Cancer Letters xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Cancer Letters

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

Polymeric nanotheranostics for real-time non-invasive optical imaging of breast cancer progression and drug release

Shiran Ferber [a,1], Hemda Baabur-Cohen [a,1], Rachel Blau [a,1], Yana Epshtein [a], Einat Kisin-Finfer [b], Orit Redy [b], Doron Shabat [b], Ronit Satchi-Fainaro [a,*]

[a] Department of Physiology and Pharmacology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel
[b] School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences, Tel-Aviv University, Tel-Aviv, Israel

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Theranostics
NIR fluorophore
Polymeric nanomedicines
Tumor targeting
Paclitaxel
Breast cancer

ABSTRACT

Polymeric nanocarriers conjugated with low molecular weight drugs are designed in order to improve their efficacy and toxicity profile. This approach is particularly beneficial for anticancer drugs, where the polymer–drug conjugates selectively accumulate at the tumor site, due to the enhanced permeability and retention (EPR) effect. The conjugated drug is typically inactive, and upon its pH- or enzymatically-triggered release from the carrier, it regains its therapeutic activity. These settings lack information regarding drug-release time, kinetics and location. Thereby, real-time non-invasive intravital monitoring of drug release is required for theranostics (***thera***py and diag***nostics***). We present here the design, synthesis and characterization of a theranostic nanomedicine, based on *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer, owing its fluorescence-based monitoring of site-specific drug release to a self-quenched near-infrared fluorescence (NIRF) probe. We designed two HPMA copolymer-based systems that complement to a theranostic nanomedicine. The diagnostic system consists of self-quenched Cy5 (SQ-Cy5) as a reporter probe and the therapeutic system is based on the anticancer agent paclitaxel (PTX). HPMA copolymer–PTX/SQ-Cy5 systems enable site-specific release upon enzymatic degradation in cathepsin B-overexpressing breast cancer cells. The release of the drug occurs concomitantly with the activation of the fluorophore to its Turn-ON state. HPMA copolymer-SQ-Cy5 exhibits preferable body distribution and drug release compared with the free drug and probe when administered to cathepsin B-overexpressing 4T1 murine mammary adenocarcinoma-bearing mice. This approach of co-delivery of two complementary systems serves as a proof-of-concept for real-time deep tissue intravital orthotopic monitoring and may have the potential use in clinical utility as a theranostic nanomedicine.

1. Introduction

In the past few years, tremendous efforts have been employed in monitoring cancer treatment, detecting response to drugs and measuring real-time accumulation of the drug within the tumor. Numerous nanocarriers have been developed (e.g., polymers, liposomes, micelles, dendrimers, etc.) and exploited as delivery vehicles for anticancer drugs to improve the drugs' biodistribution, solubility, half-life, and thus efficacy and safety [1]. Clinically-available fluorescence-based imaging contrast agents (e.g., indocyanine green and fluorescein) hold many of the limitations as chemotherapeutic agents including low molecular weight, short half-life and poor selectivity [2,3]. Consequently, monitoring slow processes, such as drug accumulation at a tumor site, is challenging. Combining therapeutic and diagnostic modalities on the same delivery system, thereby forming a theranostic (***thera***py and diag***nostic***) nanomedicine, may overcome these limitations, while enabling simultaneous monitoring and treatment of angiogenesis-dependent diseases, such as cancer [4]. Information obtained from theranostic nanomedicines is exploited for fine-tuning the therapeutic dose, while monitoring the progression of the diseased tissue, treatment efficacy and delivery kinetics [5,6]. This, from a clinical prospective, should enhance early diagnosis and treatment and may eliminate drugs under- or over-dosing, resulting in a more personalized treatment.

Among different imaging modalities (e.g., radiography, magnetic resonance imaging and ultrasound), optical imaging holds several advantages. Fluorescent molecular probes are highly

* Corresponding author. Address: Department of Physiology and Pharmacology, Sackler School of Medicine, Room 607, Tel-Aviv University, Tel-Aviv 69978, Israel. Tel.: +972 3640 7427; fax: +972 3640 9108.
E-mail address: ronitsf@post.tau.ac.il (R. Satchi-Fainaro).
[1] These authors contributed equally to the manuscript.

http://dx.doi.org/10.1016/j.canlet.2014.02.022
0304-3835/

sensitive, possess a high spatial resolution, enable simultaneous multicolor imaging and specificity, by signal activation in the tissue of interest they may possess high target to background ratio (TBR), and are relatively inexpensive [5,7]. Furthermore, they do not hold long term health risks, like other commonly-used computed tomography (e.g., PET-positron emission tomography and SPECT-single-photon emission computed tomography), which expose the patient to ionizing radiation.

An ideal theranostic nanomedicine system should hold (*i*) long circulation time in the body, (*ii*) high specificity to the target tissue, (*iii*) an efficient release mechanism, (*iv*) an imaging probe that enables monitoring its activity, (*v*) deep tissue penetration, and (*vi*) high TBR ratio [8]. High specificity can be obtained via passive targeting, by exploiting the enhanced permeability and retention (EPR) effect or via an additional functional targeting moiety [9,10].

In contrast to thin layer imaging of cells or surfaces, the signal from fluorescent probes *in vivo* is impeded by the emitted fluorescence from tissues and biomolecules (e.g., water, melanin, proteins and hemoglobin), which absorb photons in the wavelengths range of 200–650 nm (i.e., low signal-to-noise ratio) [11,12]. In addition, tissues contribute to reflection, refraction and scattering of incident photons, thus increasing the background and blur of the obtained image [13–15]. The 'imaging wavelength window' left for *in vivo* imaging in order to overcome these obstacles is at the near infra-red (NIR) range (i.e., 650–1450 nm). In this range, auto-fluorescence is minimal and scattering of light is reduced, enabling deep tissue penetration and facilitating non-invasive monitoring [16].

One way to maximize the signal from the target and to minimize the signal from background (i.e., high TBR ratio), is the use of activatable optical probes. The fluorescent signal is silenced/"OFF" under physiological conditions, and is Turned-ON at a designated site and/or under specific conditions [5,17]. Although numerous classes of Turn-ON optical probes have been described in the literature for detection of chemical and biological factors [18–20], to this point, most polymer-based theranostic nanomedicines studies utilize an 'always ON' theranostic systems. In these systems, fluorescent signal is obtained from the background and desired signal at once, resulting in low TBR. Among methods used to obtain a selective Turn-ON mechanism, Förster resonance energy transfer (FRET) is the most common and efficient. In this process, following excitation of the donor, the acceptor will absorb the emission energy of the donor and will turn off the fluorescent signal. The donor and the acceptor are required to have overlapping emission and absorbance spectra, as well as close proximity between them. A FRET-based probe is Turned-ON upon distance that results in the diffusion of the donor fluorophore away from the acceptor, and generation of a measurable fluorescent signal [17,21].

This process includes two approaches, fluorophore–fluorophore (self-quenching) and fluorophore–quencher activation. The donor is always a fluorophore, however the acceptor can be either a quencher – a dye with no native fluorescence (FRET) or a second fluorophore (self-quenching) [22]. When a FRET fluorophore–quencher process occurs, the excited fluorophore can transfer its emission energy to the nearby quencher. When the excitation and emission picks overlap, like in the case of Cy5, the fluorophore can serve as a quencher and adsorb the excitation energy. Under these circumstances the emitted energy from one fluorophore is absorbed by another fluorophore [23]. A third approach is to employ changes in the push-pull conjugated π-electron system of the dye [18,24]. The energy that was absorbed or emitted during a fluorescence process occurs as a result of the specific small gaps of conjugated π electrons energy levels. This, in turn, enables the electron movement to a higher energy level by excitation (absorbed energy). Subsequently, the loss of energy occurs by internal vibration and relaxation to the lowest energy level, finally returning to the ground state level, which results in energy emission. In order to create an activatable fluorescence sensor, the fluorophore π electron conjugated system can be modified to suppress its fluorescence. Once modified, the fluorophore will be in its "OFF" state and energy emission will not occur. Applying reversible changes in the π-electron system, like enzymatically-cleavable group, will transform the dye into a Turn-ON fluorescent probe upon meeting the suitable conditions (e.g. a specific enzyme or analyte) and re-conjugating the π-electron system.

We present here a dual approach which permits simultaneous drug release and imaging ability. We designed and synthesized two polymeric systems that together, generate a theranostics nanomedicine system. The diagnostic system consists of an efficient high-loading, self-quenched Turn-ON system with the NIR fluorescent dye Cy5 (SQ-Cy5). The therapeutic system includes the potent chemotherapeutic agent PTX. Both polymeric systems are composed from the water-soluble, non-toxic at the required concentration for activity and stable *N*-(2-Hydroxypropyl)methacrylamide (HPMA) copolymer [25,26]. Both Cy5 and paclitaxel (PTX) were conjugated to HPMA copolymer through a Gly-Phe-Leu-Gly (GFLG) linker, cleaved by cathepsin B, a lysosomal cysteine protease overexpressed in several tumor types, mainly in the lung, colon, prostate, melanoma and breast [27,28]. Moreover, cathepsin B also exhibits pro-angiogenic activity, specifically in the formation of tubular structure and secretion of vascular endothelial growth factor (VEGF) [29,30]. Under pathological conditions, an excessive amount of cathepsin B is secreted or exposed at the cell surface, by both the cancerous tissue and its vasculature. This, in turn, leads to the degradation of the extracellular matrix (ECM), the activation of other proteases and thereby promotes tumor progression, invasion, migration and angiogenesis [31]. Taken together, the high levels of cathepsin B can be harnessed as leverage for specific activation at the tumor site. Based on this rational, HPMA copolymer-SQ-Cy5 and HPMA copolymer–PTX were designed to report the release of PTX by a simultaneous fluorescent signal, enabling detection of the tumor, treatment and monitoring the therapeutic effect.

2. Materials and methods

2.1. Materials

HPMA copolymer–Gly-Phe-Leu-Gly–ethylenediamine (HPMA–GFLG–en) incorporating 10 mol% of the Gly-Phe-Leu-Gly–ethylenediamine was obtained from Polymer Laboratories (Church Stretton, UK). HPMA copolymer–GFLG–en has a molecular weight of 31,600 Da and a polydispersity of 1.66. PTX was purchased from Petrus Chemicals and Materials 1986 (LTD) (China). RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin, nystatin, L-glutamine, HEPES buffer and sodium pyruvate were purchased from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). EGM-2 medium was from Cambrex, USA and endothelial cells growth supplement (ECGS) from Zotal (Israel). All other chemical reagents, including salts and solvents, were purchased from Sigma-Aldrich (Rehovot, Israel). All reactions requiring anhydrous conditions were performed under a $N_{2(g)}$ atmosphere. Chemicals and solvents were either AR grade or purified by standard techniques.

2.2. Synthesis of HPMA Copolymer-Cy5 conjugate

Cy5-COOH was synthesized as previously described [22]. Next, Cy5-COOH fluorophore was conjugated with HPMA copolymer–GFLG–en in two-step synthesis. First, Cy5-COOH (15.1 mg; 0.023 mmol) was dissolved in 0.7 ml anhydrous *N*,*N*-Dimethylformamide (DMF). *N*-Hydroxysuccinimide (NHS) (5.3 mg; 0.046 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (9.5 mg; 0.046 mmol) were added in order to activate the free carboxylic group of the fluorophore, for further coupling to HPMA copolymer. The reaction mixture was stirred at room temperature (RT) in the dark for 12 h. Then, HPMA-GFLG-en copolymer (21.1 mg; 0.114 mmol) was dissolved in 0.5 ml anhydrous DMF and added to the reaction mixture. Following the reaction by High Pressure Liquid Chromatography (HPLC) (UltiMate® 3000 Nano LC systems, Dionex), the precipitate was washed with acetone and dried under vacuum. Purification of the conjugate by size exclusion chromatography (SEC) was performed using AKTA/FPLC system (Pharmacia/GE Healthcare), HiTrap Desalting

Please cite this article in press as: S. Ferber et al., Polymeric nanotheranostics for real-time non-invasive optical imaging of breast cancer progression and drug release, Cancer Lett. (2014), http://dx.doi.org/10.1016/j.canlet.2014.02.022