Evaluation of new bi-functional terpolymeric nanoparticles for simultaneous in vivo optical imaging and chemotherapy of breast cancer

Alireza Shalviri · Ping Cai · Andrew M. Rauth · Jeffery T. Henderson · Xiao Yu Wu

Abstract Successful development of a nanoparticulate system for cancer chemotherapy requires detailed knowledge of its biodistribution, clearance and anti-tumour efficacy in vivo. Herein we developed new bi-functional nanoparticles for simultaneous in vivo optical imaging and delivery of the anticancer drug doxorubicin (Dox) for enhanced chemotherapy. Two types of nanoparticles were synthesized, namely preformed nanoparticles (PF-NPs) and self-assembled nanoparticles (SA-NPs). The PF-NPs were prepared by cross-linking graft polymerization of methacrylic acid and polysorbate 80 with starch (PMAA-PS 80-g-St) and then loading the particles with Dox. The SA-NPs were formed upon addition of Dox to non-cross-linked PMAA-PS 80-g-St. A near infrared fluorescent probe was conjugated with the PMAA unit of the nanoparticles. The biodistribution, tumour targeting and pharmacokinetics of the Dox-loaded nanoparticles in mice were determined by in vivo/ex vivo fluorescence imaging and ex vivo fluorescence microscopy. The anti-tumour efficacy of the nanoparticles was investigated using a murine orthotopic breast cancer model. PF-NPs had an average hydrodynamic diameter and zeta potential of 137±3 nm and −38±1 mV, respectively. These values were measured at 62±5 nm and −35±5 mV for SA-NPs. PF-NPs exhibited a porous morphology while the SA-NPs appeared to have a denser structure. SA-NPs outperformed the PF-NPs in terms of blood circulation, tumour uptake and penetration. PF-NPs and SA-NPs exhibited no systemic toxicity and inhibited tumour growth significantly better than the free Dox solution with SA-NPs being the best, attributable to their excellent tumour uptake and penetration. This work demonstrates the usefulness of these bi-functional nanoparticles as nanotheranostics.

Keywords Bi-functional nanoparticles · In vivo fluorescence imaging · Doxorubicin · Biodistribution · Theranostics

Introduction

Cancer is a multi-factorial disease affecting a diverse array of cellular systems. Classical chemotherapy is frequently constrained by several factors, including but not limited to dose-limiting systemic toxicity and sub-therapeutic drug concentrations within the tumour [1]. Most conventional anticancer agents suffer from less than optimal pharmacokinetics and biodistribution with respect to tumour versus host tissues. Due to their low molecular weight, a majority of the chemotherapeutic agents are rapidly cleared from the circulation and do not accumulate in tumour tissue. As a result of their large volume of distribution, drugs build up and cause toxicity in various healthy tissues. This non-specificity in biodistribution generally leads to severe side effects, lowering the maximum tolerable dose and reducing therapeutic efficacy [1, 2].

Nanotechnology offers advantages over standard chemotherapeutic approaches. A well-designed nanoparticulate system is able to deliver the drug to the tumour and control the rate of drug release. Owing to enhanced permeation and retention (EPR) effect, nanoparticles of suitable size and...
appropriate surface properties can passively target tumours
[3, 4]. Nanoparticle formulations are also capable of protecting
their drug cargo from harsh physiologic environments and enzyme-mediated metabolic effects, thus enhancing
drug efficacy [5].

In addition to therapeutic applications, interest innano-
particles as diagnostic agents has increased. Solid lipid–
polymer hybrid nanoparticles, as well as antibodies, li-
posomes, micelles, polymers, containing imaging reagents
such as radionuclides, fluorescent probes, and MRI contrast
agents, are being designed and used to visualize tumours
and to monitor the drug delivery processes in real time
[6–10]. Owing to their huge surface to volume ratios,
well-designed nanoparticulate systems containing multi-
functional groups can simultaneously accommodate a vari-
ety of diagnostic and therapeutic agents via chemical
conjugation or physical encapsulation. This has led to cre-
tion of therapeutic–diagnostic or “theranostic” nanopa-
ticles, a platform useful for both diagnostic and therapeutic
functions [2, 11, 12]. Nanotheranostics can be used to non-
invasively assess the biodistribution and accumulation of
therapeutic agents, to monitor and quantify drug release
and to facilitate therapeutic interventions via triggered drug
release. It can also predict therapeutic response and longitu-
dinally monitor the efficacy of therapeutic intervention [7,
11, 13, 14]. Specific examples of each of the above appli-
cations have been extensively reviewed by others [2, 12].

In vivo fluorescence imaging offers several advantages
over other imaging modalities applied to oncology, in par-
ticular in preclinical settings. For instance, MRI suffers
from a low inherent sensitivity while in the case of positron
emission tomography, potentially genotoxic radioisotopes
are employed [15]. In contrast, fluorescence imaging meth-
ods exhibit high sensitivity, do not induce genotoxic damage
and can be rapidly imaged using relatively inexpensive
equipment. However, biological tissues absorb and scatter
emitted fluorescent photons, and depending upon the detec-
tion wavelengths utilized, individual tissues can generate
significant auto-fluorescence signatures in the ultraviolet
and visible spectrum. Each of these factors imposes partic-
ular limits on signal collection and quantification. Hence,
image acquisition is typically acquired at depths limited to a
few millimetres beneath the skin surface [16, 17]. Use of
near infrared (NIR) fluorophores, which emit in the range of
650–900 nm, substantially reduces the extent of light scat-
tering, absorbance and auto-fluorescence within tissues, en-
hancing the utility of fluorescent probes and providing an
opportunity for optical investigations at a greater depth in
vivo with practical penetration depths of 1–2 cm [17, 18].
For this reason, NIR-tuned semiconductor quantum dots and
NIR organic dyes have been developed and extensively
studied for in vivo tumour imaging [17–21]. However,
soluble NIR organic dyes suffer from their intrinsically fast
clearance and poor tumour accumulation. Hence, their con-
jugation to macromolecular and nanoparticulate systems has
been proposed [17].

Previously, we developed terpolymeric polymer and nanopa-
ticles based on starch, polymethacrylic acid (PMAA), and polysorbate 80 (PS 80) using a modified aqueous dispersion polymerization method which enabled simultaneous grafting and nanoparticles formation in one pot. The details of the synthesis, characterization and the effect of various processing parameters on the particle size have been published recently [22]. Starch, PS 80 and PMAA are approved pharmaceutical excipients. Starch is a food ingredient degradable by amylase in the body, and hence, this nanoparticle system is expected to possess good biocompatibility and biodegradability. Moreover, polysaccharide coatings have recently been considered as an alternative to PEG coatings for giving “stealth” properties to nanoparticles [23]. Certain polysaccharides such as heparin, dextran and hydroxyethyl starch have been shown to prolong the nanoparticle cir-
culation in blood and minimize their interaction with
blood proteins and phagocytotic cells [24–26].

The carboxylic groups in PMAA are expected to provide binding sites for cationic anticancer drugs such as Dox as suggested in previous studies using other carboxyl-containing and negatively charged polymers [27, 28]. Moreover, the carboxylic groups provide a reaction site for covalent conjugation of various chemical moieties such as fluorescent probes.

PS 80 is a polyethylene sorbitol ester with a molecular
weight of 1,310 Da. It is widely used as an emulsifier/sur-
factant/stabilizer in various pharmaceutical formulations
[29, 30]. It consists of a sorbitan ring with ethylene oxide
polymers attached at three different hydroxyl positions and
contains a mixture of fatty acid side chains which are at-
tached through an ester linkage to the ethylene oxide oxy-
gen. The major fatty acid component of the side chain is
oleic acid contributing to more than 60 % of the total
composition of the side chain [30]. We observed previously
that PS 80 was required to form stable poly(methacrylic
acid), polysorbate 80 and starch (PMAA-PS 80-g-St) nano-
particles by the aqueous dispersion polymerization due to
the fatty acid side chains which when grafted onto starch
imparts sufficient hydrophobicity leading to nanoparticles
formation by nano-precipitation [22].

Herein we have developed new bi-functional terpoly-
meric nanoparticles of PMAA-PS 80-g-St. The system ena-
bles simultaneous loading of doxorubicin and covalent
linkage of NIR fluorophores. Two types of nanoparticles
were prepared: (a) preformed nanoparticles (PF-NPs) con-
structed by cross-linking graft polymerization of PMAA-PS
80-g-St followed by conjugation of HiLyte Fluor™ 750 and
loading of Dox and (b) self-assembled nanoparticles (SA-
NPs) prepared by adding hydrophobic moieties (e.g., Dox and HiLyte Fluor™ 750) to soluble PMAA-PS 80-g-St polymer. We then evaluated the biodistribution, tumor accumulation and antitumor efficacy of these theranostic nanoparticles in a murine orthotopic model of breast cancer.

**Materials and methods**

**Chemicals**

Soluble corn starch (M_w=11,000 g/mol), methacrylic acid (MAA), N,N′-methylenebisacrylamide (MBA), sodium thiosulfate (STS), potassium persulfate (KPS), PS 80 and sodium dodecyl sulphate (SDS), fluoresceinamine isomer I (FA), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and all other chemicals unless otherwise mentioned were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). HiLyte™ Fluor 750 hydrazide was purchased from AnaSpec (Fremont, CA, USA). Doxorubicin hydrochloride (Dox) was purchased from Polymed Therapeutics (Houston, TX, USA). All cell culture plasticware was purchased from Sarstedt (Montreal, QC, Canada). Cell culture medium, α-modified minimal essential medium and phosphate-buffered saline (PBS) were obtained from the (Ontario Cancer Institute Media Laboratory, Toronto, ON, Canada). Foetal bovine serum (FBS) and trypsin were purchased from Invitrogen, Inc. (Burlington, ON, Canada).

Preparation of dual mode nanoparticles

The schematic structure of PF-NPs and SA-NPs and their preparation procedures are illustrated in Fig. 1 and prepared as indicated below.

Preparation of PF-NPs

The PMAA-PS 80-g-St nanoparticles were initially synthesized using a modified dispersion polymerization method developed previously in our laboratory [22]. Briefly, starch (1.5 g) was dissolved in distilled deionized water (DDIW) by heating to 95 °C for 30 min. The solution was cooled down to 65 °C and purged with N2 for 30 min to remove any dissolved oxygen. Subsequently, 0.25 g of SDS, 0.4 g of PS 80, 0.18 g of KPS and 0.12 g of STS were added to the starch solution while stirred at 65 °C. After 15 min, the reaction was started by adding 0.45 g of nitrogen purged MAA and 0.45 g of MBA. Opalescence appeared after 5 min, and the reaction was continued under a nitrogen blanket for 8 h at 65 °C to ensure complete grafting. The product was washed extensively with warm water and methanol followed by ultracentrifugation (96,000×g) to remove any unreacted materials and homo-polymers. The purified particles were then freeze-dried and stored in a desiccator for future use. The composition of the terpolymer was confirmed using 1H NMR (Supplementary materials).

Next, 100 mg of purified NPs was dispersed in 2 ml of DDIW, and 30 mg of EDC and 30 mg NHS were added. After 30 min, 0.2 ml of HiLyte Fluor™ 750 hydrazide (1.25 mg/ml in DDIW) was added while under stirring. The mixture was protected from light and stirred at room temperature for 24 h. Finally, the product was neutralized to pH 7.4 using 0.1 N NaOH and purified by extensive washing with water and centrifugation (96,000×g). The amount of dye conjugated to the nanoparticles was determined by measuring the absorbance of known concentration of the dye conjugated nanoparticles at λ_{abs}=754 nm. This value was baseline corrected by subtracting the absorbance of the dye-free nanoparticles of the same concentration at the same wavelength. Subsequently, the baseline corrected absorbance values were related to the dye concentration using the beer’s law:

\[
A = \varepsilon bc
\]

where \(A\) is the absorbance, \(b\) is the path length (1 cm) and \(\varepsilon\) is the extinction coefficient which was reported to be 275,000 M^{-1} cm^{-1} according to the manufacturer.

The nanoparticles were then loaded with Dox. Briefly, 50 mg of lyophilized nanoparticles was suspended in 10 ml of DDIW. Dox was added in concentration of 2.5 mg/ml to the suspension and incubated with the nanoparticle for 48 h. The particles were then ultra-centrifuged at 96,000×g for 30 min and washed trice with DDIW. The PF-NPs were then freeze-dried and store at 4 °C for future use.

Preparation of SA-NPs

The PMAA-PS 80-g-St soluble polymer was synthesized using the method described above with slight modifications. There was no cross-linker (MBA) and the amount of PS 80 was increased to 1 g.

Next, HiLyte Fluor™ 750 was covalently linked to the polymer using the method described above. Finally, to prepare the SA-NPs, 8 mg of the polymer was dissolved in 1.8 ml of sterile 5 % dextrose. The polymer solution was then placed in an ice bath, and while under ultrasonication, using a Hielscher UP100H probe ultrasonicator (Hielscher USA, Inc., Ringwood, NJ, USA), 170 μl (5×34 μl) of doxorubicin solution (12 mg/ml in 5 % dextrose) was added in small increments to the polymer solution every 30 s. The ultrasonication continued for another 10 min. Addition of the Dox resulted in spontaneous formation of nanoparticles. The SA-NPs were then passed through ion exchange resins, Sephadex G50 fine (GE Healthcare, Piscataway, NJ, USA) to remove unbound Dox.
The drug was extracted from the nanoparticles by dispersing 5 mg of the Dox-loaded nanoparticles (PF-NPs and SA-NPs) in 10 ml of 0.01 N HCl. The Dox content was assayed using an ultraviolet-visible (UV–vis) spectrometer at 495 nm. Subsequently, the drug loading content (LC%) and loading efficiency (LE%) were calculated using the following equations:

$$LC\% = \frac{Wt \text{ of drug in nanoparticles}}{Wt \text{ of nanoparticles} + Wt \text{ of drug}} \times 100\%$$ \hspace{1cm} (2)

$$LE\% = \frac{Wt \text{ of drug in nanoparticles}}{Initial \ Wt \text{ of drug in the loading solution}} \times 100\%$$ \hspace{1cm} (3)

Dynamic light scattering, electrophoretic mobility measurements and transmission electron microscopy measurements. All size and ζ-potential measurements were performed using Malvern ZetaSizer Nano ZS (Worcestershire, UK). Each measurement was performed in triplicate, and the average±standard deviations are reported. The particle size distribution was evaluated using polydispersity index (PdI) calculated by the DTS software (Malvern Instruments Ltd., Worcestershire, UK). Generally, particles with PdI values smaller than 0.12 are considered to have a narrow size distribution.

Transmission electron microscopy (TEM) was used to investigate the morphology of Dox-loaded nanoparticles. Nanoparticles suspended in DDIW were stained with ammonium molybdate and placed on carbon coated grid. The samples were blotted with filter paper and left to dry. Transmission electron micrographs were acquired on a Hitachi H7000 electron microscope (Mississauga, ON, Canada) with an accelerating voltage of 100 kV.

Drug release studies

Drug-loaded nanoparticle suspensions or solutions containing free drug (0.15 M Tris buffer, pH 7.4) were sealed in
dialysis tubing ($M_w$ cutoff of 25 kDa) and placed in 200 ml of 0.15 M Tris buffer solution at 37 °C with continuous magnetic stirring. The release medium was replaced with fresh buffer every 24 h. At selected time intervals, 200 μl of aqueous solution was withdrawn from the release media. The Dox content was assayed using an UV–vis spectrometer at 495 nm. Each experiment was repeated three times, and the mean ± standard deviations were reported.

Cell lines

The murine breast carcinoma cell line EMT6/WT was initially provided by Dr. Ian Tannock (Ontario Cancer Institute, Toronto, ON, Canada), and aliquots maintained as monolayers cultures in 75 cm² polystyrene tissue culture flasks at 37 °C in 5 % CO₂/95 % in a humidified incubator. Cells were maintained in α-minimal essential medium (Ontario Cancer Institute Media Laboratory, Toronto, ON, Canada), supplemented with 10% foetal bovine serum (Cansera Inc., Etobicoke, ON, Canada) defined as growth medium. Cells grown to confluence were trypsinized using 0.05% trypsin–EDTA (Invitrogen Inc., Burlington, ON, Canada) and reseeded where necessary at 1:10 dilutions in fresh growth medium. Periodically cell cultures were renewed from frozen stocks and checked for mycoplasma.

Animal model and in vivo treatment protocol in tumour-bearing mice

All animal handling and procedures were conducted under protocols approved by the Animal Care committee at the Ontario Cancer Institute following guidelines set forth by the Canadian Council on Animal Care. Eight- to ten-week-old Balb/c mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Orthotopic xenograft breast tumours were established by injecting $7\times10^5$ EMT6/WT murine breast cancer cells into the inguinal mammary fat pads of Balb/c mice using the sub-iliac node as an injection landmark. The use of the injection landmark ensured greater reproducibility of the tumours induced. Tumours were monitored for growth by measuring tumour dimensions using a digital calliper. The tumour volume ($V$) was calculated using the following equation [31]:

$$V = \frac{LW^2}{2}$$

where $W$ is the tumour measurement at the widest point and $L$ the tumour dimension at the longest point. The treatment started when tumours reached approximate volume of 60 mm³. The treatment groups consist of mice receiving intravenous injections via the tail vein of 5 % dextrose as a vehicle control (5 % dextrose, $n=2\times4$), 2×10 mg/kg dose equivalent SA-NPs (SA-NPs, $n=2\times3$), 2×10 mg/kg dose equivalent PF-NPs (PF-NPs, $n=2\times4$) or 2×10 mg/kg free doxorubicin (Free Dox, $n=2\times4$) with injections being administered 1 week apart. Body weight and tumour volume of all mice were measured three times weekly. Mice were euthanized upon either a 20% weight loss or when tumour volume reached 600 mm³. Cumulative survival curves were compared using Kaplan–Meier analysis followed by the log rank test. Statistics was calculated using GraphPad Prism 5. Tumour growth delay (TGD) was calculated from the median survival times of each group using the following equation:

$$TGD\% = \frac{(T_{\text{Treatment}} - T_{\text{control}})}{T_{\text{control}}} \times 100\%$$

where $T_{\text{control}}$ and $T_{\text{treatment}}$ refer to the median survival times for control and treatment groups. Immediately after euthanasia, intact hearts, livers, lungs and kidneys were fixed in 10% neutral-buffered formalin, paraffin-embedded and stained with haematoxylin and eosin (H&E) for morphological evaluation.

Real-time in vivo and ex vivo near-infrared fluorescence imaging

Real time in vivo fluorescence imaging

Tumour-bearing mice were injected with 200 μl of suspension of SA-NPs or PF-NPs containing HiLyte Fluor™ 750=1.8 nmol via the lateral tail veins and anaesthetized with isoflurane for imaging. Images were acquired using a Xenogen IVIS Spectrum whole animal imager (Caliper Life Sciences Inc., Hopkinton, MA, USA) using an excitation wavelength, $\lambda_{\text{ex}}$, of 745 nm and emission wavelength, $\lambda_{\text{em}}$, of 820 nm. Particle accumulation in and disappearance from the tumour and the whole body was quantified using the Living Image software supplied by the manufacturer. Regions of interest (ROI) were identified for the whole body and tumours and the fluorescence intensity (expressed as average radiant efficiency) calculated to construct plots of fluorescence intensity overtime. In the case of tumour, these plots were used to calculate the tumour associated nanoparticle accumulation and clearance using area under the curve (AUC), half-life ($t_{1/2}$) and elimination rate ($k_{el}$) via a one-compartment model where $t_{1/2}$ was the time it took to reach one half the maximal fluorescence intensity (FI) and $k_{el}=\text{ln}2/t_{1/2}$. AUC was calculated by the sum of trapezoids method (Eq. 6):

$$\sum_{x=1}^{n} \left[ (t_x - t_{x-1}) \left( \frac{FI_{x-1} + FI_x}{2} \right) \right]$$

where $t_x$ and $FI_x$ are the time and fluorescence intensity at each time point, respectively, and $t_{x-1}$ is time before $t_x$.

$$t_{1/2} = \frac{\ln 2}{k_{el}}$$

$$TGD\% = \frac{(T_{\text{Treatment}} - T_{\text{control}})}{T_{\text{control}}} \times 100\%$$

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$$t_{1/2} = \frac{\ln 2}{k_{el}}$$

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Ex vivo near infrared studies

Tumour-bearing mice were injected with SA-NPs or PF-NPs (HiLyte Fluor™ 750=1.8 nmol). At pre-determined time points, the injected mice were euthanized by CO₂ asphyxiation, and major organs (including blood) and tumours were dissected from the mice. NIR fluorescence images of dissected organs and tumours were obtained using the Xenogen IVIS Spectrum imager (Caliper Life Sciences, Inc., Hopkinton, MA, USA). Organ fluorescence intensities were then quantified by drawing ROI using the analysis software package (Living Image 4.0) supplied by the manufacturer. The tissue distribution of the nanoparticles was then evaluated by measuring the ratio of NIR fluorescence intensity of particle-injected tissue to untreated tissue per gram of tissue. To measure relative nanoparticle concentration in the blood, 1 ml of blood was drawn from mice subjected to heart puncture at a pre-determined time-point, and fluorescence intensities were measured at 820 nm. Quantitative results were presented as the fluorescence intensity ratio of particle-injected blood to untreated blood per 1 ml of blood. All values are expressed as means±standard deviation of six animals (n=2×3).

Results

Properties of the nanoparticles

Two different types of nanoparticles (PF-NPs, SA-NPs) based on the terpolymer of PMAA, Starch and PS 80 were prepared. ¹H NMR was used to confirm the terpolymer composition (Supplementary materials). Based on the area under the ¹H NMR peaks, the molar ratios of MAA/PS 80/St were calculated to be 1:0.01:0.37 and 1:0.02:0.34 for PF-NPs and SA-NPs, respectively. The carboxylic acid contents were measured to be 6 and 5.4 mmol/g for PF-NPs and SA-NPs, respectively, using a potentiometric titration method described previously [22]. The weight averaged molecular weight of the soluble polymer was measured to be 47,000 g/mol using a static light scattering method described elsewhere [32].

As illustrated in the schematic in Fig. 1, in the case of PF-NPs, the NIR fluorescent probe was covalently bound and the drug was loaded into the preformed cross-linked PMAA-Ps 80-g-St nanoparticles. The SA-NPs were spontaneously formed in aqueous medium with covalent linkage of the dye followed by addition of the Dox to the soluble PMAA-PS 80-g-St polymer. The linkage of HiLyte Fluor™ 750 and ionic complexation of the doxorubicin to the carboxylic acid groups of the PMAA-PS 80-g-St polymer are believed to increase the overall hydrophobicity, resulting in the formation of dense nano-structures which is stabilized by the presence of PS 80 and ionic repulsive forces on the particle surface due to presence of negatively charged carboxylic acid groups.

SA-NPs and PF-NPs exhibit particle sizes of 62±5 nm (PdI=0.12) and 137±3 nm (PdI=0.07), respectively (Fig. 2; Table 1). TEM photographs illustrate that the particle size are in good agreement with the DLS data and suggest different morphology between SA-NPs and PF-NPs (Fig. 2). PF-NPs are nearly spherical with a porous cotton ball structure while the SA-NPs are less spherical and exhibit a more compact overall morphology. The surface charge of the nanoparticles was found to be negative, with ζ-potential values of −38±1 (SA-NPs) and −35±5 (PF-NPs)
No statistically significant difference in zeta potential values was found between the dye conjugated and dye-free nanoparticles (data not shown).

High drug loading and sustained drug release were achieved. As shown in Table 1, a high loading efficiency of Dox was achieved with 88.2 % for SA-NPs and 99.9 % for PF-NPs. The drug loading contents were 21.2±0.3 and 49.7±0.3 % for SA-NPs and PF-NPs, respectively.

Figure 3 shows the sustained release profiles of SA-NPs and PF-NPs using free Dox as a reference considering the barrier effect of dialysis tubing membrane. The difference in release rate between free Dox and the nanoparticles is attributable to the prolonged release of Dox from the nanoparticles. Drug release from the PF-NPs was much slower compared to the SA-NPs with only 6.5 % of the Dox being released from PF-NPs after 24 h compared to 27 % of Dox released from the SA-NPs. The slower drug release observed in PF-NPs is likely due to larger particle size and highly cross-linked nature of these structures.

Distribution and tumour accumulation of the PF-NPs and SA-NPs in whole animals in vivo

Non-invasive real-time fluorescence imaging was utilized to track the biodistribution and tumour accumulation of nanoparticles in Balb/c mice bearing orthotopic murine EMT6 breast tumours. Owing to the near NIR emission of HiLyte Fluor™ 750 ($\lambda_{ex}=754$ nm, $\lambda_{em}=778$ nm) and the high

### Table 1 Summary of physicochemical properties of SA-NPs and PF-NPs

<table>
<thead>
<tr>
<th>Formulation</th>
<th>LC%</th>
<th>LE%</th>
<th>HiLyte Fluor 750 (μmol/g)</th>
<th>Size (nm)</th>
<th>Polydispersity index</th>
<th>Zeta (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA-NPs</td>
<td>21.1±0.3</td>
<td>88.2±1.2</td>
<td>4.3±0.01</td>
<td>62±5</td>
<td>0.12±0.05</td>
<td>−38±1</td>
</tr>
<tr>
<td>PF-NPs</td>
<td>49.7±0.3</td>
<td>99.9±0.1</td>
<td>3.9±0.02</td>
<td>137±3</td>
<td>0.07±0.02</td>
<td>−35±5</td>
</tr>
</tbody>
</table>

Particle diameter refers to the number-weighted diameter of readings averaged over 5 min. Loading efficiency (LE%) is the fraction of originally added drug that was incorporated into the NPs, whereas drug loading content (LC%) is the percent of drug weight to total weight of the nanoparticles. All values are described as the mean±standard deviation of three independent trials.
fluorescence intensity of these nanoparticles, it is possible to set detection limits such that background levels of autofluorescence can be reliably excluded (Fig. 4a).

Figure 4a presents whole body images of mice at time zero (baseline) and following injection of each formulation into the lateral tail vein at various times. At 1 h post-injection, a clear fluorescence signal is detectable throughout the body, due to distribution of nanoparticles within the blood, skin, organs and subcutaneous fat. As depicted in Fig. 4a, SA-NPs and PF-NPs exhibited distinct biodistribution profiles. One hour following injection of SA-NPs, strong fluorescence signal was observed in the urinary bladder (Fig. 4a, upper panels), indicating excretion by the renal route. The bladder signal reduced significantly 6 h post-injection; however, strong fluorescence could still be detected throughout the body suggesting both a fast and slow component to the elimination of the SA-NPs. Importantly, mice receiving SA-NPs exhibited a strong fluorescence signal within tumour tissue, while other perfused tissues such as the liver showed substantially lower levels of nanoparticle accumulation. Consistent with this, inoculated tumours could be delineated from the surrounding histologically normal tissue, suggesting a relatively great accumulation of SA-NPs within tumours. Importantly, such fluorescence signatures persisted beyond 1 week.

A different pattern of biodistribution was observed for PF-NPs. Substantial accumulation was noted in liver and spleen at 1 h post-injection, as revealed by the strong fluorescence observed in these organs. Moreover, these particles were not excreted efficiently via the renal route as no significant fluorescence accumulation was detected during the first 6 h in the bladder. Although not studied systematically here, it is worth mentioning that higher levels of fluorescence in faecal matter were observed in those mice injected with the PF-NPs (personal observation), suggesting clearance of these particles through a hepatobiliary route largely. At 1 h post-injection, the tumour could be differentiated from the surrounding tissue, but the extent of PF-NP accumulation in the tumour appeared to be substantially lower than SA-NPs.

The time-dependent excretion profiles of SA-NPs and PF-NPs were further quantified using the Xenogen IVIS system and plotted in Fig. 4b. SA-NPs initially showed a rapid clearance phase, possibly due to clearance through urinary excretion, followed by a slower clearance at later time points. The average whole body NIR fluorescence intensity of SA-NPs at 15 min post-injection was \( 1.5 \pm 0.2 \times 10^8 \) (p/s/cm\(^2\)/sr)/\((\mu W/cm^2)\). It rapidly decreased to \( 0.87 \pm 0.05 \times 10^8 \) (p/s/cm\(^2\)/sr)/\((\mu W/cm^2)\) by 6 h. The body fluorescence was measured to be \( 0.63 \pm 0.09 \times 10^8 \) and \( 0.50 \pm 0.05 \times 10^8 \) (p/s/cm\(^2\)/sr)/\((\mu W/cm^2)\) for 24 and 72 h time points, respectively. These values returned to baseline 14 days (336 h) post-injection. However, the whole body fluorescence intensity of PF-NPs decreased at much slower rate in the whole body. The body fluorescence was measured at \( 1.28 \pm 0.26 \times 10^8 \) (p/s/cm\(^2\)/sr)/\((\mu W/cm^2)\) 15 min post-particles administration and slowly decreased to \( 0.63 \pm 0.09 \times 10^8 \) and \( 0.50 \pm 0.05 \times 10^8 \) (p/s/cm\(^2\)/sr)/\((\mu W/cm^2)\) at 24 and 72 h, respectively.

The results indicate that SA-NPs undergo a fast initial elimination by excretion through the renal route followed by a slower elimination phase where the particles are cleared from the body within a time span of 14 days. Due to larger size and their highly cross-linked nature, the PF-NPs are not cleared by the renal route and are eliminated at substantially slower rate possibly via the hepatobiliary transport mechanism.

Real-time pharmacokinetics of nanoparticles in tumour tissue

The pharmacokinetic parameters for the nanoparticles in tumour were extracted from the data in Fig. 4c and summarized in Table 2. These parameters pertaining to the accumulation, retention and elimination of nanoparticles in tumours are generally predictive of the therapeutic efficacy of nanoscale drug delivery systems. In general, the SA-NPs accumulated to a higher degree within tumours but cleared at a faster rate compared to that observed for PF-NPs. The peak fluorescence intensities in the tumour were measured to be \( 6.15 \pm 1.04 \times 10^8 \) (p/s/cm\(^2\)/sr)/\((\mu W/cm^2)\) for SA-NPs and \( 0.15 \pm 0.25 \times 10^8 \) (p/s/cm\(^2\)/sr)/\((\mu W/cm^2)\) for PF-NPs. The tumour AUC for SA-NPs was 3.5-fold larger than PF-NPs reflecting the more extensive accumulation of SA-NPs than PF-NPs in the tumour. The greater tumour accumulation of the SA-NPs is supported by their longer blood.
circulation and smaller size compared to the PF-NPs. However, PF-NPs exhibited a slower tumour clearance rate reflected by the large $t_{1/2}$ value of $277\pm33$ h and the smaller $k_{el}$ of $0.0025\pm0.0003$ h$^{-1}$, while the $t_{1/2}$ and $k_{el}$ for the SA-NPs were $92\pm12$ h and $0.0075\pm0.0006$ h$^{-1}$.

Blood circulation and organ distribution of the nanoparticles determined by ex vivo imaging

Tissue distribution and tumour accumulation were also evaluated from ex vivo NIR fluorescence analysis of collected blood and dissected tumours and organs, including liver, lung, kidney, spleen, skin, intestine and heart at various time points. The data are presented as the ratio of fluorescent intensity normalized to tissue average baseline values (Fig. 5). SA-NPs were detected at higher concentrations and for longer time in the blood compared to the PF-NPs. The fluorescence intensity for the regions of interest was recorded as average radiant efficiency. Data are presented as means±standard deviation ($n=2\times3$).

**Table 2** Tumour associated pharmacokinetic data derived from the tumour average fluorescence intensity versus time curve for SA-NPs and PF-NPs

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>SA-NPs</th>
<th>PF-NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>$92.5\pm11.9$</td>
<td>$277.2\pm33.3$</td>
</tr>
<tr>
<td>$k_{el}$ (h$^{-1}$)</td>
<td>$0.0075\pm0.0006$</td>
<td>$0.0025\pm0.0003$</td>
</tr>
<tr>
<td>AUC (p/sec/cm$^2$/sr)/(μw/cm$^2$)/h×10$^{10}$</td>
<td>$8.03\pm1.60$</td>
<td>$2.24\pm0.50$</td>
</tr>
</tbody>
</table>

The values represent the mean±standard deviation ($n=2\times3$)
and 230 min for PF-NPs and SA-NPs were calculated. The PF-NPs exhibited substantially higher levels of accumulation in liver, spleen, lungs, heart and intestine while significantly higher levels of SA-NPs could be detected in kidney and tumour. The high level of fluorescence in the intestines of mice treated with PF-NPs suggests that particles accumulating in the liver are excreted through faeces. This elimination mode is generally slower than the elimination by urine. For this reason, fluorescence from the liver remains very strong over an extended period of time. These results are in good agreement with the whole body live animal imaging.

Microscopic imaging of tumour tissue demonstrated extravasation of the nanoparticles in the tumour

Tumour distribution of SA-NPs and PF-NPs at the microscopic level was examined using fluorescence microscopy. Tissue sections of vehicle-only (5 % dextrose) infused tumours were imaged over FITC (excitation 460–490 nm, emission 500–535 nm) emission window to determine the relative level of auto-fluorescence. As shown in Fig. 6, low levels of auto-fluorescence were observed in some regions of vehicle-treated tissues. Closer examination of the nature of this spectral emission demonstrated a broad wavelength distribution, in contrast to the more sharply defined emission peak of FITC-labelled nanoparticles. Such auto-fluorescence arises in tissues containing lipofucin, collagen and extended pi orbital systems such as heme. Therefore in order to definitively distinguish auto- versus nanoparticle-mediated fluorescence within the FITC emission window, tissue sections were imaged over both FITC and TRITC (excitation 540–565 nm, emission 575–620 nm) emission windows. Merging of these results allowed clear delineation of auto-fluorescent (yellow green) versus nanoparticle (green) mediated fluorescence as shown in Fig. 6. As indicated in the figure, at 4 h post-administration, the SA-NPs accumulated to a greater extent in the mouse tumour tissue compared to that seen in PF-NPs and extravasated to a much greater extent from the tumour microvasculature into tumour-bearing tissues. In contrast, PF-NPs showed a more
limited distribution within bulk tumour parenchyma. PF-NPs were predominantly confined to larger elements of the tumour vascular and associated perivascular regions. 

Anti-tumour efficacy of the nanoparticles in a murine breast cancer tumour model

PF-NPs and SA-NPs were next evaluated for their anti-tumour activity with 5% dextrose and free Dox included as the control and the comparator groups. Each group received two treatments (2×10 mg/kg, Dox) or control (5% dextrose) 1 week apart. This dosing schedule was selected based on the maximum tolerable dose of free Dox as determined by a series of preliminarily studies. The tumour volume and the survival times of the mice were monitored and presented in Fig. 7. In general, the nanoparticle formulations reduced tumour growth more significantly than free Dox (Fig. 7a). However, the SA-NPs outperformed PF-NPs, which can be seen in the substantial increase in animal survival (Fig. 7b). The median survival time for mice treated with 5% dextrose was 28 days and that with free Dox slightly increased to 35 days. In contrast, the median survival time for PF-NPs and SA-NPs were 47 and 59.5 days, respectively. These survival values were translated into TGD of 25, 68 and 112% for free Dox, PF-NPs and SA-NPs, respectively. Of the SA-NPs and PF-NPs treated groups, 33.3 and 25% of the animals were tumour free, respectively, at 62 days post-tumour implantation, whereas only 12.5% of animals in the free Dox group were tumour free by that time. The overall trend of increased survival ship was statistically significant (Mantel–Cox, p<0.0033). However, pair-wise comparison of the free Dox with PF-NPs and SA-NPs revealed only a statistically significant difference between free Dox and SA-NPs (Gehan–Breslow–Wilcoxon, p=0.0434).

Preliminarily assessment of toxicity of the nanoparticles

Longitudinal recording of animal body weight was used as a general measure of animal viability, with losses of 20% of total initial body weight determined as a toxic limit requiring euthanasia. Figure 8 shows the profiles of the body weight for mice before and after receipt of treatment administered in Fig. 7. In no instance did mice die or lose 20% of their body weight before tumours reached the cutoff size of

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**Fig. 6** Microscopic distribution of nanoparticles within tumours. For each photomicrograph, levels of relative fluorescence intensity over both FITC (green) and TRITC (red) emission windows were obtained for each specimen and merged to form the final figure. Shown are the fluorescence profiles observed in tumours treated with vehicle only, FA-labelled SA-NPs and FA-labelled PF-NPs. Tumours arising from orthotopically implanted EMT6/WT cells were allowed to grow for 8 days prior to injection of the nanoparticles. Four hours post-injection, animals were sacrificed and the distribution of particles assessed within both core and peripheral regions. The white arrows indicate representative nanoparticle in the tumour tissue.
600 mm³. All Dox groups showed some evidence of body weight loss over the first 20 days after the start of treatment. In general, treatment with free Dox, SA-NPs and PF-NPs resulted in over 1 g (approximately 5 %) body weight loss in only a few mice after 30 days of the start of treatment. No changes in eating, drinking, grooming, exploratory behaviour, activity or other physical features were noted in any of the treatment groups, suggesting a lack of general toxicity due to nanoparticles at the doses administered.

As a preliminarily evaluation of possible toxicity of Dox-loaded nanoparticles, a histological analysis was carried out of the major organs (liver, heart, kidney and lung). As seen in Fig. 9, no disturbances of tissue morphology were observed in the liver, lung or kidneys of mice treated with the SA-NPs or PF-NPs compared to the 5 % dextrose group. Liver macrophages showed no evidence of proliferation. Additionally there were no histological changes in cardiac tissue in animals treated with SA-NPs; however, some cardiomyocytes showed evidence of vacuole formation in animals treated with PF-NPs and the free Dox compared to the 5 % dextrose and SA-NPs groups with the number and severity of these vacuoles being greater in the animals in the free Dox group. Measurements of blood enzymes such as lactate dehydrogenase, alanine transaminase and creatine kinase as well as more specialized methods (e.g. assessment of left ventricular function) are required to further evaluate the toxicity of these nanoparticles.

Discussion

Utilization of the nanoparticles in cancer chemotherapy is based on the premises that a well-designed nanoparticulate system can preferentially accumulate within the tumour via the EPR effect or other targeting mechanisms and hence results in higher therapeutic efficacy, while minimizing the toxicity in normal tissue. To successfully develop a nanoparticulate drug delivery system, complete knowledge of its
biodistribution, clearance and tumour accumulation is required. For this reason, multifunctional nanoparticles enabling simultaneous in vivo imaging and drug delivery are desirable. We have developed new nanoparticles, which were able to simultaneously load Dox and conjugate a near infrared fluorescence probe and demonstrated their usefulness for real time non-invasive monitoring of biodistribution, tumour accumulation and excretion, as well as enhanced anti-tumour efficacy. The diameters of these nanoparticles are below the pore size of the permeable vasculature found in many solid tumours, suggesting that such nanoparticles should be capable of accumulating within solid tumours by means of the EPR effect.

The nanoparticles were able to load Dox effectively. The high drug loading efficiency and contents can be ascribed to strong ionic interaction of Dox with the PMAA-PS 80-g-St polymer [22]. In addition, the Dox release from the nanoparticles was sustained which could be explained in terms of hydrophobic nature of the drug–polymer complex and Dox interaction with hydrophobic domains within the core of the nanoparticles slowing down the drug release from the nanoparticles. The slow release of Dox from the nanoparticle for a long period is beneficial for delivering the cytotoxic drug to tumour tissue and releasing it at its intended site of action.

When nanoparticles are systemically administered, a variety of serum proteins, termed “opsonins”, could bind to their surface, which are recognized by scavenger receptors on the macrophage cell surface and are removed from circulation [33]. The protein binding and subsequent recognition by the mononuclear phagocyte system depend on particle’s properties such as particle size, hydrophilicity and charge [34–37].

The net surface charge of the nanoparticles has a pronounced effect on their stability as well as the adsorption of different physiological lipoproteins in the systemic circulation and could play a critical role in the clearance of the nanoparticles in vivo [35]. In general, neutral and negatively charged particles are expected to exhibit reduced uptake by liver and spleen and hence longer blood circulation [35]. Both PF-NPs and SA-NPs possess negative charge, attributable to the presence of the carboxylic acid groups, which imparts colloidal stability.

Surface composition and particle size also play a significant role in the biofate of nanoparticles such as opsonisation, hepatic filtration, tissue extravasation and kidney excretion [36–38]. PEGylation of the nanoparticles is commonly used to reduce opsonisation and improve their blood circulation. Recently, polysaccharides have been proposed as an alternative to PEG for improving the nanoparticles stability in vivo [23–26]. The circulation half-life for PF-NPs and SA-NPs were 63 and 230 min, respectively, which were significantly higher compared to the circulation half-life of only 3–5 min for bare polymethacrylic acid nanoparticles (250–300 nm) reported previously [39]. As a control experiment, we also evaluated the blood circulation half-life of cross-linked PMAA-PS 80 nanoparticles (150 nm) without starch. The blood circulation half-life of these particles was approximately 10 min (see supplementary materials, Figure S2). It appears that the presence of PS 80 slightly improves the blood circulation of the nanoparticles [40]. It is
possible that the presence of PS 80 on the particle surface allows for adsorption of certain serum proteins such as Apo-E while limiting the adsorption of other complement proteins and hence changing the opsonisation profile of the nanoparticles. Adsorption of Apo-E by PS 80-coated nanoparticles has been reported by other research groups [41]. Nevertheless, the starch grafted nanoparticles (PF-NPs and SA-NPs) exhibited significantly longer circulation life time compared to the starch free particles. Hence, it is possible that hydrophilic starch component may reduce particle up-take by mononuclear phagocyte system. Additional studies are required to understand the role of grafted starch in minimizing the nanoparticles uptake by liver and spleen.

Particle size is also an important factor in determining the blood circulation time of nanoparticles in the body. Generally, larger particles tend to accumulate more into the liver as compared to the smaller ones [38], which may explain higher splenic and hepatic uptake of PF-NPs compared to the SA-NPs. The data on the binding of opsonins to the liposomal surface suggest that opsonisation is dependent on the particle size and that the enhanced uptake by the liver is governed by the extent of opsonisation [42]. Additionally, it has been suggested that smaller particles allow the selective adsorption of some serum proteins that are known as dysopsonins, i.e. plasma components which are believed to prevent opsonisation. In other words, it may be the selective adsorption and not the prevention of adsorption that alters the pharmacokinetics and fate of nanoparticles [42–44].

A strong fluorescent signal was detected in the bladder within the first 6 h post-administration of SA-NPs, suggesting renal clearance of the formulation. Since the fluorescence dye was covalently bound with the polymer by an amide bond which is considered to be stable under the physiological conditions, its cleavage is highly unlikely, particularly in relatively short time frame of less than 6 h. In addition, no leakage of the dye from the particles was detected by an in vitro dye leakage test in 0.9 % NaCl+10 % FBS. Thus, it is reasonable to speculate that a fraction of SA-NPs are being cleared via renal filtration. Although the average diameter of SA-NPs were measured to be ~60 nm, it
is quite possible that there was a sub-population of particles with size below 10 nm formed by SA-NPs dissociation (due to Dox release), which can be readily cleared by the renal route.

The antitumor efficacy of the Dox-loaded nanoparticles was evaluated in an orthotopic murine breast cancer model. The cell line chosen, while not human, provided animal model of aggressive breast cancer in mice, demonstrating rapid growth rates with short mean survival times in untreated animals. This tumour model has been extensively studied by different research groups [45, 46]. The cells were orthotopically implanted into mammary fat pad to induce a primary breast cancer tumour model. Host microenvironment is an important determinant of tumour gene expression and microvascular functions; hence, orthotopic breast tumour models are more useful for obtaining clinically relevant information than non-orthotopic tumour models [47].

In line with the biodistribution results, the SA-NPs exhibited superior tumour accumulation and antitumor efficacy compared to the PF-NPs. Possible mechanisms underlying the enhanced antitumor efficacy of the NPs over free Dox may include the prolonged retention of Dox-loaded nanoparticles in the tumour which continuously released the drug within tumour mass. It has been reported that the antitumor activity of chemotherapeutics depends on the dose and exposure time, and extended exposure of cancer cells to constantly released Dox appeared to benefit the therapeutic outcome [48].

Our data also showed inter-mouse variations in treatment response. Variation in therapeutic efficacy of Dox-based formulations has been reported by other groups as well [49, 50]. Like in humans, such variation in mice may be ascribed to various factors in the tumour morphology and microenvironment, as well as host immunity. Although the same number of cancer cells was injected to nearly same position in each animal to inoculate tumours, the resultant tumours could be very different. In our own laboratory, we have observed tumours consisting of one or more nodules in different shapes [51]. The tumour morphology and microenvironment could affect blood supply and drug distribution within tumour tissue, thereby leading to different therapeutic efficacies. Another explanation may be the difference in the extent of immunity among animals induced by Dox treatment [52–54]. Dox treatment was reported to enhance tumour antigen-specific proliferation of CD8 T cells, and the therapeutic efficacy of Dox treatment was found to depend on CD8 T cells and IFN-γ production which varies from subject to subject [52, 53]. Because of the heterogeneity of tumour morphology, microenvironment and host immune response to chemotherapy, it is difficult to find a universal approach to rendering full response in all studied animals. Future investigations that combine imaging tools and immune response analysis with therapeutic assessment are required to delineate the correlation between the above described factors and therapeutic efficacy. Perceivably an individually tailored treatment may be needed in order to achieve more homogeneous response among animals.

Conclusions

We have developed and evaluated a new bi-functional terpolymer nanoparticle system that can simultaneously carry a NIR fluorescence probe and an anticancer drug. The system enables non-invasive evaluation of the biodistribution of drug loaded nanoparticles in vivo using fluorescence imaging. The results of this work indicate that enhanced antitumour efficacy of the nanoparticles can be linked with their biodistribution and tumour accumulation determined by optical imaging; therefore, the new bi-functional terpolymer nanoparticle system is useful as a theranostic tool for simultaneous image guided drug delivery in cancer.

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Ethical standards All animal handling and procedures were conducted under protocols approved by the Animal Care committee at the Ontario Cancer Institute following guidelines set forth by the Canadian Council on Animal Care.

References


