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Near infrared fluorescent nanoparticles based on hyaluronic acid: Self-assembly, optical properties, and cell interaction

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ABSTRACT

Fluorescent imaging agents that can specifically highlight tumor cells could have a significant impact on image-guided tumor removal. Here, fluorescent nanoparticles (NPs) derived from hyaluronic acid (HA) are investigated. HA is a ligand for the receptor CD44, which is a common biomarker present on many primary tumor cells, cancer-initiating cells, and tumor-associated fibroblasts. In addition, a family of enzymes that degrade HA, called hyaluronidases (HYALs), are also overexpressed with increased activity in many tumors. We report the design and development of a panel of targeted imaging agents using the near-infrared (NIR) dye, Cy7.5, that was directly conjugated to hydrophobically-modified HA. Two different molecular weights of HA, 10 kDa and 100 kDa, and three different degrees of hydrophobic moiety conjugation (0, 10, and 30 mol%) were utilized to develop a panel of NPs with variable size that ranged from 50 to 400 nm hydrodynamic diameter (HD) depending HA molecular weight, extent of fluorescence quenching (25–50%), kinetics of cellular uptake, and targeting to CD44+ cells. The kinetics and energy-dependence of cellular uptake in breast and prostate cancer cell lines, MDA-MB 231 and PC-3 cells, respectively, showed increased uptake with longer incubation times (at 4 and 8 h compared to 1 h), as well as uptake at 37 °C but not 4 °C, which indicated energy-dependent endocytosis. NP uptake studies in the presence of excess free HA showed that pre-treatment of cells with excess high molecular weight (MW) free HA decreased NP uptake by up to 50%, while no such trend was observed with low MW HA. These data lay the foundation for selection of optimized HA-derived NPs for image-guided surgery.

Statement of Significance

Here, hyaluronic acid (HA), a well-studied biomacromolecule, is modified with a near infrared fluorophore and a hydrophobic moiety. The significance of this work, especially for imaging applications, is that the impact of HA molecular weight and the hydrophobic moiety conjugation degree on fluorescence and cell interaction can be predicted. With respect to existing literature, the eventual use of these HA-based NPs is image-guided surgery; thus, we focus on the dye, Cy7.5, for conjugation, which is more NIR than most existing HA literature. Furthermore, HA is a ligand for CD44, which is associated with cancer and tumor microenvironment cells. Systematic studies in this work highlight that HA can be tuned to maximize or minimize CD44 binding

1. Introduction

Surgery is a primary method of treatment for more than 50% of patients with some form of cancer [1]. Current diagnostic imaging techniques are primarily based on computed tomography, positron emission tomography, magnetic resonance imaging, and ultrasound.

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These can be limited in their ability to differentiate benign and malignant lesions, especially for small tumors [2]. Detection of small malignant lesions and residual cancer cells remaining at the surgical margin impacts tumor recurrence, follow-up treatment, and ultimately, patient survival.

Use of NIR fluorescence imaging in cancer surgery could be paradigm shifting, resulting in decreased healthcare costs and better patient survival. NIR light can penetrate tissue on a millimeter scale, while visible light can only penetrate on the order of micrometers. NIR fluorophores offer several advantages over UV–Vis dyes, such as deeper tissue penetration due to lower absorbance and scatter from hemoglobin and water, decreased autofluorescence from endogenous fluorophores, and minimum photo damage to the native tissue [2–4]. Intraoperative NIR imaging enables visualization of superficial tumors (<8 mm below surface of tissue) and local metastatic lesions, is relatively safe for patients and surgeons, and has the potential for specific targeting [5]. NIR dyes have been used to explore various applications regarding in vitro and *in vivo* imaging, including, pH changes, biomolecules, reactive oxygen and reactive nitrogen species signaling, metal ions and enzymes [3]. Currently available FDA-approved NIR dyes, indocyanine green (ICG) and methylene blue, are non-specific [6,7]. Demand for new probes that have higher photo-stability, quantum yield, Stoke's shift, and ease of modification for various applications is required for the next generation of specifically targeted NIR fluorescent bioconjugates. For example, IR800-CW (LI-COR Biosciences, Lincoln, NE) conjugated to bevacizumab (against VEGF) is being studied in patients in Phase I clinical trials [8].

NPs have gained recent attention in biomedical imaging and especially for image-guided surgery [9–11]. Fluorescent dyes and drugs embedded in the core of nanomaterials have been shown to possess higher chemical stability (against enzymes or ROS) and photo stability, improved water solubility, and targetability. Furthermore, macromolecules in general, including NPs, can preferentially accumulate in tumors due to enhanced vascular permeability and poor lymphatic drainage [12]. Specificity can be enhanced by conjugation of targeting ligands to improve intratumoral accumulation, drug efficacy, and reduced off-target toxicity [13,14]. To that end, HA, a non-sulfated glycosaminoglycan comprised of disaccharide repeat units of alternating (1–3)-β linked *N*-acetyl-D-glucosamine and (1–4)-β linked D-glucuronic acid, is a biopolymer uniquely suited for biomedical applications. HA is biocompatible and biodegradable by HYALs, which are overexpressed in many tumors [15–17]. Of note, HA is a ligand for CD44, which is receptor commonly present on many primary tumor cells, cancer-initiating cells, and tumor-associated fibroblasts [18,19].

Several groups have taken advantage of CD44's endogenous ligand, HA, to develop NPs that can target multiple types of cancer for drug and/or imaging agent delivery [20–22]. One common method of NP formation using HA is modification with hydrophobic ligands and polymers. For example, ceramide, bile acids, or polymers such as poly [lactide-(co-glycolic acid)] (PLGA), poly (β-amino ester) have been conjugated or grafted to HA to drive self-assembly into nanoparticles for targeted delivery of therapeutic moieties [20,21,23–28]. Likewise, we have recently described HA-derived NPs that entrap ICG, termed NanoICG, for image-guided surgery using human breast tumor xenografts in mice [28]. In this case, HA was modified with different hydrophobic ligands to form self-assembled micelle-like structures that could entrap ICG. Although NanoICG demonstrated significantly higher contrast to noise compared to ICG alone, the physical entrapment of ICG has inherent limitations in determining the role and distribution of amphiphilic HA, as ICG can also associate with serum proteins [7,29].

Furthermore, it has been shown that for a polymer, molecular weight plays a large role in fundamental NP properties such as size, toxicity and *in vivo* behavior [30,31]. This work presents an initial analysis into the effect of HA MW and hydrophobic ligand content on NP properties. Accordingly, we have designed and developed a panel of HA-derived nanoparticles using the NIR dye Cy7.5 directly conjugated to HA. We report here the effect of HA molecular weight, hydrophobic ligand content, and direct dye conjugation on the physical, chemical, optical, and biological properties of HA-derived nanoparticles in vitro. These studies lay the foundation for the next generation of HA-based NIR imaging agents for image-guided surgery.

2. Materials and methods

2.1. Materials

HA (10 and 100 kg/mol) was purchased from LifeCore Biomedical (Chaska, MN). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and N,N-dimethylformamide (DMF) were purchased from Fisher Scientific. 5-beta cholanamide (5βCA) was synthesized from 5-beta cholanic acid (Fisher Scientific) using a previously published procedure [22,28]. All reagents were used without further purification unless specified otherwise. Cy7.5-amine was obtained from Lumiprobe Corporation (Hallandale Beach, FL). Desalting PD10 columns and dialysis membranes (3500 MWCO and 6000–8000 MWCO) were purchased from GE healthcare and Fisher Scientific, respectively. NMR was performed on a 500 MHz Bruker or 600 MHz Varian system using a 5 mm probe at room temperature. Size exclusion chromatography (SEC) was performed in an aqueous mobile phase containing 0.1 M sodium phosphate (NaH_2PO_4) with 250 ppm sodium azide in water at a flow rate 0.45 mL/min. HA-conjugates dissolved in the mobile phase were separated using Ultrahydrogel™ 250 and 1000 columns (Waters Corporation). The SEC system was equipped with a Waters 2998 photodiode array, 2414 refractive index detector, and a Wyatt miniDAWN TREOS multiangle laser light scattering detector. Data was recorded and analyzed using the ASTRA (version 6.1) software.

DMEM, fetal bovine serum, and penicillin/streptomycin were obtained from Cell and Viral Vector lab at Wake Forest Health Sciences. PBS was obtained from Hyclone™ Laboratories Inc. (GE Healthcare). Human breast (MDA-MB 231) and prostate (PC-3) cancer cell lines were obtained from ATCC (Manassas, VA).

2.2. General procedure for conjugation of 5-beta cholanamide to hyaluronic acid

Sodium hyaluronate (100 mg of 10 or 100 kg/mol) was dissolved into 12.5 mL nanopure water. HA polymer conjugates or nanoparticles derived from 10 kDa HA are identified with subscript "10", while those from 100 kDa HA are identified with subscript "100". Next, EDC and NHS (78–154 mmol each) were dissolved into the HA/nanopure water solution to give a ten-fold molar excess of coupling agent. HA solution was stirred at room temperature for 30 min to allow activation of carboxylic acid groups before further functionalization and then 12.5 mL of DMF was slowly added to this solution. Zero (no ligand, subscript Ø), 10 (low content, subscript L), or 25–30 (high content, subscript H) mol% 5βCA was dissolved into 12.5 ml of DMF under stirring and low heat. After cooling to room temperature, 12.5 ml of nanopure water was added to the 5βCA/DMF solution. Finally, 5βCA solution was added to the activated HA mixture at room temperature and stirred for 12–15 h. The reaction mixture was dialyzed against 1:1 ethanol: ultrapure water for one day and ultrapure water for