Polymer nanoassemblies with solvato- and halo-fluorochromism for drug release monitoring and metastasis imaging

Background: Theranostics, an emerging technique that combines therapeutic and diagnostic modalities for various diseases, holds promise to detect cancer in early stages, eradicate metastatic tumors and ultimately reduce cancer mortality. **Methods & results:** This study reports unique polymer nanoassemblies that increase fluorescence intensity upon addition of hydrophobic drugs and either increase or decrease fluorescence intensity in acidic environments, depending on nanoparticle core environment properties. Extensive spectroscopic analyses were performed to determine optimal excitation and emission wavelengths, which enabled real time measurement of drugs releasing from the nanoassemblies and *ex vivo* imaging of acidic liver metastatic tumors from mice. **Conclusion:** Polymer nanoassemblies with solvato- and halo-fluorochromic properties are promising platforms to develop novel theranostic tools for the detection and treatment of metastatic tumors.

Cancer is the second leading cause of death in the USA next to heart attack, and more than 90% of cancer-related deaths are attributed to metastasis [1]. Metastasis is a disease state in which cancer cells migrate from primary tumors to other tissues through a variety of pathological events that often cause irreversible damage to vital organs [2]. Surgery, chemotherapy, radiotherapy and heat ablation are not effective at treating metastatic tumors, even though they are the main options commonly used to treat primary tumors [3,4]. One of the pathological phenotypes that make metastasis difficult to treat is the heterogeneous tissue microenvironment where cancer cells grow in mixture with normal cells and form micrometastatic colonies [5]. The micrometastatic colonies within parenchyma are often too small and too abundant for clinicians to detect early in development. Eradicating cancer cells effectively without damaging normal organ functions is difficult late in metastatic tumor development when metastasis can be detected [6]. Therefore, there is a growing need to develop new methods to improve detection and treatment of metastatic tumors and thus reduce mortality in cancer patients.

Theranostics, a combination of therapy and diagnostics of a disease, is an emerging technique that may allow clinicians to detect cancer early and treat metastatic tumors more efficiently. Additionally, theranostics may also allow the monitoring of disease progression over the course of treatment [7]. The successful development of theranostics that can offer these benefits for metastasis treatment depends on the logical selection of therapeutic and diagnostic elements. As first-line therapy widely used for various types of cancer in the clinic, chemotherapy is suitable for the therapeutic elements of theranostics. Applying existing treatment regimens and following existing safety guidelines for chemotherapy can provide proven therapeutic elements to these techniques [8,9]. However, chemotherapy has several limitations associated with its use. Chemotherapy often uses small molecule anticancer drugs that are poorly soluble in water, are not selective at entering tumor tissues and are cleared from the body too quickly to maintain effective concentrations for prolonged periods of time [10]. In order to improve safety and patient outcomes of chemotherapy, clini-

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Key terms

Theranostics: A combination of therapy and diagnostics of a disease that may allow for earlier detection and more efficient treatment of the disease while monitoring progress over the course of treatment.

Ex vivo imaging: Diagnostic method of identifying abnormalities in tissue collected from the body and evaluating their physical characteristics, such as size and location of tumors.

Solvato-fluorochromism: Property of fluorescent dyes that causes fluorescence-emitting spectra and quantum yields to change based on solvent polarity.

Halo-fluorochromism: Property of fluorescent dyes that causes fluorescence-emitting spectra and quantum yields to change depending on solution pH.

Polymer nanoassemblies: Nanoscale macromolecules created from self-assembling or cross-linked copolymers.

cians must carefully balance the efficacy and toxicity of anticancer drugs.

Pharmacokinetic studies are performed to initially predict accumulation, distribution, metabolism and elimination (ADME) of drugs [11]. Clinicians generally determine effective and safe drug doses to metastatic tumors based on the results of pharmacokinetic studies in a group of patients. Although these doses are determined for groups of patients, chemotherapeutic efficacy and toxicity often vary for each individual patient. Real-time monitoring of the ADME of drugs in the body is expected to provide timely, personalized regimens during the course of treatment while maximizing accumulation of anticancer drugs in metastatic tumors [12]. However, even if a sufficient amount of anticancer drugs are delivered to metastatic tumors, not all drugs are effective at killing cancer cells in micrometastatic colonies [13]. Metastatic tumor cells that grow quickly produce a large amount of acidic byproducts, such as lactic acid, during the conversion of glucose into energy [14]. This tissue acidifying process, also known as the Warburg effect, can reduce chemotherapy efficacy, because many anticancer drugs lose activity in acidic conditions [15,16]. By understanding the ADME of anticancer drugs in real time, factors that affect drug efficacy, like drug concentrations in tumor tissue and anticancer drug selection, can be used to maximize therapeutic outcomes while keeping treatments safe.

The acidic tissue environment can also be negatively diagnostic, the second key to the successful theranostics of metastatic tumors. Fluorescence is an established spectroscopic method that has been used to develop diagnostic and imaging tools for many years [17]. However, many fluorescent dyes can provide false signals during imaging assays unless used carefully. Theranostics for tumor metastasis using fluorescence must consider signal-to-noise ratios, quantum yields and photo-bleaching during their develop-

ment [18]. Fluorescent dyes often change fluorescence-emitting spectra and quantum yields based on solvent polarity (**solvato-fluorochromism**) and pH (**halo-fluorochromism**) [19]. Solvato- and halo-fluorochromic properties are determined by electronic mobility and conformation of fluorophores in the environment where the dyes are present [20,21]. Fluorochromism is generally troublesome for the development of biomarkers that must provide reliable, quantitative imaging results. However, fluorochromic dyes have great potential to provide a unique opportunity for the development of theranostic tools. For example, Nile Blue (NB) is a solvato- and halo-fluorochromic compound that emits strong fluorescence in nonpolar solvents and is quenched in water while changing fluorescence as a function of pH [22]. Fluorescence quenching of NB in water is useful to suppress background signals induced by free dyes or autofluorescence of tissues in the body. Despite its unique optical properties, NB is a small molecule that can be cleared quickly from the body, similar to the fate of small molecule anticancer drugs. Therefore, the pharmacokinetic profiles of anticancer drugs and imaging dyes need to be controlled to improve theranostics of metastatic tumors. In order to control these profiles, it is imperative to develop carriers that can deliver therapeutic and diagnostic agents concurrently to metastatic tumors in a selective manner [23].

Tumor-selective delivery of small molecules can be achieved by using biocompatible nanomaterials as carriers that can preferentially go through leaky tumor blood vessels while sparing well-defined vasculature in normal organs [24]. One type of nanomaterial that is capable of selective delivery of small molecules to tumors is **polymer nanoassemblies** created from self-assembling or cross-linked copolymers, such as polymer micelles, polymer vesicles and polyionic complexes. Physicochemical properties of polymer nanoassemblies, such as shape, size, surface charge and chemical functions, can be controlled, allowing the pharmacokinetic profiles of their payload to be modulated [25]. Controlling the size (<100 nm in diameter) and surface charge (neutral or slightly anionic) of polymer nanoassemblies is often desired to allow efficient tumor accumulation. Polymer nanoassemblies are typically engineered to have a hydrophobic core enveloped by a hydrophilic shell. The hydrophobic core is used to entrap drugs with low-water solubility, while the hydrophilic shell is used to protect the drugs from precipitation, degradation or nonspecific binding to plasma proteins in the blood. The shell can also prevent the drug-loaded polymer nanoassemblies from being taken up by the mononuclear phagocytic system and immune cells [26]. Modulating properties of the

nanoparticle core, such as charge density and lipophilicity, can control drug release from polymer nanoassemblies [27]. Previous studies have demonstrated that polymer nanoassemblies with these desirable properties can be used to deliver a variety of therapeutic, diagnostic and imaging molecules separately or in combination to tumors [28,29]. We hypothesize that these parameters can be used to modulate optical properties of fluorophores for the development of theranostics for metastatic tumors.

Therefore, the purpose of this study is to confirm the feasibility of real-time quantification of drugs in carriers and tissue pH-sensitive imaging of metastatic tumors by using polymer nanoassemblies with solvato- and halo-fluorochromic properties. As shown in Figure 1, we synthesized polymer nanoassemblies by tethering hydrophilic polymer chains (PEG) onto a single polymer backbone (branched poly[ethylene imine]; PEI) while modifying the core of the nanoassemblies with lipophilic pendant groups (palmitate: PAL) to modulate fluorochromism of NB. This simplified design of polymer nanoassemblies allowed us to focus on optical properties of NB-conjugated carriers for theranostic applications. Two types of polymer tethered nanoassemblies (TNAs) used in this study: PEG-PEI-NB (2PN) and PEG-PEI-PAL-NB (3PN). 2PN has ionizable moieties in the core that can attract water and quench NB fluorescence, while 3PN has a hydrophobic core modified with PAL to increase entrapment of hydrophobic anticancer drugs. Carfilzomib (CFZ), a US FDA approved proteasome inhibitor, was used in this study as a model anticancer drug that is clinically relevant, nonfluorescent, practically insoluble ($\log p = 4.2$) and unionizable in physiological conditions ($pK_a = 3.5$). Traditionally, carfilzomib has been used to treat hematological malignancies such as multiple myeloma, but there is growing interest in the use of carfilzomib to treat solid tumors and tumor metastasis [30,31]. The NB-conjugated TNAs were designed to monitor the amount of drug remaining in the hydrophobic core of polymer nanoassemblies in solution in real time, and detect metastatic tumors in a tissue pH-dependent manner.

Materials & methods

Chemicals & cells

α -methoxy- ω -NHS activated PEG (5 kDa) was purchased from NanoCS (NY, USA). Branched PEI, palmitoyl chloride and chloroacetyl chloride were purchased from Sigma Aldrich (MO, USA). Fisher Scientific (MA, USA) provided acridine orange (AO), NB, HEPES buffer (pH 8.0, 1 M), phosphate buffered saline (PBS; pH 7.4, 150 mM), dialysis membrane with molecular weight cut-off (MWCO) of 8 and 100 kDa and other organic solvents, such as methanol, ethanol,

tetrahydrofuran (THF), isopropanol (IPA), DMF, acetone, DMSO, pyridine and N-Methyl-2-pyrrolidone (NMP). Carfilzomib was purchased from LC Laboratories (MA, USA). A human colon cancer HCT 116 cell line expressing green fluorescent protein (GFP) was purchased from American Type Culture Collection (VA, USA). Green fluorescent protein expression was verified by fluorescence microscopy before use (data not shown). McCoy's 5A, 0.05% trypsin/EDTA and PBS were purchased from GE Healthcare (UT, USA). Fetal bovine serum was purchased from Atlanta Biologicals (GA, USA). HCT 116 cells were cultured in McCoy's 5A media supplemented with 10% fetal bovine serum according to all American Type Culture Collection recommendations. Cells were maintained at logarithmic growth in a humidified environment with 5% CO₂ at 37°C.

Polymer tethered nanoassembly synthesis

Polymer nanoassemblies were synthesized following Figure 2. Branched PEI was purified before use by dialysis using a 100 kDa MWCO membrane in water, in order to remove small impurities. Other materials were used without further purification. PEI was reacted with 5 kDa PEG NHS ester in a mixed solution of DMSO and HEPES (1:1) at room temperature for 2 days. The reaction produced PEG-PEI (2P), which was purified by dialysis using a 100 kDa MWCO membrane for 5 days in water, and collected by freeze drying. Half of PEG-PEI was reacted with palmitoyl chloride in THF at 40°C for 2 h. Pyridine was added after 30 min into the reaction to scavenge hydrochloric acid byproducts. The reaction produced PEG-PEI-PAL (3P), which was precipitated in diethyl ether and subsequently dialyzed in water prior to freeze drying. 2PN and 3PN were synthesized using a two-step reaction scheme. First, 2P and 3P were reacted with chloroacetyl chloride in THF at 40°C for 2 h. Then, NB was dissolved in DMF and added to the reaction solution. The temperature was increased to 60°C and the reaction was allowed to proceed for an additional 3 h. Pyridine was added after 30 min into this reaction to scavenge hydrochloric acid byproducts. Both 2PN and 3PN were purified by dialysis using 8 kDa MWCO membranes in a mixed solution of methanol and water (9:1) for 3 days and then in pure water for an additional 3 days. Purified products were collected by freeze drying. Gel permeation chromatography (GPC) and HPLC were used to confirm absence of free dyes and other impurities in purified TNAs. The chemical compositions of TNAs were determined using NMR spectroscopy (Varian 400 MHz, DMSO-*d*₆). Nile blue conjugation was confirmed by measuring absorbance and fluorescence with a microplate reader (SpectraMax M5, CA, USA).

Figure 1. Polymer TNAs prepared through unimolecular self-assembly of polymer conjugates (A). TNAs with solvato- and halo-fluorochromic properties for real-time determination of drug concentrations (B) and acid-sensitive monitoring of metastatic tumors (C). TNA: Tethered nanoassembly.

Validation of TNA synthesis

The diameter and surface charge of TNAs were determined by dynamic light scattering analysis and zeta-potential measurement (Zeta-sizer Nano, Malvern, UK). Diameters were determined by measuring 173 degree back-scattered light from a He-Ne laser (4 mW, 633 nm) and calculated using the Stokes-Einstein equation. Zeta potentials were determined by measuring scattered light while applying voltage (automatic voltage selection) and calculated using the Smo-

luchowski equation. The molecular weight and uniformity of TNAs were determined by GPC (Asahipak GF-7M column, PBS mobile phase, 0.5 ml/min, 40°C, calibrated using PEG standards).

Solvato-fluorochromic properties of Nile Blue

Nile Blue was dissolved in various solvents used widely in the laboratory for analysis and chemical reactions: deionized water, methanol, ethanol, THF, IPA, DMF, acetone, DMSO, pyridine and NMP. Each NB solu-

Figure 2. Synthesis protocols of TNAs with a hydrophilic core (2PN) and hydrophobic core (3PN).
 2PN: PEG-PEI-NB; 3PN: PEG-PEI-PAL-NB; TNA: Tethered nanoassembly.

tion (200 μ l, 100 μ g/ml) was added to a clear glass vial for imaging changes in light absorbance and fluorescence at various excitation (Ex) and emission (Em) wavelengths. To determine fluorescent properties, NB solutions were serially diluted (250, 125 and 62.5 ng/ml) and imaged over a wide range of wavelengths (500 nm Ex/620 nm Em, 605 nm Ex/660 nm Em and 640 nm Ex/700 nm Em) using an IVIS Spectrum imaging system (Caliper Life Sciences, MA, USA).

Drug loading of TNAs

TNAs were loaded with CFZ using a thin film evaporation method. Stock solutions of carfilzomib (1 mg/ml) and TNAs (100 mg/ml) were prepared in ethanol. The TNA solution (100 μ l) was mixed with the carfilzomib solution (1 ml) in a round bottom flask, arbitrarily aiming for 10% drug loading. The mixed solution was evaporated under reduced pressure to form a thin film on the bottom of the flask. The film was rehydrated with deionized water while applying heat and sonication. The solution containing CFZ-loaded TNAs was centrifuged to remove unloaded drugs and other insoluble impurities. The supernatant was lyophilized for storage. Drug loading was quantified by HPLC (Agilent Eclipse XDB-C18 column, 55:45 v/v acetonitrile/water with 0.1% formic acid mobile phase, 1 ml/min, 40°C, 200 nm, 2.01 min retention time). For each sample, 10 μ l was injected. From these measurements, drug loading efficiency was calculated as the ratio of drug mass in solution to TNA mass in solution.

Halo-fluorochromic properties of TNAs with & without drug loading

Initially, nonfluorescence of CFZ was verified by dissolving 1 mg/ml CFZ in ethanol and measuring fluorescence emission spectra for several excitation values between 400 and 700 nm (data not shown). TNAs (10 mg/ml) were dissolved in buffer solutions at pH 7.4, 7.0, 6.0 and 5.0. Drug-loaded TNAs were used for comparison to determine the effect of hydrophobic drugs entrapped in the core on optical properties of TNAs. Free dyes (NB [2 μ g/ml] and AO [1 μ g/ml]) were also dissolved in buffer solutions with and without drug. Each solution (100 μ l) was added to a 96-well plate, which was initially analyzed using fluorescence emission spectroscopy at multiple excitation wavelengths to determine fluorescence excitation and emission pairs. These pairs were further imaged using IVIS (500 nm Ex/620 nm Em and 640 nm Ex/700 nm Em) and a plate reader (500 nm Ex/600 nm Em and 640 nm Ex/680 nm Em).

Drug release of TNAs

Drug-loaded TNAs were dissolved in PBS. One ml of drug-loaded TNA solution (10 mg/ml) was added to dialysis cassettes. Each cassette was dialyzed for 48 h in 1 L of PBS. The temperature was maintained at 37°C throughout the study, in order to mimic physiological conditions. Samples (100 μ l) were removed at 0, 1, 3, 6, 24 and 48 h. Samples were analyzed by HPLC, using the same conditions used to measure drug load-

Key term

Orthotopic tumor models: Biological models that establish tumors in physiologically relevant locations in the body.

ing. Samples were also analyzed by fluorescence spectroscopy. 2PN fluorescence was measured at 610 nm Ex/670 nm Em, while 3PN fluorescence was measured at 580 nm Ex/640 nm Em. These excitation and emission pairs were chosen to maximize fluorescence signal. Drug release profiles were curve-fitted to a first order drug release model using GraphPad Prism 5 (CA, USA). Drug release measurements were excluded from curve fitting if previous HPLC measurement indicated less than 5% initial drug remaining.

Ex vivo imaging of metastatic tumors with TNAs

Athymic NCr nude mice (6–8 weeks old, male) were purchased from Taconic (NY, USA) and handled following the Institutional Animal Care and Use Committee (IACUC) protocol at University of Kentucky. *Ex vivo* imaging of liver metastatic colon tumors was performed with an IVIS by using tissue samples harvested from animals. An **orthotopic tumor model** of liver metastatic colon cancer was developed by injecting 100 μ l of 5×10^6 HCT 116 cells/ml into the spleen capsule of 6–8 weeks old male athymic NCr nude mice. HCT 116 cells stably expressing GFP were used. Mice were allowed to develop metastatic tumors in the liver for 6 weeks, prior to intravenous injections of 3PN (10 mg/kg) into the tail vein. 6 h after the injection, mice were euthanized to harvest livers for *ex vivo* imaging with an IVIS to detect fluorescence from GFP and 3PN at 480 nm Ex/520 nm Em and 640 nm Ex/700 nm Em, respectively. As negative controls, healthy mice were treated with either 3PN (10 mg/kg) or PBS (300 μ l) and euthanized to harvest metastasis-free livers for *ex vivo* imaging.

Results

Synthesis & physiochemical characterization of TNAs

TNAs were synthesized as shown in Figure 2. All TNAs were uniform and contained no impurities as confirmed by a single peak shown on GPC (Figure 3). Complete removal of free NB was achieved by dialysis of TNAs using a 9:1 mixed solution of methanol and water. Absence of free NB in TNA samples was also confirmed by performing centrifugal filtration of TNA solutions. Absorbance and fluorescence of the filtrate was measured, corresponding to free NB filtered from the samples. A lack of absorbance and fluorescence compared with water indicated complete removal of

NB from TNA solutions. DLS (dynamic light scattering) and zeta-potential measurements confirmed that 2PN and 3PN were less than 40 nm in diameter and had a neutral surface charge (Table 1). These results indicate that NB and palmitate are present in the core of TNAs and the PEG shell efficiently shielded the charge, dye and hydrophobic groups. Modification of the core with palmitate groups did not seem to affect the surface properties of TNAs, although it altered lipophilicity and molecular conformation of the core. Polydispersity measurements indicate that 3PN has a greater dispersity than 2PN, despite GPC and DLS measurements suggest that both TNAs have narrow size distributions. This suggests that nanoparticles may weakly interact during polydispersity measurements, but that these interactions are not observed during chromatographic measurements.

GPC revealed that the molecular weights of 2PN and 3PN were 165 and 176 kDa, respectively. The molecular weights of intermediates 2P and 3P were also measured to be 129 and 159 kDa, respectively. By comparing the molecular weight of each reactant to the amount of primary amines available to react on a PEI molecule, the percentage of reacted binding sites on each TNA could be calculated. For 2PN, approximately 10% of the binding sites were conjugated with PEG. For 3PN, approximately 10 and 55% of binding sites on PEI were conjugated with PEG and PAL, respectively. GPC also estimated the amount of NB conjugated to 2PN or 3PN. Due to solvato-fluorochromic effects of NB, quantification of NB based on spectroscopic comparisons of free NB to NB-conjugated TNAs can cause false results, based on inaccurate assumptions regarding the nanoparticle core environment. As a result, quantification was based on changes in molecular weight. Our estimations, based on the molecular weights of NB-conjugated TNAs compared with unconjugated TNAs, suggest that approximately 50% of initial binding sites on 2P were conjugated with NB, while 25% of initial binding sites on 3P were conjugated with NB. These estimations indicate that 55% of remaining primary amines on 2P were conjugated with NB, while 71% of remaining primary amines on 3P were conjugated with NB. Although core modifications decreased the amount of binding sites for NB conjugation, core modifications increased the reaction yield for these remaining binding sites.

PEG and PAL conjugation were further confirmed by NMR spectroscopy. 2PN NMR had major peaks corresponding to PEG (3.54 ppm) and PEI (2.32, 2.44, 2.55, 2.60 and 2.66 ppm). 3PN NMR had major peaks corresponding to PEG (3.54 ppm), PEI (2.32, 2.44, 2.55, 2.60 and 2.66 ppm) and PAL (1.20 ppm). By quantifying peak sizes and comparing to peak sizes

Figure 3. Gel permeation chromatographs for 2PN (A) and 3PN (B). Peak absorbance was measured by UV-VIS detector at 220 nm. Molecular weights of TNAs were determined using PEG standards. 2PN: PEG-PEI-NB; 3PN: PEG-PEI-PAL-NB; TNA: Tethered nanoassembly.

of PEI peaks, the percentage of reacted binding sites could be confirmed. For 2PN and 3PN, our estimations were in agreement with calculated NMR values or reacted binding sites.

Solvato-fluorochromic & halo-fluorochromic properties of free NB & TNAs

Figure 4 shows varying colors of NB in various solvents. NB was blue in polar solvents, such as water, methanol, ethanol, IPA, acetone and DMSO, pale pink in THF, and bright pink in DMF, pyridine and NMP. In comparison to color, fluorescence of NB changed in a wavelength-dependent manner. NB fluorescence was observed in DMF, acetone, pyridine and NMP at 500 nm Ex, and in methanol, ethanol, IPA and DMSO at either 605 nm or 640 nm Ex. Importantly, fluorescence of NB remained quenched in water under all conditions tested in this study. Similarly, NB-conjugated TNAs dissolved in water showed a red shift in fluorescence compared with NB in water. 3PN with a lipophilic core showed greater fluorescence than

2PN with a hydrophilic core that is capable of attracting water and inducing NB fluorescence quenching. 2PN did not show a significant red shift, although it fluoresced more strongly than free NB. These results indicate that the core of 2PN is slightly hydrophobic, while the core of 3PN is more hydrophobic than 2PN.

Figure 5 shows the optical properties of TNAs and free dyes as measured by an IVIS. Acridine orange, a commonly used biological pH indicator with a pKa of 3.2, was used as a negative control because its fluorescence was not quenched in water. At 500 nm Ex/620 nm Em, AO increased fluorescence intensity at pH 5.0 compared with other pH values, while fluorescence of NB was negligible. At 640 nm Ex/700 nm Em, NB increased fluorescence intensity at pH 5.0 compared with other pH values, while fluorescence of AO was negligible. In the presence of drug, fluorescence of AO and NB was minimally affected, as confirmed by fluorescence measurements using a plate reader. Changes in lipophilicity and pH could be detected using 2PN and 3PN. Drug-loaded TNAs showed halo-fluoro-

Table 1. Physiochemical properties of tethered nanoassemblies as measured by dynamic light scattering and HPLC.

TNA	Diameter (nm)	Zeta potential (mV)	Polydispersity index	Drug loading efficiency (%)
2PN	26.5 ± 8.8	-0.6 ± 0.4	0.32 ± 0.04	2.96 ± 0.09
3PN	34.3 ± 3.5	-1.3 ± 0.3	0.69 ± 0.07	8.66 ± 0.35

Values are presented as mean ± standard deviation for three measurements. 2PN and 3PN measurements were compared using a t-test for each parameter. Size and zeta potential measurements are not statistically different ($p > 0.05$), while polydispersity index and drug loading efficiency measurements are statistically different ($p < 0.01$).

2PN: PEG-PEI-NB; 3PN: PEG-PEI-PAL-NB; TNA: Tethered nanoassembly.

chromism while increasing fluorescence intensities in the presence of CFZ. These results suggest that NB in the core of the TNA behaved as a sensor of the environmental conditions, such as changes in lipophilicity and pH, without directly interacting with drug molecules. This indicates that pH-dependent differences in fluorescence of TNAs are significant enough to detect *ex vivo*.

Figure 6 shows pH-dependent changes in fluorescence of TNAs, as quantified by a plate reader. Results confirmed the observed fluorescence behavior of TNAs

in Figure 5. Empty 2PN and 3PN showed no difference in pH-dependent fluorescence at 500 nm Ex and 600 nm Em, but showed pH-dependent differences in fluorescence at 640 nm Ex and 680 nm Em. Empty 2PN gradually decreased fluorescence as pH decreases, while empty 3PN increased fluorescence as pH decreases. Drug-loaded 2PN and 3PN showed stronger fluorescence at 500 nm Ex and 600 nm Em than empty 2PN and 3PN. However, drug-loaded 3PN decreased in fluorescence at acidic conditions. 3PN entrapping drug decreased fluorescence slightly in acidic condi-

Figure 4. Optical properties of Nile Blue (NB) in various solvents at high concentrations (A) and at low concentrations (B). Fluorescence was measured under three detection conditions (C, D & E) with varying excitation (Ex) and emission (Em) wavelengths at concentrations from (B). Optical properties of NB depend on solvent environment and wavelength of detection. Fluorescence of NB in water was not observed.

Figure 5. Wavelength-dependent changes in solvato-fluorochromic and halo-fluorochromic properties of free dyes (AO and NB) and TNAs measured by IVIS. Samples were dissolved in each buffer solution at 1 $\mu\text{g/ml}$ (AO), 2 $\mu\text{g/ml}$ (NB) and 10 mg/ml (2PN and 3PN). 2PN: PEG-PEI-NB; 3PN: PEG-PEI-PAL-NB; AO: Acridine orange; NB: Nile Blue; TNA: Tethered nanoassembly.

tions, presumably due to partial changes in molecular conformation or ionization state of CFZ in the core of TNAs. CFZ ionization may also bring water into the core of TNAs, decreasing fluorescence. Empty 2PN and 3PN showed pH-dependent differences in fluorescence at 640 nm Ex and 680 nm Em. Empty 2PN gradually decreased fluorescence as pH decreases from 7.4 to 5.0. Based on IVIS observations and plate reader measurements, 3PN was selected as a formulation for further *ex vivo* imaging studies.

Drug release from TNAs

Figure 7 shows drug release profiles from 2PN over 24 h and from 3PN over 48 h. Drug release was determined by measuring drug remaining using an HPLC (HPLC method) and by monitoring fluorescence changes of

the TNA solution (solvato-fluorochromic method). Because CFZ is not fluorescent over the range of Ex wavelengths used on this study, changes in fluorescence of TNA solution are attributed to changes in solvato-fluorochromism of TNAs. Both HPLC and solvato-fluorochromic method indicate that drug remaining decreased exponentially over 48 h. In order to compare drug release measurement methods, these measurements were fit to first order drug release models. Kinetic parameters, such as half-life and drug release rate, were obtained using HPLC and solvato-fluorochromic method measurements (Table 2).

2PN and 3PN released drugs at different rates according to HPLC and solvato-fluorochromic methods. HPLC method indicates that drug release half-lives are much greater than solvato-fluorochromic method indicates.

Figure 6. Solvato-fluorochromic and halo-fluorochromic properties of tethered nanoassemblies quantified by plate reader. 2PN and 3PN were used at 10 mg/ml for all measurements. Fluorescence of TNAs was measured in the absence and presence of a model drug (CFZ). Data were analyzed by two-way ANOVA with Bonferroni post-test correction. For all measurements, * represents $p < 0.05$ in comparison with pH 7.4, ** represents $p < 0.01$ in comparison with pH 7.4 and *** represents $p < 0.001$ in comparison with pH 7.4. 2PN: PEG-PEI-NB; 3PN: PEG-PEI-PAL-NB; CFZ: Carfilzomib; TNA: Tethered nanoassembly.

Also, HPLC method indicates that drug release rates are much slower than the solvato-fluorochromic method indicates. HPLC method shows that drug release was only negligibly slowed in 3PN compared with 2PN, but solvato-fluorochromic method indicates that 3PN drug release occurs much slower than 2PN. Differences in measured kinetic parameters by HPLC and fluorescence can be attributed to differences in measured drug populations. HPLC method measures drug outside and inside nanoparticles, while solvato-fluorochromic method measures only drug inside the nanoparticle core. Therefore, the palmitate in the nanoparticle core may slow

drug release from the TNA core, but drug may remain bound to polymers on the outside of the TNAs. Solvato-fluorochromic method appears to determine drug concentrations in the core of polymer nanoassemblies more directly than HPLC methods.

Ex vivo imaging of liver with & without metastatic tumors

Figure 8 shows *ex vivo* imaging of metastatic tumors. Control mice without liver metastasis received intravenous injections of 3PN or PBS. HCT 116 metastasis was confirmed with GFP imaging at 480 nm Ex and

Figure 7. Drug release measurements for CFZ-loaded 2PN (A) and CFZ-loaded 3PN (B) at pH 7.4. Drug release was measured by HPLC (circles, solid line) and by measuring solvato-fluorochromic changes in TNAs (squares, dashed line).

2PN: PEG-PEI-NB; 3PN: PEG-PEI-PAL-NB; CFZ: Carfilzomib; TNAs: Tethered nanoassemblies.

520 nm Em. When wavelengths were switched to 640 nm Ex and 700 nm Em, 3PN emitted strong fluorescence in metastatic tumors and not in control mice. Interestingly, the areas where 3PN fluorescence was detected did not overlap completely with tumoral areas confirmed by GFP signals. These results suggest that some areas of metastatic tumors are acidic enough to trigger pH-sensitive fluorescence of 3PN and not all tumor cells are present in such highly acidic tissue areas. Tumor macrophages may also take up acid-sensitive nanoparticles emitting strong fluorescence, altering their location in metastatic tumors. Although further study is necessary to deliver drugs to all metastatic tumor cells more efficiently, our results demonstrated that 3PN was effective to detect metastatic tumors as small as a few millimeters in diameter and significantly improve sensitivity and specificity of imaging of metastasis.

Discussion

Fluorochromism is an optical property that allows fluorophores to change light-emitting wavelengths and intensities as functions of various environmental factors such as solvent polarity (solvato-fluorochromism) and pH (halo-fluorochromism) [32]. NB was selected as a model fluorophore with fluorochromism that changes fluorescence wavelengths depending on the amount of drugs entrapped in nanoparticles as well as the pH of solvents in which nanoparticles are present. Solvato-fluorochromic molecules have been used to detect changes in environmental polarity [33]. Pyrene is a fluorochromic dye commonly used to detect the hydrophobic micelle core environment [34]. The fluorochromic properties of pyrene change slightly when the dye is entrapped in a nonpolar environment. By measuring a ratio of fluorescence intensities, the polarity change between water and the micelle core

Table 2. Kinetic constants of drug release measurements calculated from a first order drug release model.

TNA	Detection method	$t_{1/2}$ (h)	k (h^{-1})
2PN	HPLC	4.89 ± 0.80	0.14 ± 0.02
	Solvato-fluorochromic	1.89 ± 0.10	0.37 ± 0.03
3PN	HPLC	4.66 ± 0.90	0.15 ± 0.03
	Solvato-fluorochromic	2.68 ± 0.35	0.26 ± 0.04

Values are presented as mean $\pm$ standard deviation for three measurements. 2PN and 3PN measurements were compared using a t-test for each parameter. Drug release constants measured using HPLC method are not statistically different ($p > 0.05$), while drug release constants measured using solvato-fluorochromic method are statistically different ($p < 0.05$).

2PN: PEG-PEI-NB; 3PN: PEG-PEI-PAL-NB; TNA: Tethered nanoassembly.

Figure 8. *Ex vivo* imaging of liver metastatic tumors of colon cancer (HCT 116). Livers were harvested from mice with liver metastases treated with 3PN (A), healthy mice treated with 3PN (B) and healthy mice treated with PBS (C). The top image indicates fluorescence from HCT 116 cells expressing GFP in liver metastasis. The bottom image indicates fluorescence from 3PN. 3PN: PEG-PEI-PAL-NB; GFP: Green fluorescent protein; PBS: Phosphate-buffered saline.

can be detected. NB undergoes significant fluorescence changes in solvents with different polarities. Because of this increased sensitivity, NB was able to detect changes in hydrophobicity due to the addition of hydrophobic drugs, in addition to changes in nanoparticle core environment.

The addition of lipophilic pendant groups to TNAs affects the drug loading and fluorochromic properties of the TNA. Palmitate increases drug loading of TNAs and increases fluorescence of drug-loaded and empty TNAs. Also, PAL causes fluorescence of TNAs to increase in acidic conditions, rather than decrease. The addition of PAL decreased drug release rates, measured by solvato-fluorochromism. Comparison of 2PN and 3PN indicates that the design, synthesis and chemical properties of polymer nanoassemblies are critical to the successful use of fluorochromic dyes.

Solvato-fluorochromism is important to the design of nanoparticles for the detection of metastatic tumors. Traditional fluorophores are successful in detecting

solid tumors because accumulation of dye-containing nanoparticles in solid tumors can increase fluorescence signals and overcome interference from free dyes or circulating nanoparticles [35]. However, metastatic tumors are not expected to significantly accumulate dye-containing nanoparticles, and free dyes and circulating nanoparticles could interfere with imaging [36]. Therefore, the development of nanoparticles that can suppress free dye and circulating nanoparticle signals, while enhancing nanoparticle signals in tumor tissue, is important to the detection of metastatic tumors.

Our results show that NB-conjugated TNAs fluoresce greater than free NB in water due to the solvato-fluorochromism of NB. Because of this, any potential fluorescence from free NB that may be removed from the TNA through *in vivo* metabolism will not contribute significantly to fluorescence imaging. This allows us to detect metastatic tumors without interference from free dyes. Solvato-fluorochromic measurements of drug release improve on traditional *in vitro* drug

release measurement methods. Traditional drug release measurement methods are used to estimate *in vivo* release by making careful assumptions regarding thermodynamic and kinetic constants obtained from these methods. Although this approach has been important to understanding drug release from nanoparticles, it does not provide direct measurements of *in vivo* drug release rates. In this study, hydrophobic anticancer drugs were successfully entrapped in TNAs and their release profiles were determined directly by monitoring fluorochromic changes of drug-loaded TNAs in buffer solutions over time. Our evidence suggests that this system can be used to measure drug release rates in real time. Measuring drug release rates using solvato-fluorochromism has the potential to directly measure drug release rates *in vivo*, which can be used to better understand real-time biodistribution of drugs in patients by measuring changes in fluorescence over time in a desired location. Toward this goal, the future development of signal normalization approaches, such as peak ratio methods or the addition of an internal fluorescent standard, are crucial to separating the solvato- and halo-fluorochromic effects of TNAs from concentration-based and time-based fluorescence effects *in vivo*.

Other detection methods, such as fluorescence resonance energy transfer, have also been used to measure drug release from nanoparticles in real time [37]. This method requires a specific energetic interaction between drugs and fluorescent dyes, which limits the types of drugs and fluorescent dyes that can be used in these systems [38]. Because solvato-fluorochromic properties do not require the drug to have specific optical properties, these TNAs have the potential to measure drug release for various applications [39]. The use of solvato-fluorochromic fluorophores to measure drug release rates has some limitations. First, drugs that absorb and fluoresce light in the same range as these TNAs may interfere with drug release measurements. Although this is a significant limitation, many hydrophobic anticancer drugs do not absorb or fluoresce light at the values considered in this study. Additionally, anticancer drugs that are not hydrophobic may bring water into the nanoparticle core, reducing fluorescence measurements. Therefore, the scope of this system must be narrowed to hydrophobic drugs. Although further feasibility tests are necessary *in vivo*, these results suggest that *in situ* determination of drug concentrations in metastatic tumors or other targeted sites might be possible by using fluorochromic nanoparticles. In combination with other known factors, such as metabolism and clearance, this system has the potential to allow ADME of a drug to be understood

in real time. Toward this goal, developing a method that can normalize drug concentrations with respect to drug carrier concentrations is needed.

Halo-fluorochromic properties of molecules often depend on ionization of a specific location on the molecule resulting in fluorescence shifts [40]. The pKa of this ionization process often shifts greatly depending on the solvent environment of the molecule [41]. The addition of hydrophobic groups to the core of TNAs caused differences between 2PN and 3PN halo-fluorochromic properties. While 2PN decreased fluorescence in acidic conditions, 3PN and NB increased fluorescence in acidic conditions. Interactions between NB and ionized moieties on PEI may cause a shift in NB fluorescence peaks, resulting in this difference. The addition of hydrophobic drugs to the TNA core does not remove the halo-fluorochromic properties of the TNA. Addition of hydrophobic drug increased fluorescence values at all pH values, but did not alter increases due to pH in buffered solutions. This indicates that both solvato-fluorochromic properties and halo-fluorochromic properties are observed from TNAs. However, the addition of drugs that can undergo ionization in biological ranges may affect the halo-fluorochromic properties of the TNAs. Therefore, the use of these TNAs should be narrowed to drugs without pKa values between 5.0 and 7.4.

Fluorochromic molecules are often used to identify biological components in imaging assays. For example, AO is a dye used widely in biochemistry to detect nucleic acids [42]. Acridine orange changes Ex and Em maximum wavelengths when binding to DNA (Ex 502 nm/Em 525 nm) and RNA (Ex 460 nm/Em 650 nm). In acidic conditions, AO can also undergo a blue shift in excitation wavelength [43]. Despite its potential, fluorophores like AO are not capable of differentiating metastatic tumors from normal tissue as well as the locations where dyes are present such as vascular, interstitial and intracellular space. NB has many advantages over other fluorochromic molecules. In comparison with AO that fluoresces in water, NB quenches its fluorescence in water and changes fluorescence intensities when entrapped in nanoparticles. Such a unique optical property of NB allowed TNAs entrapping NB to be able to provide real-time information on the amount of drugs remaining in the particles during drug release and tissue pH of colon cancer tumors metastasized to the liver. The sensitivity and specificity of the pH-enhanced imaging of metastatic tumors were significantly improved as opposed to other existing diagnostic options that can only detect relatively large tumors.

Measuring *in vivo* drug release from nanomaterials is important for clinical applications [44]. Clinicians

need to accurately determine drug concentrations in metastatic tissue to treat patients effectively and safely. Our results show that solvato-fluorochromic measurement methods predict faster drug release rates and shorter drug release half-lives than traditional HPLC measurement methods predict. It is possible that solvato-fluorochromic methods overestimate *in vivo* drug release rates or that HPLC methods underestimate *in vivo* drug release rates. Overestimation of *in vivo* drug release rates results in lower anticancer drug concentrations in metastatic tumor tissue, resulting in ineffective treatment. However, underestimation of *in vivo* drug release rates results in higher anticancer drug concentrations in metastatic tumor tissue and healthy tissue, resulting in toxicity. In order to estimate the clinical accuracy of both measurement methods, the significance of measured drug populations to *in vivo* drug release must be considered. Solvato-fluorochromic methods measure drugs remaining in the nanoparticle core, while HPLC methods measure drugs inside the nanoparticle core and drugs outside the core, which may be bound to polymers or remain in the aqueous environment [45]. Drugs bound to polymers or in solution are not expected to affect *in vivo* drug release rates, even though they can significantly affect HPLC measurements [46]. Although mechanistic modeling has been used to correct for these interactions in HPLC methods, solvato-fluorochromic methods provide a unique opportunity to directly measure drug release from the nanoparticle core during drug release measurements. Because solvato-fluorochromic measurements only consider drugs in the core, they are expected to accurately measure drug release for clinical applications. Further studies to determine the relationship between solution pH, HPLC drug release measurements and solvato-fluorochromic drug release measurements may allow for better understanding of *in vivo* drug release rate measurements.

It must be noted that NB-conjugated TNAs did not detect tumor cells directly but likely provide silhouette of metastatic tumors by detecting acidic tissue areas. Biomarkers using fluorescence-tagged antibodies or aptamers are certainly more selective in detecting cancer cells than fluorochromic nanoparticles that detect metastatic tumors based on indirect information such as the acidic tumor tissue environment [47]. However, nanoparticles circulating in the body for a prolonged period of time can extend the time span for clinicians to observe metastatic tumors while performing surgery or administering other therapeutic options in combination to patients [48]. Tissue pH of metastatic tumors has been elusive although numerous attempts have been made in the past years to determine the pH

of tumor tissues. One of the obstacles to measuring tumor tissue pH is rapid clearance of biomarkers and alteration of optical properties of reporter molecules within the tumor tissue. In contrast, NB-loaded TNAs can be used as imaging tools that provide real-time information about the tumor tissue pH because they can stay in tumors for an extended time. Our results suggest that some areas of metastatic tumors in the liver are highly acidic ($\text{pH} < 6.0$), which can deactivate many anticancer drugs that lose activity in acidic conditions. However, further study of tumor tissue pH in multiple metastasis models is necessary to understand the key factors that affect metastatic tumor tissue pH.

The therapeutic impact of metastatic tumor tissue acidity is an interesting topic for future study. Additionally, the size of a tumor affects the pH of tumor tissue [49]. Determining the minimum size necessary to detect a metastasis with this system using multiple metastasis models will indicate the potential to detect micrometastasis using pH-enhanced imaging. Metastatic tumors are not considered suitable for passive targeting because they do not develop as complete of a vascular system as primary tumors due to their small-tissue size [50]. Our observations revealed that TNAs with a particle diameter smaller than 40 nm were capable of accumulating in metastatic tumors. Because nanoparticles often accumulate in the liver, detection of metastatic tumors in the liver would be difficult unless detection signals from nanoparticles in metastatic tumors could be distinguished from signals in healthy liver tissue [51]. Our results demonstrate that tissue pH-enhanced imaging would be promising to reduce background signals from healthy liver tissue while increasing signals from metastatic tumors.

MRI and computed tomography are imaging techniques typically used in the clinic to detect metastasis by monitoring structural abnormalities in normal tissues [52]. However, structural abnormalities cannot be detected when metastatic tumors are small. Even when structural abnormalities were detected in organs of interest, potential tumor metastasis areas are generally analyzed further to determine pathologically if the area is a metastasis or another type of structural abnormality that is not cancer. In comparison with these traditional diagnostic approaches, fluorochromic TNAs report not only structural abnormalities (such as leaky blood vessels) but also tissue pH, a hallmark of metastatic tumors, while emitting fluorescence that can be monitored in real time. Because of this, fluorochromic TNAs successfully detected tumor metastasis located in the liver without interference of signals from healthy liver tissue.

Conclusion

In this study, we synthesized polymer nanoassemblies by tethering hydrophilic polymer chains (PEG) onto a branched polymer scaffold (PEI) and then conjugating the core of the nanoassemblies with fluorochromic molecules (NB) and lipophilic chains (PAL). NB was selected because its solvato-fluorochromic and halo-fluorochromic properties offered several advantages over traditional fluorophores, such as increased fluorescence in the nanoparticle core. Two types of polymer TNAs were evaluated as potential drug carriers and tissue pH sensors: 2PN and 3PN. NB was initially used to detect the lipophilicity and acidity of the nanoparticle core. In comparison with 2PN with a hydrophilic core that quenched NB fluorescence, 3PN with a hydrophobic core showed strong fluorescence in acidic conditions or in the presence of a model hydrophobic anticancer drug (CFZ). The solvato-fluorochromic properties of 2PN and 3PN were used to measure drug concentrations in TNAs during drug release. Fluorescent measurements indicated that drug release from the nanoparticle core occurred quicker than indicated by traditional measurements, suggesting traditional drug release measurements may overestimate the amount of drug remaining in nanoparticles. This result indicates that TNAs hold potential as a method of directly measuring *in vivo* drug release rates. The halo-fluorochromic properties of 3PN were also used to detect liver metastasis on mice models *ex vivo*. TNAs were able to detect metastatic tumors without any noticeable interference of background fluorescence noise. We surmise that TNAs accumulated in the tissue space surrounding a tumor, and a

pH-enhanced, silhouette image of metastatic tumors differentiated tumor tissue from normal tissue. This result demonstrates that tissue pH-enhanced imaging is useful to improve selectivity and sensitivity of metastatic tumor imaging. The findings in this study suggest that further experiments with tethered nanoassemblies may validate their potential as diagnostic tools with the potential to deliver chemotherapeutic drugs.

Future perspective

Fluorescent imaging will become an important diagnostic method for cancer, due to its simplicity and versatility. Initially, fluorescent imaging will use traditional fluorescent dyes to detect the location of solid tumors. As fluorescent imaging becomes commonly used, fluorochromic dyes will begin to replace multiple step diagnostic methods that require biopsy and further analysis before treatment. Early detection of metastasis will allow traditional treatment approaches to successfully treat cancer metastasis, ultimately reducing cancer mortality.

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Executive summary

- Two types of polymer tethered nanoassemblies were synthesized: PEG-PEI-NB (2PN) and PEG-PEI-PAL-NB (3PN). 2PN and 3PN were small (<40 nm diameter), had neutral surface charge and uniform size distribution.
- 2PN fluoresced weakly, likely due to water quenching fluorescence in the nanoparticle core. 3PN fluoresced greatly, likely due to hydrophobic pendant groups in the nanoparticle core.
- Solvato-fluorochromic and halo-fluorochromic properties of 2PN and 3PN could be detected by fluorescence imaging. Fluorescence differences in 2PN and 3PN were easier to distinguish than differences in free dyes.
- 2PN and 3PN increased fluorescence in response to incorporation of model hydrophobic chemotherapeutics to the nanoparticle core. Addition of hydrophobic drugs increased nanoparticle core polarity, which could be measured by solvato-fluorochromism.
- 2PN decreased fluorescence in response to acidity, while 3PN increased fluorescence in response to acidity. Ionized groups present in 2PN core may have altered its halo-fluorochromic properties.
- Drug release from 2PN and 3PN was measured in real time using solvato-fluorochromism. Solvato-fluorochromic methods indicate that drug release from nanoparticle core occurred quicker than HPLC methods indicate, suggesting that traditional drug release measurements overestimate drug remaining in the nanoparticle core.
- *Ex vivo* tumor imaging detected liver metastasis on mice models. 3PN accumulated near metastatic tumors and increased fluorescence in acidic tumor metastasis, creating a pH-induced silhouette corresponding to tumor metastasis.

References

- 1 Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell* 147(2), 275–292 (2011).
- 2 Bravo-Cordero JJ, Hodgson L, Condeelis J. Directed cell invasion and migration during metastasis. *Curr. Opin. Cell Biol.* 24(2), 277–283 (2012).
- 3 Xing M, Kooby DA, El-Rayes BF, Kokabi N, Camacho JC, Kim HS. Locoregional therapies for metastatic colorectal carcinoma to the liver – an evidence-based review. *J. Surg. Oncol.* 110(2), 182–196 (2014).
- 4 Li D, Kang J, Golas BJ, Yeung VW, Madoff DC. Minimally invasive local therapies for liver cancer. *Cancer Biol. Med.* 11(4), 217–236 (2014).
- 5 Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* 501(7467), 346–354 (2013).
- 6 Haeno H, Gonen M, Davis Meghan B, Herman Joseph M, Iacobuzio-Donahue Christine A, Michor F. Computational modeling of pancreatic cancer reveals kinetics of metastasis suggesting optimum treatment strategies. *Cell* 148(1–2), 362–375 (2012).
- 7 Liu Y, Feng L, Liu T *et al.* Multifunctional pH-sensitive polymeric nanoparticles for theranostics evaluated experimentally in cancer. *Nanoscale* 6(6), 3231–3242 (2014).
- 8 Shelley MD, Cleves A, Wilt TJ, Mason MD. Gemcitabine chemotherapy for the treatment of metastatic bladder carcinoma. *BJU Int.* 108(2), 168–179 (2011).
- 9 Pivot X, Schneeweiss A, Verma S *et al.* Efficacy and safety of bevacizumab in combination with docetaxel for the first-line treatment of elderly patients with locally recurrent or metastatic breast cancer: results from AVADO. *Eur. J. Cancer* 47(16), 2387–2395 (2011).
- 10 Wang Z, Yang J, Kirk C *et al.* Clinical pharmacokinetics, metabolism, and drug–drug interaction of carfilzomib. *Drug Metab. Dispos.* 41(1), 230–237 (2013).
- 11 Alsina M, Trudel S, Furman RR *et al.* A Phase I single-agent study of twice-weekly consecutive-day dosing of the proteasome inhibitor carfilzomib in patients with relapsed or refractory multiple myeloma or lymphoma. *Clin. Cancer. Res.* 18(17), 4830–4840 (2012).
- 12 Gotta V, Widmer N, Decosterd LA *et al.* Clinical usefulness of therapeutic concentration monitoring for imatinib dosage individualization: results from a randomized controlled trial. *Cancer Chemother. Pharmacol.* 74(6), 1307–1319 (2014).
- 13 Carloni V, Mazzocca A, Mello T, Galli A, Capaccioli S. Cell fusion promotes chemoresistance in metastatic colon carcinoma. *Oncogene* 32(21), 2649–2660 (2013).
- 14 Justus CR, Dong L, Yang LV. Acidic tumor microenvironment and pH-sensing G protein-coupled receptors. *Front. Physiol.* 4, 354 (2013).
- **Authors describe the physiological causes and biological implications of the acidic tumor microenvironment, and then discuss the therapeutic implications of targeting the acidic tumor microenvironment in cancer therapy.**
- 15 Kato Y, Ozawa S, Miyamoto C *et al.* Acidic extracellular microenvironment and cancer. *Cancer Cell Int.* 13(1), 89 (2013).
- 16 Barar J, Omid Y. Dysregulated pH in tumor microenvironment checkmates cancer therapy. *Bioimpacts* 3(4), 149–162 (2013).
- 17 Key J, Leary JF. Nanoparticles for multimodal *in vivo* imaging in nanomedicine. *Int. J. Nanomedicine* 9, 711–726 (2014).
- 18 Hu H, Huang P, Weiss OJ *et al.* PET and NIR optical imaging using self-illuminating (64)Cu-doped chelator-free gold nanoclusters. *Biomaterials* 35(37), 9868–9876 (2014).
- 19 Jose J, Burgess K. Benzophenoxazine-based fluorescent dyes for labeling biomolecules. *Tetrahedron* 62(48), 11021–11037 (2006).
- **The solvato- and halo-fluorochromic properties of Nile Blue and other benzophenoxazine-based fluorescent dyes are discussed and compared.**
- 20 Yao Z, Hu X, Huang B *et al.* Halochromism of a polythiophene derivative induced by conformational changes and its sensing application of carbon dioxide. *ACS Appl. Mater. Inter.* 5(12), 5783–5787 (2013).
- 21 Marini A, Muñoz-Losa A, Biancardi A, Mennucci B. What is solvatochromism? *J. Phys. Chem. B.* 114(51), 17128–17135 (2010).
- 22 Jose J, Ueno Y, Burgess K. Water-soluble Nile Blue derivatives: syntheses and photophysical properties. *Chem. Eur. J.* 15(2), 418–423 (2009).
- 23 Svenson S. Theranostics: are we there yet? *Mol. Pharm.* 10(3), 848–856 (2013).
- **The author describes the advantages and limitations of theranostic carriers in clinical development, while also emphasizing key characteristics for successful theranostic nanomaterials.**
- 24 Maeda H, Nakamura H, Fang J. The EPR effect for macromolecular drug delivery to solid tumors: improvement of tumor uptake, lowering of systemic toxicity, and distinct tumor imaging *in vivo*. *Adv. Drug Del. Rev.* 65(1), 71–79 (2013).
- 25 Ponta A, Bae Y. PEG-poly(amino acid) block copolymer micelles for tunable drug release. *Pharm. Res.* 27(11), 2330–2342 (2010).
- 26 Steichen SD, Caldorera-Moore M, Peppas NA. A review of current nanoparticle and targeting moieties for the delivery of cancer therapeutics. *Eur. J. Pharm. Sci.* 48(3), 416–427 (2013).
- 27 Lee HJ, Bae Y. Brushed block copolymer micelles with pH-sensitive pendant groups for controlled drug delivery. *Pharm. Res.* 30(8), 2077–2086 (2013).
- **By developing a library of block copolymers with lipophilic chains of increasing length, the authors show that hydrophobic nanoparticle core modifications can modulate drug release properties.**
- 28 Chen F, Nayak TR, Goel S *et al.* *In vivo* tumor vasculature targeted PET/NIRF imaging with TRC105(Fab)-conjugated, dual-labeled mesoporous silica nanoparticles. *Mol. Pharm.* 11(11), 4007–4014 (2014).

- 29 Ferber S, Baabur-Cohen H, Blau R *et al.* Polymeric nanotheranostics for real-time non-invasive optical imaging of breast cancer progression and drug release. *Cancer Lett.* 352(1), 81–89 (2014).
- 30 Baker AF, Hanke NT, Sands BJ, Carbajal L, Anderl JL, Garland LL. Carfilzomib demonstrates broad anti-tumor activity in pre-clinical non-small cell and small cell lung cancer models. *J. Exp. Clin. Cancer Res.* 33, 111 (2014).
- 31 Mehta A, Zhang L, Boufraqueh M *et al.* Carfilzomib is an effective anticancer agent in anaplastic thyroid cancer. *Endocr. Relat. Cancer.* 22(3), 319–329 (2015).
- 32 Boens N, Leen V, Dehaen W. Fluorescent indicators based on BODIPY. *Chem. Soc. Rev.* 41(3), 1130–1172 (2012).
- 33 Uchida Y, Maezawa Y, Uchida Y, Hiruta N, Shimoyama E. Molecular imaging of low-density lipoprotein in human coronary plaques by color fluorescent angiography and microscopy. *PLoS ONE* 7(11), e50678 (2012).
- 34 Thapa U, Ismail K. Urea effect on aggregation and adsorption of sodium diocytlsulfosuccinate in water. *J. Colloid Interface Sci.* 406, 172–177 (2013).
- 35 Cheng L, Yang K, Chen Q, Liu Z. Organic stealth nanoparticles for highly effective *in vivo* near-infrared photothermal therapy of cancer. *ACS Nano.* 6(6), 5605–5613 (2012).
- 36 Maeda H. Vascular permeability in cancer and infection as related to macromolecular drug delivery, with emphasis on the EPR effect for tumor-selective drug targeting. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 88(3), 53–71 (2012).
- **The limitations of passive targeting approaches to nanoparticle drug delivery to metastatic tumors are discussed, and methods of overcoming these limitations are considered.**
- 37 Zou P, Chen H, Paholak HJ, Sun D. Noninvasive fluorescence resonance energy transfer imaging of *in vivo* premature drug release from polymeric nanoparticles. *Mol. Pharm.* 10(11), 4185–4194 (2013).
- 38 Wang J, Gao PP, Yang XX, Wang TT, Wang J, Huang CZ. Real-time imaging of intracellular drug release from mesoporous silica nanoparticles based on fluorescence resonance energy transfer. *J. Mater. Chem. B.* 2(27), 4379–4386 (2014).
- 39 Lai J, Shah BP, Garfunkel E, Lee K-B. Versatile fluorescence resonance energy transfer-based mesoporous silica nanoparticles for real-time monitoring of drug release. *ACS Nano.* 7(3), 2741–2750 (2013).
- 40 Zhou J, Fang C, Chang T, Liu X, Shangguan D. A pH sensitive ratiometric fluorophore and its application for monitoring the intracellular and extracellular pHs simultaneously. *J. Mater. Chem. B.* 1(5), 661–667 (2013).
- 41 Sanli S, Altun Y, Guven G. Solvent effects on pKa values of some anticancer agents in acetonitrile–water binary mixtures. *J. Chem. Eng. Data.* 59(12), 4015–4020 (2014).
- 42 Sarnat HB. Immunocytochemical markers of neuronal maturation in human diagnostic neuropathology. *Cell Tissue Res.* 359(1), 279–294 (2015).
- 43 Liu J, Zang L, Wang Y, Liu G. Preparation of acridine orange-doped silica nanoparticles for pH measurement. *J. Lumin.* 147(0), 155–158 (2014).
- 44 Moore T, Chen H, Morrison R, Wang F, Anker JN, Alexis F. Nanotechnologies for noninvasive measurement of drug release. *Mol. Pharm.* 11(1), 24–39 (2014).
- 45 Modi S, Anderson BD. Determination of drug release kinetics from nanoparticles: overcoming pitfalls of the dynamic dialysis method. *Mol. Pharm.* 10(8), 3076–3089 (2013).
- 46 Ponta A, Fugit KD, Anderson BD, Bae Y. Release, partitioning, and conjugation stability of doxorubicin in polymer micelles determined by mechanistic modeling. *Pharm. Res.* (2014).
- 47 Goetz M, Hoetker MS, Diken M, Galle PR, Kiesslich R. *In vivo* molecular imaging with cetuximab, an anti-EGFR antibody, for prediction of response in xenograft models of human colorectal cancer. *Endoscopy* 45(6), 469–477 (2013).
- 48 Hill TK, Abdulhad A, Kelkar SS *et al.* indocyanine green-loaded nanoparticles for image-guided tumor surgery. *Bioconjugate Chem.* 26(2), 294–303 (2015).
- 49 Estrella V, Chen T, Lloyd M *et al.* Acidity generated by the tumor microenvironment drives local invasion. *Cancer Res.* doi:10.1158/0008-5472.CAN-12-2796 (2013) (Epub ahead of print).
- 50 Schroeder A, Heller DA, Winslow MM *et al.* Treating metastatic cancer with nanotechnology. *Nat. Rev. Cancer* 12(1), 39–50 (2012).
- 51 Maawy AA, Hiroshima Y, Zhang Y, Luiken GA, Hoffman RM, Bouvet M. Polyethylene glycol (PEG) linked to near infrared (NIR) dyes conjugated to chimeric anti-carcinoembryonic antigen (CEA) antibody enhances imaging of liver metastases in a nude-mouse model of human colon cancer. *PLoS ONE* 9(5), e97965 (2014).
- **The authors show that although liver metastasis can be detected using dye-labeled antibodies targeting carcinoembryonic antigen, fluorescence signals from other organs are also observed.**
- 52 Reiner CS, Stolzmann P, Husmann L *et al.* Protocol requirements and diagnostic value of PET/MR imaging for liver metastasis detection. *Eur. J. Nucl. Med. Mol. Imaging* 41(4), 649–658 (2014).