Acta Biomaterialia 75 (2018) 323–333

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Hyaluronic acid formulation of near infrared fluorophores optimizes surgical imaging in a prostate tumor xenograft ☆

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ARTICLE INFO

Article history:
Received 8 February 2018
Received in revised form 6 June 2018
Accepted 7 June 2018
Available online 8 June 2018

Keywords:
Polymer conjugate
Self-assembly
Nanoparticle
Intraoperative imaging
ICG
Cy7.5

ABSTRACT

The presence of positive surgical margins confers an increased risk of biochemical relapse and need for salvage therapy in men undergoing radical prostatectomy. Image-guided surgery using near-infrared (NIR) fluorescent contrast agents is a potential method to detect remaining cancerous tissue. The objective of this study was to evaluate three hyaluronic acid (HA) nanoparticle (NP) formulations loaded with NIR fluorophore for their ability to contrast-enhance prostate cancer. HA was modified by conjugation with the hydrophobic ligand, aminopropyl-1-pyrenebutanamide to drive nanoparticle self-assembly. Indocyanine green (ICG) was physicochemically entrapped in the HA-NP, termed NanoICG. Alternatively, Cy7.5 was directly conjugated to amphiphilic HA, termed NanoCy7.5. NanoCy7.5 was synthesized with two HA molecular weights to determine the HA size contribution to delivery to PC3 prostate tumor xenografts. Contrast-enhancement of the tumors and relative biodistribution were assessed by a series of fluorescence imaging, image-guided surgery with spectroscopy, and microscopic techniques. Intravenously administered NanoICG improved tumor signal-to-noise ratio (SNR) at 24 h over ICG by 2.9-fold. NanoCy7.5 with 10 kDa and 100 kDa HA improved tumor SNR by 6.6- and 3.1-fold over Cy7.5 alone, respectively. The PC3 xenograft was clearly identified with the image-guided system providing increased contrast enhancement compared to surrounding tissue for NanoICG and NanoCy7.5 with 10 kDa HA. NIR fluorescence microscopy showed that Cy7.5 in NPs with 10 kDa HA were distributed throughout the tumor, while NanoCy7.5 with 100 kDa HA or NanoICG delivered dye mainly to the edge of the tumor. CD31 staining suggested that PC3 tumors are poorly vascularized. These studies demonstrate the efficacy of a panel of HA-derived NPs in identifying prostate tumors *in vivo*, and suggest that by tuning the structural properties of these NPs, optimized delivery can be achieved to poorly vascularized tumors.

Statement of Significance

We have demonstrated the potential of a panel of near-infrared fluorescent (NIRF) nanoparticles (NPs) for image-guided surgery in a prostate cancer xenograft model. Image-guided surgery and imaging of organs *ex vivo* showed greater tumor signal and contrast when mice were administered NIRF dyes that were covalently conjugated to ($NanoCy7.5_{10k\text{-}PBA}$) or physicochemically entrapped in ($NanoICG_{PBA}$)

Abbreviations: AUC, Area under the curve; FIGS, fluorescence image-guided surgery; ICG, indocyanine green; IGS, image-guided surgery; HA, hyaluronic acid; NIR, near-infrared; NIRF, near-infrared fluorescent; NP(s), nanoparticle(s); PBA, aminopropyl-1-pyrenebutanamide; RARP, robot-assisted radical prostatectomy.

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https://doi.org/10.1016/j.actbio.2018.06.016

hyaluronic acid (HA) NPs, compared to free dyes. These results show the potential to use these NPs as tools to detect the margins of tumors and to differentiate healthy and tumor tissue intraoperatively. Moreover, this project provides insight into selecting optimal formulation strategies for poorly vascularized tumors.

1. Introduction

Prostate cancer is the most diagnosed cancer in men, accounting for almost 1 in 5 new cancer diagnoses, and is the third leading cause of death resulting from cancer in men in the United States [1]. Nearly 80% of new prostate cancer diagnoses have localized disease [1], with the most common interventions being surgery, radiotherapy, and monitoring or surveillance. Population-based data sets show that 40–50% of newly diagnosed men with localized disease undergo radical prostatectomy [2,3], making it the most common treatment in this setting. However, treatment for prostate cancer can have adverse effects on urinary, bowel, or sexual function.

Up to 40% of men have biochemical recurrence after radical prostatectomy upon 10–15 year follow-up [4]. In one study, over 30% of men undergoing open radical prostatectomy had positive margins, and the presence of a positive margin was associated with increased risk of biochemical recurrence, local recurrence, and need for salvage therapy [5]. Surgery for prostate cancer has evolved, with open radical prostatectomy being largely replaced with robot-assisted radical prostatectomy (RARP) [6], with numbers of RARPs increasing from 1.8% to 85% in the United States during the period of 2003–13 [7]. A meta-analysis reported that positive surgical margins for RARPs ranged from 6.5% to 32% (mean 15%) [8]. Positive surgical margin rates for RARPs have been reported to be at least equivalent to radical retropubic prostatectomy in a meta-analysis [9], and a randomized, controlled phase 3 study showed no statistically significant difference in positive surgical margin rates between radical retropubic prostatectomy vs RARP [10].

Fluorescence image-guided surgery (FIGS) has the potential to differentiate normal versus diseased tissue and provide real-time guidance for pathology on resected specimens. FIGS approaches often make use of the "NIR window", which allows for imaging in wavelengths of approximately 700–1000 nm, where light has its maximum depth of penetration in tissue due to relatively lower tissue absorption of light by water and hemoglobin [11]. The FDA- and EMA-approved NIR fluorophore, indocyanine green (ICG), has been used in tens of thousands of patients with low side effect rate (<0.15%) [12]. ICG primarily reflects initial vascular distribution (through binding to albumin and other plasma proteins) and subsequent hepatobiliary excretion, and thus has been used for tests of cardiac output, hepatic function and liver blood flow, as well as ophthalmic angiography [12].

Fluorescence imaging has also been demonstrated clinically during cancer surgery for a number of cancer types, utilizing fluorophores such as ICG, methylene blue, and 5-aminolevulinic acid [13]. Fluorescence-enhanced RARP using ICG injection directly into the prostate of patients has been performed for sentinel lymph node mapping [14–16]. Further, direct injection of multimodal ICG-^{99m}Tc-NanoColl into the prostate was used for sentinel lymph node mapping [17]. These studies showed fluorescence surgery to be feasible and safe, but displayed variable levels of specificity and sensitivity for detection of nodal metastasis.

Targeted NIR dyes and nanoparticles (NPs) are being developed as novel contrast agents for IGS. Several types of fluorescent NPs have been developed for cell imaging and biomedical applications [18,19]. Fluorescent NPs take advantage of the enhanced permeability and retention (EPR) effect of tumors, can be specifically targeted to tumors, and can be modified to avoid clearance [20]. In this study we assessed the delivery of NIR dyes using hyaluronic acid (HA)-based NPs. HA is a glycosaminoglycan found throughout the body, can have a variety of sizes composed of repeat units of β(1,4) D-glucuronic acid and β(1,3)N-acetyl-D-glucosamine, and is capable of binding to a number of receptors, including CD44, LYVE1, and Stabilin-2 (HARE) [21,22]. With its biocompatible, non-immunogenic characteristics, and potential to target overexpressed CD44 on some tumors or accumulate passively by EPR, HA has been used in synthesis of a number of NPs for imaging and drug delivery applications [23–28]. A common method of NP formulation is to modify the hydrophilic HA backbone with hydrophobic ligands to drive self-assembly [29–31].

In this study, we report the *in vivo* imaging evaluation of a panel of three HA-derived near-infrared fluorescent (NIRF) NPs that used physicochemically entrapped ICG or covalently conjugated Cy7.5. The "NanoICG" formulation used 10 kDa HA polymers and the "NanoCy7.5" formulation used 10 kDa and 100 kDa HA polymers. The NPs were delivered to nude mice bearing PC3 tumors and examined at 24 h. Image-guided surgery, tumor contrast, biodistribution, and distribution through tumors were analyzed.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (10 kDa and 100 kDa) was purchased from Lifecore Biomedical (Chaska, MN, USA). 1-Pyrenebutyric acid, 1,3-diaminopropane, and ICG was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cy7.5-amine was purchased from Lumiprobe Corporation (Hallandale Beach, FL, USA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), methanol, and dialysis membranes (3500 MWCO and 6000–8000 MWCO), were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethanol was purchased from the Warner-Graham Company (Cockeysville, MD, USA) or the General Supply at the University of Nebraska Medical Center. Desalting PD10 columns were purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). D_2O [99.9% D] and DMSO-D_6 [99.8% D] were purchased from EMD Millipore. All water was obtained from a Barnstead NANOpure Diamond or Barnstead GenPure system (Thermo Fisher Scientific) producing 18.2 MΩ water.

2.2. Cell lines and culture

PC3 prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). PC3 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 I.U. penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37 °C in a humidified incubator with 5% CO_2.