, after 5 days of incubation (Figure 5).

**Cellular Uptake and Toxicity Studies**

Cellular uptake of TAMRA loaded core-shell nanoparticles was then studied in TNBC cell lines (Supplemental Figure 9). As shown from the fluorescent microscope images, P(B5AMA) and PEG coated core-shell nanoparticles are well-up taken and tend to localize in the cytoplasm of triple negative breast cancer cells, indicating their potential usage as a drug delivery carrier.

The toxicity of bare core-shell nanoparticles was then studied in primary and metastatic triple negative breast cancer cell lines. The stealth polymers functionalized core-shell nanoparticles showed essentially nontoxic profile at 50 and 100 µg/mL and cell viabilities of > 60% were calculated at high concentrations (200 µg/mL) of nanoparticles in both MDA-MB231 and MDA-MB 231-BR cell lines. Irradiation of bare nanoparticles treated cells with 808 nm laser did not show significant hyperthermia induced cell death at all studied concentrations (50-200 µg/mL) in both primary and metastatic TNBCs (Figure 6, Supplemental Figures 10 & 11), possibly due to the low power density of the laser used for the study. (Liu et al. 2022)

Paclitaxel (PTX) loaded core shell nanoparticles were then synthesized, using previously established procedure optimized for TAMRA encapsulation and were tested for their cytotoxicity in MDA-MB231 and in metastatic brain cell line (MDA-MB231-BR), in the presence and absence of laser irradiation. In this study, PTX treated TNBCs showed ~50% cell viability at 100 nM concentration (Supplemental Figure 12). The toxicity of core-shell nanoparticles in MDA-MB231 cells at 200 µg/mL nanoparticle concentrations were reduced from 80 to 45% and from 60 to 22% for PEG@PDA-NPs and P(B5AMA)@PDA-NPs, respectively (Figure 6B). Interestingly, MDA-MB231-BR cells showed relatively higher susceptibility to the nanoparticles.
treatment at all studied concentrations (100 and 200 µg/mL) and cell viabilities were 18% and 2% for PTX loaded PEG@PDA-NPs and p(B5AMA)@PDA-NPs, respectively (Figure 6C). The irradiation of drug loaded nanoparticles treated cells with 808 nm laser source showed limited effect on cell viabilities and toxicity profile of nanoparticles was mainly dominated by the presence of chemotherapeutics.

Discussion

Starch is the most abundant polysaccharide that is non-toxic, biodegradable and has been extensively studied to develop drug delivery carriers. (Li et al. 2016, Ismail et al. 2017, Qi et al. 2017, El-Naggar et al. 2015 & Odeniyi et al. 2018) The water-soluble starch loaded drug nanoparticles are well-explored for the capability to encapsulate hydrophobic cargo molecules and for their improved antibacterial properties. (Li et al. 2016) Polydopamine coated starch nanoparticles were optimized as a function of concentration of starch and dopamine, pH of the reaction and reaction time and near monodisperse nanoparticles obtained at starch: dopamine w/w ratio of 0.25 pH 8.5 and after 2.5 hours of reaction time showed complete encapsulation of starch in nanoparticles core, as was analyzed by I2-KI assay and by FTIR analysis. Previous studies indicated that at high pH dopamine undergoes rapid oxidation into Dopamine Quinone (DQ) and leukodopaminechrome (LDC) that subsequently forms 5,6-dihydroxyindole (DHI), hence yielding smaller nanoparticles. (Ho et al. 2013) The faster conversion of dopamine into PDA nanoparticles during PDA-SNPs synthesis, however led to poor encapsulation of starch in core-shell nanoparticles, as was seen by the presence of white precipitates of starch in the reaction mixture, post-nanoparticles synthesis.

The nanoparticles synthesized were modified with stealth layer to improve the colloidal stability of the nanoparticles. PEG is the most studied stealth polymer that is well documented to improve physiological stability of nanocarriers. (Pelosi et al. 2021) In addition to PEG, hydrophilic
polymers such as poly(sulfobetaine methacrylate) p(SBMA), and poly(carboxybetaine methacrylate) p(CBMA) and poly(2-methacryloxyethyl phosphorylcholine) p(MPC) have received much attention as alternative polymeric coatings. (Schlenoff et al. 2014, Jensen et al. 2021 & Bekale et al. 2015) Recently, we have developed poly(B5AMA) of predetermine molecular weight by RAFT polymerization approach and have documented their antifouling and non-toxic behavior. (Combita et al. 2021 & Nazeer et al. 2021) Thiol and amine terminated PEG chains are well documented to undergo Michael addition/Schiff base reaction under alkaline conditions with the amino group of PDA. Poly(B5AMA) however, is hydroxyl groups rich telechelic polymer, and interact with PDA shell via hydrogen bonding and hydrophobic interactions. (El Yakhlifi et al. 2020) P(B5AMA)@PDA-SNPs and PEG@PDA-SNPs showed superior physiological stability in the presence of serum proteins and net negative surface charge and the results obtained were similar to a recent study, where PHBV embedded core-shell PDA nanoparticles showed size of ~ 250 nm with net negative surface potential of -20 mV. (Wu et al. 2021) The reduced size of nanoparticles in the presence of serum proteins, in comparison with deionized water has been reported earlier and was attributed to the compact corona of nanoparticles in the presence of serum proteins. (Ahmed et al. 2011)

Evaluation of UV-vis NIR spectra in NIR region of polydopamine nanoparticles as a function of concentration is well documented to demonstrate photothermal properties. (Tian et al. 2019) PDA coated core-shell starch nanoparticles showed absorption spectra similar to PDA nanoparticles, however, lower absorption capacity of P(B5AMA)@PDA-SNPs, when compared to other nanoparticles may be related to their smaller sizes (analyzed by both DLS and TEM), possibly due to reduced thickness of PDA core in the presence of poly(B5AMA) stealth layer. The reduction in absorption potential poly(methacrylate) functionalized PDA nanoparticles, in
comparison with bare PDA nanoparticles was reported by others and was attributed to the reduced thickness of PDA shell in the case of polymer coated nanoparticles. (Tian et al. 2019)

The core-shell nanoparticles prepared in this study showed absorbance in the NIR region (800nm-1000nm) indicating potential photothermal properties of nanoparticles when irradiated with a near infrared, 808nm, laser.

Photothermal therapy (PTT) is a minimally-invasive treatment in which NIR laser light is absorbed by a photothermal agent converting optical energy into thermal energy, which results in tissue destruction. (Tian et al. 2019) The laser power, beam dimension(s) and irradiation time are the key irradiation parameters for determining the resultant heating of the photothermal agents such as polydopamine nanoparticles. (Liu et al. 2021, Zhang et al. 2015 & Liu et al. 2022)

The nanoparticles irradiated with 1.4 W/cm² laser showed similar increase in temperature, indicating that complex nanoparticles morphology in the case of stealth layer coated core-shell nanoparticles did not compromise photothermal conversion capabilities of PDA-NPs. Liu et al. and others have studied laser power density dependent change in temperature, in the presence of PDA-NPs and showed that laser power density of 0.125 W/cm² to 1 W/cm² resulted in temperature change from 0°C to 25°C indicating the power density dependent increase in temperature. (Liu et al. 2021, Zhang et al. 2015 & Liu et al. 2022)

The encapsulation and release of cargo molecules from dopamine nanoparticles was studied and all the nanoparticles synthesized showed similar encapsulation and release efficacies of TAMRA dye. The encapsulation of drugs in nanoparticles is variable (in the range of 25-85%) and is dependent on the morphology of the nanoparticles and on the type of drug molecule studied. (Zhu et al. 2017, Li et al. 2021 & Liu et al. 2022) TAMRA is a zwitterionic highly stable dye that was chosen for its excellent photostability and quantum yield that is essentially unaffected at acidic pH. (Christie et
al. 2009 & Herce et al. 2014) PDA-NPs, when compared with PEG@PDA-SNPs and P(B5AMA)@PDA-SNPs showed no significant difference in the release of cargo at all studied pH, indicating that polymeric stealth layer does not interfere with the release of encapsulated molecules from the core of nanoparticles. The pH-dependent drug release efficacies of nanoparticles, observed in this study are consistent with others, where slow release of cargo from dopamine nanoparticles was attributed to the zwitterionic property of PDA that increase the binding of anionic cargo molecules with the nanoparticles surface at acidic pH. (Poinard et al. 2018 & Pan et al. 2020) pKa of TAMRA is < 5, as carboxyl group of molecules are fully protonated under acidic pH that mediate stronger interactions with anionic PDA-NPs, hence demonstrating reduced drug release at acidic pH. (Christie et al. 2009 & Herce et al. 2014) A handful of studies demonstrated pH-dependent disintegration and release of cargo drugs from PDA-NPs. (Li et al. 2021, Liu et al. 2022, Tian et al. 2019, & Wu et al. 2019) The acidic pH was shown to perturb Π-Π interactions between the drug and PDA moieties, showing faster drug release at low pH. In order to evaluate the degradation of PDA core-shell nanoparticles and release of starch under acidic pH, Iodine-KI assay was performed 48 hours post-incubation of nanoparticles at different pH. The analysis of nanoparticles as a function of pH and time demonstrated less then 5% release of starch from PEG@PDA-SNPs and poly(B5AMA)@PDA-SNPs over 48 hour time period at pH of 5.5 and 7.4, indicating the stability of core-shell nanoparticles at different pH. (Pan et al. 2020 & Zhao et al. 2019)

PTX is a natural broad spectrum antineoplastic drug that is capable of mediating tubulin polymerization resulting in abnormal arrangement of cell bundles, causing cell death and is extensively used in clinics for TNBC treatment. (Wu et al. 2021) PTX is documented for its inhibitory concentration (IC\textsubscript{50} values) in TNBCs at concentrations ranging from 1.5-500 nM in
vitro. (Kenicer et al. 2014) PTX loaded core-shell nanoparticles showed concentration dependent cytotoxicity and significant decrease in cell viabilities was observed compared to bare nanoparticles at all studied concentrations and for both cell lines.

Conclusions and Future Directions

PDA coated starch nanoparticles synthesized in one-pot, template free approach and coated with poly(B5AMA) and PEG stealth layers yielded near monodisperse nanoparticles of 300-400 nm. The core-shell nanoparticles showed excellent absorption capacities in NIR region and exhibited photothermal properties, upon NIR laser irradiation. The photothermal properties of core-shell nanoparticles were comparable to PDA-NPs, indicating that presence of starch core and polymeric shell do not interfere with their photothermal properties. P(B5AMA)@PDA-SNPs and PEG@PDA-SNPs also exhibited excellent colloidal stability under physiological conditions, however PDA-SNPs prepared in the absence of stealth coating showed rapid aggregation in the presence of serum proteins. The nanoparticles demonstrated cellular uptake in TNBC cell lines and exhibited concentration dependent toxic effects in MDA-MB231 and MDA-MB231-BR cells. Although, limited effect of photothermal properties of nanoparticles on cell viabilities was observed, possibly due to low power of laser used for the study, the nanoparticles exhibited excellent drug loading capacity and concentration dependent toxicity in TNBC cell lines. Our study demonstrates that PDA coated starch nanoparticles has the potential to develop as delivery carrier to improve TNBC therapy.

Acknowledgements: The authors would like to thank Canadian Cancer Society Emerging Scholar Award to MA and Canadian Cancer Society’s JD Irving, Limited –Excellence in Cancer Research Fund to MS for the funding of this project.
Data Availability Statement: The authors declare that all the data supporting the findings of this study are contained within the paper" to your manuscript.

Authorship Contributions: Conducted experiments: Miranda Steeves, Diego Combita William Whelan; Contributed new reagents or analytic tools: Marya Ahmed, William Whelan; Performed data analysis: Marya Ahmed, William Whelan, Diego Combita, Miranda Steeves; Wrote or contributed to the writing of the manuscript: Marya Ahmed, Diego Combita, William Whelan, Miranda Steeves

References


**Footnotes**

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No author has an actual or perceived conflict of interest with the contents of this article.

**Legends for Tables and Figures**

**Scheme 1:** Schematics depicting the synthesis of drug loaded biodegradable, core-shell nanoparticles comprised of a starch core and PDA shell, in one pot approach.

**Figure 1:** FTIR spectra of PDA-NPs and PDA-SNPs.

**Figure 2.** TEM images and histogram analysis of core-shell nanoparticles. A) and C) PEG@PDA-SNPs (n=147) and B) and D) P(B5AMA)@PDA-SNP (n=207). Scale bar is 500nm.
Figure 3. UV-vis-NIR Spectra of nanoparticles in deionized water A) PDA-NPs, B) PDA-SNPs, C) PEG@PDA-SNPs and D) P(B5AMA)@PDA-SNP.

Figure 4. A) Demonstration of laser set up used for the photothermal experiments; the laser positioned above a sample well, and 808 nm laser beam illuminating an infrared sensor card. B) measurement of temperature change in response to 808 nm laser irradiation, in the presence of PEG@PDA-SNPs, P(B5AMA)@PDA-SNPs, PDA-NPs and water (as a control), as was measured by thermal camera. The experiments on temperature change study were repeated three times and mean is presented here.

Figure 5. Drug release profile of PEG@PDA-SNP and P(B5AMA)@PDA-SNP at pH 5.5 and 7.4 over a period of 5 days. The experiments were performed in triplicates.

Figure 6. Cytotoxicity of A) empty nanoparticles in MDA-MB231 cells, B) PTX loaded nanoparticles in MDA-MB231 cells, C) PTX loaded nanoparticles in MDA-MB231-BR cells, D) PTX loaded and empty nanoparticles at 200 µg/mL in MDA-MB231 cells, and E) drug loaded and empty nanoparticles at 200 µg/mL in MDA-MB231-BR cells, as determined by MTS assay. The brackets indicate different groups of treatments compared for their cytotoxicity and *P<0.05, Two Tailed Welch Correction T-test.
**Table 1:** Characterization of size and zeta potential of nanoparticles in deionized water and phosphate buffer saline in the presence of serum proteins.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size (nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H₂O</td>
<td>PBS + serum</td>
</tr>
<tr>
<td>PDA-SNPs</td>
<td>465 ± 53</td>
<td>330 ± 53</td>
</tr>
<tr>
<td>PEG@PDA-SNPs</td>
<td>410 ± 39</td>
<td>290 ± 53</td>
</tr>
<tr>
<td>P(B5AMA) @PDA-SNPs</td>
<td>250 ± 11</td>
<td>240 ± 41</td>
</tr>
</tbody>
</table>
Figure 2

A) and B) Images showing the morphology of nanoparticles.

C) and D) Histograms illustrating the size distribution of nanoparticles, with categories ranging from 30-59 nm to 100+ nm.
Figure 3
Figure 4

A) 

B) 

- Water
- PEG@PDA-SNPs
- P(B5AMA)@PDA-SNPs
- PDA-NPs

Temperature (°C)

0 3 6 9 12 15 18

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**Figure 5**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Encapsulation Efficacies of TAMRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDA-SNPs</td>
<td>70 ± 0.9</td>
</tr>
<tr>
<td>PEG@PDA-SNPs</td>
<td>66 ± 0.8</td>
</tr>
<tr>
<td>P(B5AMA)@PDA-SNPs</td>
<td>72 ± 0.5</td>
</tr>
</tbody>
</table>

**A)**

**B)**

![Graph showing cumulative release over time](image)
Figure 6

A) 

![Graph A]

B) 

![Graph B]

C) 

![Graph C]

D) 

![Graph D]

E) 

![Graph E]
Scheme 1

Starch Core  Polydopamine Shell

Paclitaxel

Stealth Coating
PEG or P(B5AMA)

pH 8.5, r.t, 2 hr.

Drug loaded Core-Shell Nanoparticle
Supplemental Materials

Chemotherapeutics Loaded Poly(Dopamine) Core-Shell Nanoparticles for Breast Cancer Treatment

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Supplemental Figure S1. GPC analysis of poly(B5AMA).

**GPC Analysis:**

Gel Permeation Chromatography (GPC): The molecular weights and molecular weight distributions of the polymers were obtained using an Agilent Technologies GPC 1260 Infinity system equipped with a refractive index (IR) detector and two PolarGel-M columns (8µm, 7.5 x 300mm). A LiBr solution in DMF (0.5% w/v) was used as the mobile phase with a flow rate of 0.5 mL/min and a temperature of 55 °C. Calibration of the method was performed using a set of poly(2-hydroxyethyl methacrylate) (poly(HEMA)) standards with molecular weights ranging from 2.11 kDa to 88.8 kDa (Scientific Polymer Products).
Supplemental Figure S2. Synthesis of PDA-SNPs, as was seen by the color change of reaction from transparent to dark brown.

Supplemental Table S1. Optimization of w/w ratio of dopamine/starch required for the synthesis of monodisperse nanoparticles. The concentrations indicated below are final concentrations of reactants in the reaction mixture.

<table>
<thead>
<tr>
<th>Starch (mg/mL)</th>
<th>Dopamine (mg/mL)</th>
<th>Starch/Dopamine w/w ratio</th>
<th>Size (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>0</td>
<td>330</td>
<td>0.38</td>
</tr>
<tr>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>470</td>
<td>0.19</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>0.25</td>
<td>465</td>
<td>0.18</td>
</tr>
<tr>
<td>0.1</td>
<td>1</td>
<td>0.1</td>
<td>670</td>
<td>0.16</td>
</tr>
<tr>
<td>0.05</td>
<td>1</td>
<td>0.05</td>
<td>570</td>
<td>0.19</td>
</tr>
</tbody>
</table>
Supplemental Figure S3. Standard curve of starch content in water with use of Iodine-KI at absorbance of 600nm.
**Supplemental Figure S4.** Standard curve of starch content prepared in washes of PDA nanoparticles to evaluate the interference of nanoparticles in the Iodine-KI assay. The absorbance was read at 600nm.

**Supplemental Table S2.** Effects of reaction pH on nanoparticle synthesis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sizes and Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH = 8.5</td>
<td>465 nm</td>
</tr>
<tr>
<td>pH = 10.5</td>
<td>Visible aggregation</td>
</tr>
<tr>
<td></td>
<td>1000-2000nm</td>
</tr>
<tr>
<td>pH = 13</td>
<td>white precipitates in brown solution indicating that starch is not encapsulating in dopamine core</td>
</tr>
</tbody>
</table>
**Supplemental Figure S5.** FTIR spectra of starch.

**Supplemental Figure S6.** DLS analysis of size and polydispersity of PEG@PDA-SNPs, P(B5AMA)@PDA-SNPs and PDA-NPs nanoparticles studied at different time points.
Supplemental Figure S7: Images from thermal camera demonstrating final temperature of nanoparticles, 10-minutes post-irradiation with 808 nm laser. The starting temperature of the nanoparticles solution, measured by thermal camera was 26 °C.
Supplemental Figure S8. Standard curve of TAMRA prepared in 50% water:50% TRIS buffer prepared to mimic the washes/supernatant of nanoparticles synthesized in the presence of TAMRA dye, using microplate reader. The samples were excited at 552 nm and emission wavelength was 578 nm.
**Supplemental Figure S9**: Cell viabilities of MDA-MB231 upon 24 hour post-treatment with A) different concentrations of PEG@PDA-SNPs and B) 100 ug/mL of with p(B5AMA)@PDA-SNPs, as was determined by MTS assay.
**Supplemental Figure S10:** Cell viabilities of MDA-MB231-BR upon 24 hour post-treatment with PEG@PDA-SNPs as was determined by MTS assay.

![Graph showing cell viabilities](image)

**Supplemental Figure S11:** Cell viabilities of MDA-MB231 upon 24 hour post-treatment with different concentrations PTX, as was determined by MTS assay.