Fluorocoxib A Loaded Nanoparticles Enable Targeted Visualization of Cyclooxygenase-2 in Inflammation and Cancer

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ABSTRACT. Cyclooxygenase-2 (COX-2) is expressed in virtually all solid tumors and its overexpression is a hallmark of inflammation. Thus, it is a potentially powerful biomarker for the early clinical detection of inflammatory disease and human cancers. We report a reactive oxygen species (ROS) responsive micellar nanoparticle, PPS-b-POEGA, that solubilizes the first fluorescent COX-2-selective inhibitor fluorocoxib A (FA) for COX-2 visualization in vivo. Pharmacokinetics and biodistribution of FA-PPS-b-POEGA nanoparticles (FA-NPs) were assessed after a fully-aqueous intravenous (i.v.) administration in wild-type mice and revealed 4 – 8 h post-injection as an optimal fluorescent imaging window. Carrageenan-induced inflammation in the rat and mouse footpads and 1483 HNSCC tumor xenografts were successfully visualized by FA-NPs with fluorescence up to 10-fold higher than that of normal tissues. The targeted binding of the FA cargo was blocked by pretreatment with the COX-2 inhibitor indomethacin, confirming COX-2-specific binding and local retention of FA at pathological sites. Our collective data indicate that FA-NPs are the first i.v.-ready FA formulation, provide high signal-to-noise in inflamed, premalignant, and malignant tissues, and will uniquely enable clinical translation of the poorly water-soluble FA compound.

KEYWORDS. Cancer, Inflammation, COX-2, Molecular Imaging, Nanoparticles, Reactive Oxygen Species

INTRODUCTION

Cyclooxygenases (COXs) are important biological mediators of inflammation that catalyze the biotransformation of arachidonic acid into prostaglandins and thromboxane [1]. Most normal tissues express the COX-1 isoform, which performs housekeeping functions, such

as maintenance of vascular tone, control of hemostasis, and cytoprotection of the gastric mucosa [2]. In contrast, the inducible COX-2 isoform is overexpressed in inflammation, where it modulates edema and pain, and in neoplastic diseases, where it potentiates tumor growth and metastasis [3]. Overexpression of COX-2 is an early event in carcinogenesis, and it plays a vital role in cancer progression [4], suggesting that it is a useful biomarker for both early- and late-stage cancer detection. Moreover, COX-2 inhibitors have been shown to be effective adjuvant chemotherapeutic agents in some cancers [5, 6]. Therefore, COX-2 is an ideal candidate for targeted visualization of inflammatory disease and a broad spectrum of human cancers. To this end, we discovered fluorocoxib A (FA), a fluorescent 5-carboxy-X-rhodamine- (5-ROX)-labeled COX-2-selective inhibitor, to visualize COX-2 in inflamed or cancerous tissues [7].

Clinical translation of FA has the potential to address the significant, unmet need for techniques that enable earlier detection of cancers of the skin, colon, esophagus, bladder, and oropharynx [7-10]. For example, five year survival for colorectal cancer is 90% if diagnosed while it is still localized, but falls to 68% for regional disease, and just 10% for disease with distant metastases [11]. Moreover, miss rates for colorectal neoplastic polyps can be as high as 28% by traditional white-light colonoscopy [12]. Likewise, cancers of the esophagus, bladder, and oropharynx show higher survival rates and better prognoses when detected early (before regional spreading and metastases) [13-15]. Despite the clear need, translation of optical imaging agents from basic research tools to clinically relevant contrast agents remains challenging [16]. The Molecular Imaging and Contrast Agents Database lists 1444 agents as of June 2013. Of these, only 119 agents have been FDA approved, and only 4 are optical imaging agents (all Indocyanine Green and Fluorescein derivatives).

Attempts to translate FA to the clinic have been hampered by its lack of solubility in aqueous solutions appropriate for human administration. All previous administrations of FA have been in 100% dimethyl sulfoxide (DMSO) or a mixed solvent consisting of DMSO (16%)/EtOH (33%)/propylene glycol (17%)/warm sterile saline (34%, 37.5 °C), that are not appropriate for human applications, especially by intravenous administration. Thus, a new administration strategy is necessary before the clinical potential of FA can be realized. Many promising small molecule drugs and imaging agents, such as FA, suffer from extreme hydrophobicity, limiting use in an injectable form. Polymeric nanoparticles provide a promising approach for solubilizing and altering the pharmacokinetics of such small molecules in vivo [17, 18]. For example, amphiphilic diblock polymers can be designed to self-assemble into micellar nanoparticles that enable solubilization of hydrophobic compounds by sequestering them into a hydrophobic core surrounded by a hydrated corona. Utilization of inert, hydrophilic macromolecules such as poly(ethylene glycol) (PEG) to form the corona enhances nanoparticle stealth, reducing opsonization and rate of clearance by the mononuclear phagocyte system (MPS) [19-22]. Ideal nanoparticle formulations have a hydrodynamic diameter greater than ~10 nm, which avoids rapid renal clearance, and fall into a size range (approximately 20-200 nm) that enhances passive targeting to cancer and inflammation by the enhanced permeability and retention (EPR) effect [23, 24]. Material responsiveness to environmental cues, such as changes in pH, enzyme activity, and reactive oxygen species (ROS), can then be leveraged to trigger cargo release within these tissues [25-30]. Importantly, micellar systems are being tested clinically and in some cases (e.g. Genexol-PM) have made it through clinical trials to become approved for use in humans, supporting the potential translatability of the proposed approach [31].

Herein, we report the synthesis and characterization of a novel diblock polymer that self-assembles into water-soluble micellar nanoparticles (NPs) that efficiently encapsulate / solubilize FA. We describe the pharmacokinetics and biodistribution of FA-NPs and validate COX-2-specific delivery and binding of FA, released from FA-NPs, in vivo by blocking the COX-2 active site through pre-treatment with a high-affinity inhibitor. These studies were designed to demonstrate that formulation into NPs enables IV use and clinical translation of FA for optical diagnosis of inflammatory and neoplastic diseases.

MATERIALS AND METHODS

Polymer Synthesis and Characterization

We synthesized a diblock polymer-based micellar nano-formulation of FA (Fig. 1). The chemical structure and photophysical properties of FA are described in Fig. 1A. The diblock polymer, poly(propylene sulfide)₁₀₆-b-poly[oligo(ethylene glycol)₉ methyl ether acrylate]₁₇ (PPS₁₀₆-b-POEGA₁₇), was synthesized by a combination of anionic and reversible addition-fragmentation chain-transfer (RAFT) polymerization (Fig. 1B) as described below.

Synthesis of hydroxyl end-functionalized poly(propylene sulfide) (PPS₁₀₆-OH). Poly(propylene sulfide) was prepared by anionic polymerization of propylene sulfide using DBU/1-butanethiol as an initiator and subsequently end-capped with 2-iodoethanol to yield a terminal hydroxyl group. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (6.0 mmol, 0.897 mL) was dissolved in dry tetrahydrofuran (THF) (15 mL) in a dried and nitrogen flushed 50 mL round bottom flask and degassed for 30 min before lowering the reaction temperature to 0 °C. 1-Butanethiol (2.0 mmol, 0.14 mL) in THF (5 mL) was added drop wise to the flask and allowed to react for 30 min. Later, freshly distilled and degassed propylene sulfide (120 mmol, 9.387 mL)

monomer was added to the reaction mixture, and the temperature was maintained at 0 °C for 2 h. The reaction was quenched by addition of 2-iodoethanol (6.0 mmol, 1.03 g) and stirred overnight at room temperature [32]. After stirring overnight, the polymer solution was filtered to remove precipitated salt and further purified by three precipitations into cold methanol before vacuum-drying to yield a colorless viscous polymer. ¹H NMR (400 MHz; CDCl₃, δ): 1.3-1.4 (s, CH₃), 2.5-2.8 (s, -CH), 2.8-3.1 (s, CH₂), 3.72 (t, CH₂-OH). (PPS₁₀₆-OH, M_{n, GPC} = 8,200 g/mol, PDI = 1.4).

Synthesis of poly(propylene sulfide)-4-cyano-4-(ethylsulfanylthiocarbonyl)sulfanylpentanoic acid (PPS₁₀₆-ECT RAFT macro-CTA). N,N'-Dicyclohexylcarbodiimide (DCC) (0.248 g, 1.2 mmol) was added to a solution of 4-cyano-4-(ethylsulfanylthiocarbonyl)sulfanylpentanoic acid (ECT) (2, 0.314 g, 1.2 mmol), PPS₁₀₆-OH (3.12 g, 0.4 mmol), and 4-dimethylaminopyridine (DMAP) (0.015 g, 0.12 mmol) in anhydrous dichloromethane (20 mL) at 0 °C [33]. After stirring at room temperature for 24 h, the reaction mixture was filtered to remove precipitated dicyclohexyl urea and concentrated under vacuum. The crude reaction mixture was first purified by dialysis against dichloromethane for 24 h to remove free ECT, further purified through double precipitation into cold ethanol, and characterized by ¹H-NMR spectroscopy (Fig. S1). ¹H-NMR (400 MHz; CDCl₃, δ): 1.35 (t, 3H, -S-CH₂-CH₃), 1.3-1.4 (s, 3H, CH₃), 1.85 (s-C(CN)-CH₃), 2.4-2.67 (m, -CH₂-CH₂-S), 2.5-2.8 (broad s, S-CH), 2.8-3.1 (broad s, 2H, CH₂), 3.42 (q, -S-CH₂-CH₃), 3.8 (t, -OCH₂-CH₂). (PPS₁₀₆-ECT, M_{n, GPC} = 8,200 g/mol, PDI = 1.4)

Synthesis of poly(propylene sulfide)-b-poly[oligo(ethylene glycol) methyl ether acrylate] (PPS₁₀₆-b-POEGA₁₇) diblock copolymer. The diblock copolymer PPS₁₀₆-b-POEGA₁₇ was synthesized by RAFT polymerization from a PPS₁₀₆-ECT macro-chain transfer agent (macro-

CTA) using azobisisobutyronitrile (AIBN) at a 10:1 (macro-CTA:AIBN) molar ratio as the radical initiator. In a dry round bottom flask (10 mL), PPS₁₀₆-ECT (0.743 g, 0.095 mmol, M_n = 8,200 Da), OEGA (1.21 mL, 2.86 mmol), and AIBN (1.56 mg, 9.5 μmol) in dioxane (5 mL) were degassed by nitrogen purging for 30 min. The reaction was allowed to proceed for 24 h at 70 °C. The reaction product was dialyzed against methanol for 24 h, dried under vacuum to yield a purified, milky white polymer, and characterized by ¹H-NMR spectroscopy (Figure S2). ¹H NMR (400 MHz; CDCl₃, δ): 1.35 (t, 3H, -S-CH₂-CH₃), 1.3-1.4 (s, 3H, CH₃), 1.85 (s-C(CN)-CH₃), 2.4-2.67 (m, -CH₂-CH₂-S), 2.5-2.8 (broad s, S-CH), 2.8-3.1 (broad s, 2H, CH₂), 3.42 (q, -S-CH₂-CH₃), 3.68 (m, -OCH₂-CH₂), 3.8 (t, -OCH₂-CH₂). (PPS₁₀₆-*b*-POEGA₁₇, M_{n,NMR} = 16,004 g/mol)

Polymer Characterization. ¹H NMR spectra were collected for all polymers in CDCl₃ on a Brüker 400 MHz spectrometer. Molecular weights (M_n), polydispersities (PI), and compositions were determined by either ¹H NMR (Figure S3) or gel permeation chromatography (GPC) (Agilent Technologies, Santa Clara, Ca, USA) using dimethylformamide (DMF) + 0.1 M LiBr at 60 °C as the mobile phase through three serial Tosoh Biosciences TSKGel Alpha Columns (Tokyo, Japan). Serial dilutions (10 mg/mL – 0.25 mg/mL) were scanned on a digital refractometer to determine the refractive index increment (dn/dc) of polymers in order to calculate absolute molecular weights by GPC.

Physicochemical Characterization of FA-NPs

*Preparation and characterization of PPS₁₀₆-*b*-POEGA₁₇ polymer micelles (NPs).* Micelles were formed of PPS₁₀₆-*b*-POEGA₁₇ by the bulk solvent evaporation method. PPS₁₀₆-*b*-POEGA₁₇ was dissolved in chloroform at 10 mg/ml. Polymer solution (0.1 mL) was added dropwise to 1 mL phosphate-buffered saline (PBS) (-/-) with stirring. The solution was left

stirring over night to evaporate the chloroform and allow for micelle formation. The micelle solutions were passed through a 0.45 μm syringe filter and used for hydrodynamic diameter (D_h) and zeta potential (ζ) measurements, employing a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK) equipped with a 4 mW He–Ne laser operating at $\lambda = 632.8$ nm (Figure S3). TEM samples were prepared by adding 5 μL of polymer solution to pure carbon TEM grids (Ted Pella Inc, Redding, CA, USA), blotting dry (3 s) after 60 s, and counterstaining with 3% uranyl acetate (5 μL) for 20 s. The grids were dried overnight under vacuum prior to imaging on a FEI Tecnai Osiris microscope operating at 200 kV for TEM (Figure S3).

Loading of Fluorocoxib A and 5-ROX into PPS₁₀₆-b-POEGA₁₇ polymer micelles (FA-/5-ROX-NPs). FA-NPs and 5-ROX-NPs were prepared via the bulk solvent evaporation method. Either FA or 5-ROX and PPS₁₀₆-b-POEGA₁₇ were dissolved in chloroform, separately. Then, solutions of either FA or 5-ROX (50 μL , 20 mg/mL) and PPS₁₀₆-b-POEGA₁₇ (50 μL , 200 mg/mL) were mixed together, and the resulting solution was added drop wise to 1 mL PBS (-/-) with stirring. The solution was stirred overnight to evaporate chloroform and provide either FA- or 5-ROX-loaded micelles. Any unloaded precipitates were removed by centrifugation followed by aspiration of the supernatant solution. The drug loading was quantified as described previously [34, 35]. Briefly, 50 μL of DMF was added to 50 μL aliquots of the FA-NPs in order to re-dissolve micelles. Fluorescence intensity (ex: 581 nm, em: 605 nm) was measured on a TECAN Infinite F500 micro-plate reader and compared to a standard curve of FA fluorescence in 50/50 DMF/PBS (-/-). All loading measurements were performed in triplicate (Figure S3).

Nanoparticle stability in the presence of serum and whole human blood. Förster Resonance Energy Transfer- (FRET-) pair loaded-PPS₁₀₆-b-POEGA₁₇ NPs (FRET-NPs) were assessed for particle stability in the presence of whole human blood and serum. FRET-NPs were

generated by co-loading the FRET-pair DiO (donor) / DiI (acceptor) (chosen to model FA due to high lipiphilicity) within PPS₁₀₆-*b*-POEGA₁₇ NPs and using an excitation of 480 ± 5 nm and emissions of DiO (517 ± 5 nm) and DiI (573 ± 5 nm) to calculate FRET efficiency according to the following equation:

$$FRET = \frac{I_{573}}{I_{517} + I_{573}}$$

where, I_{517} = fluorescent intensity of 517 nm emission and I_{573} = fluorescent intensity of 573 nm emission. After efficient FRET was confirmed as previously established [34], the FRET-NPs (0.25 mg/mL DiO/DiI) were incubated in whole human blood or whole human blood diluted with PBS (1:1, 1:2, 1:3, and 1:5 dilutions) for 30 min. After 30 min, samples were centrifuged and plasma was monitored on a Tecan plate reader at excitation of 480 ± 5 nm and emissions of 517 ± 5 nm and 573 ± 5 nm. FRET-NPs were also incubated with varying doses of fetal bovine serum (FBS; 10, 20, 30, 40, and 50%) and FRET kinetics were monitored for 180 min. FRET-NPs (0.25 mg/mL DiO/DiI) were added to 10, 20, 30, 40, and 50% serum in a 96-well plate and FRET was measured every 5 min on a Tecan plate reader at excitation of 480 ± 5 nm and emissions of 517 ± 5 and 573 ± 5 nm. The percent FRET remaining was calculated by comparing the FRET at each dose of FBS to FRET-NPs incubated at analogous volume percent in PBS.

To confirm that a micellar nanoparticle structure was retained in the presence of serum, PPS₁₀₆-*b*-POEGA₁₇ NPs (10 mg/mL) were incubated for 0.5, 1, 2, and 4 h in wither PBS or 10% FBS and particle size distributions were measured by DLS. After incubation with either PBS or 10% FBS, NPs were passed through a 0.45 µm syringe filter and used for hydrodynamic diameter (D_h) measurements, employing a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK) equipped with a 4 mW He–Ne laser operating at $\lambda = 632.8$ nm.

Determination of CMC and H₂O₂-dependent drug release. The CMC and H₂O₂-dependent drug release of PPS₁₀₆-*b*-POEGA₁₇ NPs were quantified as previously described [34]. In both cases, Nile Red (NR) was used as a surrogate drug due to its fluorescence properties (fluoresces strongly only while in hydrophobic environment but is minimally fluorescent when released into aqueous phase where it is poorly soluble). Different dilutions were prepared from a 1 mg/mL stock solution to obtain NP samples ranging in concentration from 0.0001 to 1 mg/mL. Then, 10 μ L of a 1 mg/mL NR stock solution in chloroform was added to 1 mL of each NP sample and incubated overnight in the dark at room temperature. The next day, samples were filtered with a 0.45 μ m syringe filter and their Nile red fluorescence was measured in 96 well plates using a micro-plate reader (Tecan Infinite 500, Tecan Group Ltd., Mannedorf, Switzerland) at an excitation wavelength of 535 ± 20 nm and an emission wavelength of 612 ± 25 nm. The CMC was defined, as previously described, as the intersection on the plot of the Nile red fluorescence versus the copolymer concentration [36]. The CMC was also confirmed by the observation of size and morphological changes on DLS.

To prepare 1% NR loaded NP solution, 50 μ L of a 1 mg/mL NR stock solution in chloroform was added to 5 mL of NP solution (1 mg/mL). The residual chloroform was removed by incubation overnight in the dark at room temperature. The next day, samples were filtered using a 0.45 μ m syringe filter prior to use. NR-loaded NPs were exposed to a range of concentrations (0 to 1000 mM) of hydrogen peroxide. Fluorescence intensity of NR was monitored in a 96 well plate using a micro plate reader (Tecan Infinite 500) at an excitation wavelength of 535 ± 20 nm and an emission wavelength of 612 ± 25 nm. Release of the dye due to NP oxidation and destabilization was assessed over time based on disappearance of NR fluorescence. The loss of fluorescence for each sample at each time point was determined by

subtracting the fluorescent value from that of the sample prior to H₂O₂ addition, and the percent fluorescence remaining was determined by normalization to the same value (before addition of H₂O₂). This value for percent fluorescence remaining was subtracted from 100% and expressed as a percent release for each sample at each time point.

Imaging FA-NPs in inflammation and cancer

Fluorescent imaging of 1483 HNSCC cancer cells in vitro. Human 1483 head and neck squamous cell carcinoma (HNSCC) cells were plated on MatTek dishes and allowed to adhere. After adhering, the cells were treated with either empty NPs (5), FA-NPs (6), or 5-ROX-NPs (7) as prepared above in PBS (-/-) for 50 min at 1.3 μ M. Cells were then rinsed 3 times with Hanks balanced salt solution (HBSS)/Tyrode's buffer, and the medium was replaced by DMEM/F12 with 5% FBS. After 50 min, the cells were rinsed 1 time with HBSS/Tyrode's buffer and imaged at 40x magnification, 0.65 objective using a fluorescence microscope (Leica DM IL LED FIM).

In vivo imaging of COX-2 in inflammation. Carrageenan (50 μ L 1% in sterile saline) was injected in the rear left footpad of Sprague Dawley rats (350-400g) or C57BL/6 mice, followed by FA-NPs (1 mg/kg FA, i.p. or i.v.) at 2 h or 24 h post-carrageenan, respectively. Rats were imaged 3 h later and mice were imaged over a timecourse of 48 h in a Xenogen IVIS 200 (DsRed filter, 1.5 cm depth, 1 s). For blocking COX-2, animals were pre-dosed with indomethacin (2 mg/kg, i.p.) 1 h prior to FA-NPs injection.

Establishment of xenografts in nude mice. Female nude mice were purchased at 6-7 weeks of age from Charles River Labs. Human 1483 HNSCC cells were trypsinized and re-suspended in cold PBS containing 30% Matrigel such that 1×10^6 cells in 100 μ L were injected subcutaneously on the left flank. Tumors were allowed to grow for 2-3 weeks.

In vivo imaging of nude mice with xenografts. Female nude mice bearing 1483 xenograft tumors (800-1000 mm³) on the left flank were dosed with FA-NPs or 5-ROX-NPs (1 mg/kg FA or 5-ROX) by i.p. or tail vein injection. The animals were lightly anesthetized with 2% isoflurane for fluorescence imaging in the Xenogen IVIS 200 with the cy5.5 filter at 1.5 cm depth and 1 s exposure (f2). For the COX-2 active site blocking experiments, nude mice bearing 1483 xenografts were pre-dosed by injection with indomethacin (2 mg/kg, i.p.) 1 h prior to dosing with FA-NPs (1 mg/kg FA, i.p.).

Pharmacokinetics and biodistribution. Wild-type CD-1 mice (4-6 weeks old, Charles River) were injected via the tail vein with FA-NPs (1 mg/kg FA). Mice were euthanized at 15, 30, 60, 120, and 240 min, blood was collected by cardiac puncture into a heparinized syringe into a 1.5 ml heparinized tube on ice followed by dissection and collection of major organs such as liver, lung, heart, kidney, and spleen. The blood samples were centrifuged at 4°C at 6000 rpm for 5 min, and the plasma was transferred to clean tubes and frozen at -80°C. FA was extracted by homogenizing plasma samples in 100 mM Tris, pH 7.0, buffer and mixing an aliquot of the homogenate with 1.2x volume of acetonitrile. The acetonitrile was removed and the samples were dried, reconstituted and analyzed via reversed phase HPLC-UV using a Phenomenex 10 x 0.2 cm C18 or a Phenomenex 7.5 x 0.2 cm Synergi Hydro-RP column held at 40°C. The samples were quantified against a standard curve prepared by adding FA to homogenates of un-dosed animals followed by the workup described. Collected organs were imaged on an IVIS Lumina III system (excitation filter: 580 ± 5 nm, emission filter: 620 ± 5 nm) and the images were analyzed for photon counts for statistical analysis.

In vivo toxicology. Wild-type CD-1 mice (4-6 weeks old, Charles River) were injected via the tail vein with either saline or FA-NPs (1, 10, and 20 mg/kg FA). Blood and major organs

(heart, lungs, liver, spleen, and kidneys) were collected 24 h after FA-NP injection. Serum was obtained by incubating whole blood at room temperature for 30 min and centrifuging at 3,000g for 5 min. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN) were measured by the Vanderbilt TPSR using a commercially available Transaminase-CII kit and Blood Urea Nitrogen Test (Wako), respectively. Major organs were fixed immediately in 10% neutral buffered formalin, embedded, sliced 5 μ m thick, and stained with hematoxylin and eosin (H&E). Slides were blindly reviewed by a board certified pathologist for organ toxicity.

Statistical methods. Treatment groups were statistically compared using the student's t-test, where a p-value < 0.05 represents a statistically significant difference between groups. All data is represented as the arithmetic mean and standard error of the indicated samples size (n).

RESULTS AND DISCUSSION

Polymer synthesis and nanoparticle physicochemical characterization

Hydroxyl monofunctional PPS ($M_n = 7800$ Da, PDI = 1.4) was synthesized by anionic polymerization. Poly(propylene sulfide)ethan-1-ol (PPS₁₀₆-OH) was conjugated with 4-cyano-4-(ethylsulfanylthiocarbonyl) sulfanylpentanoic acid (ECT) (79.5% efficiency by NMR) to form poly(propylene sulfide)-ECT (PPS₁₀₆-ECT RAFT macro-CTA, Fig. S1) [32]. The macro-CTA was then used for the RAFT polymerization of oligo(ethylene glycol) methyl ether acrylate to yield PPS-b-POEGA ($M_n = 16004$ Da, Fig. S2). PPS was utilized in this design because of its phase transition behavior from hydrophobic to hydrophilic (propylene sulfide to propylene sulfoxides and sulphones) in response to ROS [25, 34, 37, 38] so that FA would be preferentially released at inflamed sites, which are characterized by high ROS. COX-2 overexpression and increased ROS-production are naturally concomitant phenomenon, motivating the choice of PPS

as a hydrophobe for this application [39, 40]. The POEGA block forms a brush-like arrangement of molecules of 9 repeating units of ethylene glycol grafted from a hydrocarbon backbone. POEGA was chosen as the hydrophilic block because recent studies have shown that this brush-like surface architecture enhances MPS stealth and overall circulation time over linear PEG [41, 42].

Figure 1. Properties and nano-formulation of Fluorococoxib A (FA). **(A)** Chemical structure and photophysical properties ($\lambda_{\text{ex}} = 581 \text{ nm}$, $\lambda_{\text{em}} = 605 \text{ nm}$) of FA. **(B)** Synthesis of PPS₁₀₆-b-POEGA₁₇, FA-NPs and 5-ROX-NPs – Reagents and conditions: (a) DCC, DMAP, CH₂Cl₂ 25 °C, 24 h; (b) POEGA, AIBN, (CH₂)₄O₂, 70 °C, 24 h; (c)

CHCl₃, PBS, 25 °C, 24 h; (d) FA or 5-ROX, CHCl₃, PBS, 25 °C, 24 h; (e) solubilization of FA or FA-NPs in PBS – (i) FA (1 mg/mL) and (ii) FA-NPs (1 mg/mL FA).

The PPS₁₀₆-b-POEGA₁₇ (NPs) and FA-loaded PPS₁₀₆-b-POEGA₁₇ (FA-NPs) micelles were formed by self-assembly in aqueous medium by the solvent evaporation method in the absence of any small molecules (NPs), or in the presence of either FA (FA-NPs) or 5-ROX (5-ROX-NPs) (Fig. 1B). The 5-ROX was formulated as a fluorescent molecule control without the COX-2-specific binding component. The FA compound alone was insoluble in water (precipitates visible in an otherwise clear aqueous solution), whereas the FA-NPs formed a deep purple solution without any apparent turbidity, suggesting stable FA colloidal solubilization. This represents the first fully aqueous formulation of FA, a major step toward its clinical translation. The average FA loading was ~ 0.063 (wt drug / wt polymer), and encapsulation efficiency was $\sim 63\%$ (Table S1). FA-NPs have an 82.3 ± 8.4 nm average hydrodynamic diameter as determined by dynamic light scattering (DLS), and they are slightly smaller in their dehydrated form as imaged by transmission electron microscopy (TEM) (Fig. 2A, B and Table S1). The zeta potential is approximately neutral (-1.51 ± 0.6 mV) (Table S1). FA-NPs retained the excitation / emission fluorescence spectra of FA (Fig. 2C), although micelle loading resulted in a partial quenching effect of FA which diminished its fluorescent intensity ~ 13 -fold (data not shown). The critical micelle concentration (CMC) of FA-NPs was quantified using Nile Red (NR) as ~ 0.065 mg/mL, and this value was confirmed by concentration-dependent changes in nanoparticle size and morphology. (Fig. 2D, E) The CMC (~ 0.065 mg/mL) of FA-NPs is an order of magnitude below the diluted concentration of FA-NPs within the blood initially after i.v. administration (0.5 – 1.0 mg/mL) at doses used within this report. FRET-based readouts and particle morphology measurements confirmed that FA-NPs are stable and retain their cargo in the presence of serum and whole human blood (Fig. S3). The chosen solubilization strategy

using a PPS-based micelle incorporated a mechanism for preferential cargo release in response to oxidative stress. As COX-2 overexpression and ROS production are naturally connected physiologic events [39, 40], release of FA is expected to be accelerated from FA-NPs in tissues which overexpress COX-2, allowing binding and retention of FA. ROS-responsiveness of NPs was confirmed by hydrogen peroxide- (H_2O_2 -) dependent NR release at varying concentrations of H_2O_2 . NPs had minimal drug release when no H_2O_2 was present, however drug cargo was released in a dose-dependent manner upon exposure to H_2O_2 . (Fig. 2F) In sum, the physicochemical characteristics of FA-NPs (i.e. size, surface charge, drug loading, fluorescent properties, ROS-degradability, and CMC) were rigorously validated and confirm that FA-NPs are a fully aqueous, i.v.-ready formulation of FA.

Figure 2. Physical properties of FA-NPs. (A and B) DLS and TEM characterization confirms NPs are under 100 nm diameter (82.3 ± 8.4 nm hydrodynamic diameter). (C) FA-NPs retain the excitation and emission ($\lambda_{\text{ex}} = 581$ nm, $\lambda_{\text{em}} = 605$ nm) spectra of the parent FA molecule. (D and E) The critical micelle concentration of NPs was determined

as ~ 0.065 mg/mL by Nile red assay and observation of size and morphological changes by DLS. (F) NPs release drug dose-dependently in response to increasing H_2O_2 .

In vitro COX-2 labeling by FA-NPs

We initially evaluated FA-NPs for targeting COX-2 in human 1483 head and neck squamous cell carcinoma (HNSCC) human cancer cells. After pre-incubation for 50 min with NPs, FA-NPs, or 5-ROX-NPs (Fig. S4A-C), the 1483 cells were washed, incubated for 50 min in serum-containing medium, and imaged under a fluorescence microscope. FA-NP treated cells were the only group that showed fluorescence in vitro. Lysotracker experiments (not shown) suggest that the intact FA-NPs are internalized through endocytosis, but the lipophilicity of FA ($\text{LogP} = \sim 6.0$) allows for diffusion through endo/lysosomal membranes to reach COX-2 within the cytosol. The lack of measurable fluorescence in cells treated with 5-ROX-NPs confirms that specific binding to COX-2 is required for effective visualization of COX-2-overexpressing cells following a “wash-out” incubation stage.

Imaging COX-2 in inflammation and cancer after intraperitoneal injection (i.p.)

We next evaluated FA-NPs for COX-2 detection in 1483 HNSCC tumor xenografts in vivo. We initially performed COX-2 imaging studies after i.p. administration to mimic previous administration methods for FA in DMSO [7]. In mice injected i.p. with FA-NPs (1 mg/kg FA), no fluorescence was observed in the tumor during the first 30 min post-injection, but signal was reproducibly detected in the COX-2-expressing 1483 tumors at 3 to 4 h post-injection. We next evaluated whether fluorescence observed in the tumors was due strictly to nanoparticle

accumulation within the tissue by EPR effect or also by release of the FA molecule and its binding to COX-2. To this end, nude mice with 1483 xenografts were pretreated with either DMSO or indomethacin in DMSO (2 mg/kg, i.p.) prior to FA-NPs dosing (1 mg/kg FA, i.p.). At 4 h post-injection, the vehicle-pretreated mice showed strong fluorescence in their tumors, whereas the tumors of the indomethacin-pretreated mice displayed significantly lower fluorescence ($p = 0.003$, Fig. 3A-C). Lastly, 5-ROX-NPs which have no specific COX-2 binding moiety were administered and showed similar fluorescent signal to tumors in indomethacin pretreated mice and significantly lower fluorescent signal than tumors in FA-NP-treated mice ($p = 0.005$, Fig. S5). These observations collectively confirm that FA is released from FA-NPs to bind specifically with COX-2, increasing retention within tumors.

We also evaluated FA-NPs in vivo using carrageenan-induced inflammation in the rat footpad. It is well documented that COX-2-derived prostaglandins are major contributors to the acute inflammation that develops 2 h after carrageenan injection into the paw in this model [43]. The inflamed footpad model is ideal for imaging inflammation because it enables a direct comparison with the vehicle-injected contralateral footpad. After i.p. injection, FA-NPs (1 mg/kg FA) targeted the inflamed rat footpad with an average 10-fold increase in fluorescence over that of the contralateral control footpad ($p < 0.002$), and the uptake was efficiently blocked by pretreatment with the nonselective COX inhibitor indomethacin (Fig. 3D-F). The results in the rat footpad inflammation model demonstrate that FA-NPs effectively target sites of inflammation in vivo in order to visualize COX-2 in carrageenan-induced edema. The blocking study further confirms that the FA molecule is released from the nanoparticle and able to engage COX-2.

Figure 3. Targeted in vivo imaging of COX-2 in inflammation and cancer. Female nude mice bearing COX-2-expressing 1483 HNSCC tumor xenografts were dosed with FA-NPs (1 mg/kg FA, i.p.) and imaged at 4 h post-injection of NPs in a Xenogen IVIS200 optical imaging instrument. A subset of animals was pretreated with indomethacin to block the FA binding site on COX-2. (A-B) Fluorescence images of 1483 HNSCC tumor-bearing mice injected with FA-NPs with or without indomethacin pre-treatment 1 h prior to i.p. injection of FA-NPs. (C) Quantification of images showing relative tumor signal ($n = 10$, $p = 0.003$). (D-E) Sprague Dawley rats with carrageenan-induced inflammation in their right hind footpads and with or without pre-injection of competitive COX-binding molecule indomethacin (2 mg/kg) were imaged 3-h post injection with FA-NPs (1 mg/kg FA, i.p.). (F) Quantification of signal in images of inflamed (carrageenan injected) versus non inflamed footpads with and without indomethacin pre-treatment ($n = 8$, $p < 0.002$).

Evaluation of FA-NPs after intravenous injection

Effectiveness and specificity of FA-NPs for visualizing animal models of inflammation and cancer was initially confirmed using a traditional i.p. administration method for FA. However, i.p. administration is not ideal for systemic administration of nanoparticles due to lowered bioavailability and slower tissue distribution relative to i.v. injection. The biodistribution and pharmacokinetics of FA-NPs was next monitored after a single i.v. administration (1 mg/kg FA) in wild-type CD-1 mice in order to inform future clinical development. FA-NPs had a 1.1 h plasma half-life after the single administration (Fig. 4A). FA fluorescence was measured in organs of interest ex vivo at each time point after FA-NP administration. The FA-NPs biodistributed most heavily to the liver, kidney, and lungs early after administration (Fig. 4B).

Interestingly, we did not observe high fluorescence within the spleen, a major MPS organ typically associated with nanoparticle clearance, which is likely a result of tracking the FA molecule rather than a fluorophore conjugated to the polymer component of the NPs. The kinetics of organ biodistribution correlated well with FA-NP persistence in the blood, with amount of FA-NPs in all organs decreasing at similar rates to FA-NP blood clearance. Importantly, the fluorescent signal in non-targeted major organs nearly returns to baseline by 4 h post-injection, indicating that this is an ideal time point for imaging target sites with little background noise.

Figure 4. Pharmacokinetics and biodistribution of IV-injected FA-NPs. (A) FA-NPs have 1.1 h circulation half-life after i.v. administration via tail vein. (B) FA-NPs biodistribute to liver, kidney, and lungs after i.v. administration, but FA is cleared from these organs by 4 h post-injection. (C) FA imaging at 4 h after injection with FA-NPs reveals a significant increase in tumor signal relative to liver, kidney, or lungs. ($n = 9$, $p < 0.004$) (D) FA-NP signal is significantly higher in the inflamed footpad of mice over 8 h post-injection ($n = 3$, $p < 0.01$, *).

Based on this insight, we next monitored the pharmacokinetics, biodistribution, uptake, and retention of FA-NPs in animal models of cancer and inflammation after a single i.v. administration. Nude mice bearing 1483 HNSCC xenografts were administered FA-NPs (1 mg/kg FA, i.v.) and tissues of interest (tumor, liver, kidney, lung, and muscle control) were excised and imaged ex vivo after 4 h. Remarkably, maximal signal was documented in the tumor even without normalization to tissue weight, with raw tumor fluorescence intensity of over 2-fold

above raw liver fluorescence intensity (Fig. 4C). To investigate the kinetics of FA-NP uptake and retention at sites of inflammation following i.v. delivery, the inflamed footpads of C57BL/6 mice were imaged beginning 24 h after carrageenan-induced edema (100 μ L, 1%). A mouse model was chosen for this experiment due to the slow resolution of inflammation and extended time course of COX-2 expression within this model (up to 48 h compared to only 5 h in the rat model). Inflamed footpads of mice dosed with FA-NPs (1 mg/kg FA, i.v.) had up to 3-fold higher fluorescence relative to the contralateral control footpads throughout the time course (Fig. 4D). Maximal signal was detected at the first time point measured (2 h post-injection), and signal was detected above background over 8 h post-injection. The high retention of FA-NPs within inflamed footpads between 4 – 8 h is in contrast to the retention in other major organs after 4 h; this result further supports that 4 – 8 h is the optimal window for imaging in order to achieve high signal-to-noise (SNR) in the inflamed and / or cancerous tissue. Amplex Red was used to quantify H₂O₂ within both the inflamed and contralateral tissues, showing over 2-fold increase in ROS-production within the inflamed tissue (Fig. S6). The concomitant overexpression of COX-2 and ROS-production within inflamed tissue increases release of FA from FA-NPs and FA binding to COX-2 at these sites, contributing to the high SNR achieved over contralateral tissue.

The clinical translation of promising molecular imaging agents is contingent upon safety of the reagent at doses well in excess of those needed for imaging effectiveness. To test the safety profile of FA-NPs, we i.v. administered the lowest effective imaging dose (1 mg/kg FA) as well as 10x (10 mg/kg FA) and 20x (20 mg/kg FA) higher doses. Biochemical analysis of liver (ALT and AST) and kidney (BUN) toxicity and histological analysis of major organ (heart, lungs, liver, spleen, and kidneys) toxicity was performed 24 h post-injection. No significant increase in serum markers of liver and kidney toxicity were observed compared to saline treated

mice at any dose administered (Fig. 5A). Moreover, major organs were observed by a blinded, board certified pathologist and no apparent toxicity was seen within any treatment groups (Fig. 5B).

Effective detection of inflamed, premalignant, and malignant tissue with high signal to noise relative to normal tissue is challenging [44, 45]. The only optical imaging agents currently FDA approved are indocyanine green- (ICG-) or fluorescein-based dyes, which are highly nonspecific and not clinically useful for high resolution molecular imaging. The clinical translation of more recently developed agents has been hampered by cost and long-term risk of clinical failure, exemplifying the need for new reagents which are both effective and simple [46]. More recently, nanoparticle-based molecular imaging technologies have shown great promise by rationally designing agents with multi-functionality [47, 48] and high signal-to-noise [49, 50] in pathological tissue. Though the first clinically-used, fluorescent COX-2 imaging reagent is likely to be based on near infrared (NIR) fluorophores rather than rhodamine, FA was chosen as the probe of choice in these studies because it has been most rigorously validated as a molecular imaging agent for visualizing overexpression of COX-2 in inflammation [7], HNSCC tumor xenografts [7], spontaneous murine colorectal carcinomas [7], spontaneous canine colorectal carcinoma [8], canine transitional cell carcinoma xenografts [9], and non-melanoma basal cell carcinoma allografts and spontaneous basal cell carcinomas [10]. Importantly, fluoroxocibs that enable imaging in the near infrared (NIR) region are in earlier stages of development [51], and we anticipate that our delivery approach will translate seamlessly to these new compounds and yield even better SNR, potentially visualizing COX-2 at doses up to 100x lower than those safely administered to mice within the present studies (0.2 vs. 20 mg/kg). The main outcome sought from the current studies was to validate that packaging of FA within NPs enables fully aqueous

administration and that the resultant formulation is both safe and effective. Critically, these studies show a nanotechnology could be coupled with a highly specific and effective imaging compound (FA) to enable fully aqueous administration, leveraging a combination of the EPR effect and inflammation-responsive drug release to achieve safe, efficient, and COX-2-dependent visualization of inflamed, pre-malignant, and malignant tissue.

Figure 5. In vivo toxicology of FA-NPs after i.v. administration. (A) Serum chemical markers of liver (ALT and AST) and kidney (BUN) toxicity measured 24 h after i.v. administration of saline, 1 mg/kg, 10 mg/kg, or 20 mg/kg FA-NPs (FA dose). (B) H&E sections of liver and kidney at 20x magnification.

CONCLUSIONS

FA-NPs were developed to solubilize the first fluorescent COX-2 inhibitor, FA, and enabled delivery of FA in 100% aqueous solution for the first time. It was verified that through a combination of the nanoparticle-mediated EPR effect, ROS-triggered nanoparticle disassembly, and the high affinity binding of FA to COX-2, FA-NPs provide a unique, multi-stage mechanism for attaining unprecedentedly high uptake and signal-to-noise in COX-2-expressing tissues compared with normal tissues. Analysis of the pharmacokinetics and biodistribution of FA-NPs revealed an optimal imaging window of 4 – 8 h post-injection where FA is cleared from non-targeted major organs but persists within targeted tissue for high SNR imaging. FA-NP imaging of intracellular COX-2 (as opposed to nonspecific tissue accumulation), was unequivocally proven through studies showing that the fluorescent signal of FA is not retained in control tissues under any circumstances or in target tumor/inflamed tissues if the binding site of COX-2 is blocked. Finally, the FA-NP formulation was also proven to be very safe and cause no signs of toxicity even at a 20x the dose needed for COX-2 visualization. Our collective data provide strong support for the continued development of FA-NPs as a formulation strategy for detection of COX-2 in clinical settings amenable to epifluorescent optical measurements.

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