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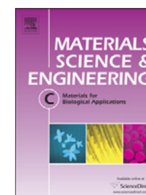

Synthesis and enzymatic cleavage of dual-ligand quantum dots

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ABSTRACT

Site directed therapy promises to minimize treatment-limiting systemic effects associated with cytotoxic agents that have no specificity for pathologic tissues. One general strategy is to target cell surface receptors uniquely presented on particular tissues. Highly specific *in vivo* targeting of an emerging neoplasm through a single molecular recognition mechanism has not generally been successful. Nonspecific binding and specific binding to non-target cells compromise the therapeutic index of small molecule, ubiquitous cancer targeting ligands. In this work, we have designed and fabricated a nanoparticle (NP) construct that could potentially overcome the current limitations of targeted *in vivo* delivery. Quantum dots (QDs) were functionalized with a poly(ethylene glycol) (PEG) modified to enable specific cleavage by matrix metalloprotease-7 (MMP-7). The QDs were further functionalized with folic acid, a ligand for a cell surface receptor that is overexpressed in many tumors, but also expressed in some normal tissues. The nanomolecular construct is designed so that the PEG initially conceals the folate ligand and construct binding to cells is inhibited. MMP-7 activated peptide cleavage and subsequent unmasking of the folate ligand occurs only near tumor tissue, resulting in a proximity activated (PA) targeting system. QDs functionalized with both the MMP-7 cleavable substrate and folic acid were successfully synthesized and characterized. The proteolytic capability of the dual ligand QD construct was quantitatively assessed by fluorometric analysis and compared to a QD construct functionalized with only the PA ligand. The dual ligand PA nanoparticles studied here exhibit significant susceptibility to cleavage by MMP-7 at physiologically relevant conditions. The capacity to autonomously convert a biopassivated nanostructure to a tissue-specific targeted delivery agent *in vivo* represents a paradigm change for site-directed therapies.

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1. Introduction

Many current cancer treatments produce nonspecific injury to cancer and normal tissues, leading to systemic toxicity. Ideally, targeted therapies would preferentially deliver anticancer agents to tumor tissues and spare normal tissues. First generation targeted therapies used site-specific ligands directed to the surface of cancer cells [1–4]. Unfortunately, nonspecific binding and specific binding to non-tumor cells diminished the effectiveness of early targeted drug delivery approaches, especially for small molecular weight ligands [5,6]. Second generation targeted therapies have evolved that employ proteolytically cleavable substrates to reduce nonspecific interactions [7–12]. These substrates have been utilized in the design of imaging agents [7–10,12] and several prodrugs [13–15].

One such substrate, the peptide sequence RPLALWRS, is cleavable by MMP-7 (also known as matrilysn). MMP-7 is a zinc-dependent metalloprotease that is involved in the degradation of extracellular matrix and tumor progression. MMP-7 is also active in the progression of breast and colon cancer [12,16,17] and has been used as a diagnostic marker for ovarian, pancreatic, esophageal and colon cancers [18–22]. Furthermore, the enzyme has been shown to be secreted at the earliest stages of cancer development by precancerous lesions [23,24]. Previously, McIntyre et al. have shown that MMP-7 cleaves RPLALWRS that has been functionalized on a PAMAM dendrimer *in vivo* [12].

Proteolytically cleavable peptides have also been conjugated to nanoparticles for use in imaging applications [25]. Nanoparticles are particularly advantageous for targeted therapies due to the large surface to volume ratio compared to molecular constructs, which allows many reactive ligands to be conjugated to the surface. A stable, covalent conjugation of an MMP-7 cleavable construct to a quantum dot (QD) has been reported by Smith et al. [25]. The MMP-7 cleavable construct consists of the RPLALWRS peptide sequence flanked by two polyethylene (PEG) groups. The PEG groups reduce the nonspecific binding of the particle [26,27]. When the construct is in the proximity of MMP-7, the peptide is cleaved, hence the construct is "proximity activated" (PA) [25].

In this work, we have extended the effort of Smith et al. [25] to create a dual-ligand QD functionalized with both the PA construct and folic acid, a tissue specific small molecule [28,29]. Folic acid receptors are over-expressed in many human cancers including breast, ovarian, brain, kidney, and lung [30]. Furthermore, a study by Hartman et al. concluded that breast cancer patients with primary tumors that express folic acid

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receptors were more aggressive and correlated to disease recurrence and reduced patient survival [31]. Folic acid receptors, however, are expressed by normal tissues such as the kidney, intestine and lung, compromising specificity as a tumor-targeting agent [30,32–35]. In the multifunctional nanoparticle designed here, the PA construct conceals the folic acid ligand until the particle is in the proximity of the tumor (Fig. 1). The folic acid is revealed upon PA cleavage, enabling highly specific tumor tissue targeting in the proximity of cleavage. A novel chemistry application has been used to synthesize a multifunctional nanoparticle (FA-**QD**-PA) with two different ligands, folic acid and the PA construct. Additionally, the cleavage of FA-**QD**-PA has been analyzed using exogeneous MMP-7 *in vitro*.

Autonomous unmasking of a targeting ligand only in the proximity of an emerging neoplasm is the critical feature of the imaging nanoconstruct described here. The construct would be administered as a PEGylated nanoparticle, projected to possess the prolonged cardiovascular half-life and limited nonspecific binding characteristic of this class of materials [26,27]. MMP-7, a protease secreted from neoplasm, cleaves a peptide that bridges the PEG and the nanoparticle core of the construct. As the PEG and peptide stub diffuse away from the construct, the targeting ligand is revealed, facilitating specific recognition of the imaging nanoparticle with the neoplastic cells in the proximity of the relatively high MMP-7 concentration. This approach requires colocalization of two different characteristics of neoplasm for specific recognition: MMP-7 secretion and folate receptor expression on the cell surface. The PEG 'barrier' offers a significant obstruction to the folate recognition that would otherwise occur on some non-target cells. The requirement for local MMP-7 to reveal the folate ligand ensures that the construct will only actively target in the proximity of a neoplasm.

2. Materials and methods

2.1. Materials

The PA construct, PEG_{3400}-[Ahx]-RPLALWRS-[Ahx]-PEG_{5000}-K(5-FAM)-NH_2, was purchased from AnaSpec Corporation (San Jose, CA). This structure consists of a cleavable 8-mer peptide substrate (RPLALWRS) flanked by PEG groups with a molecular (feed) weight of 3400 and 5000. The PA construct also contains aminohexanoic acid (Ahx) groups that function as spacer molecules as well as a fluorescent tag (5-carboxyfluorescein, 5-FAM) for cleavage detection. The N-terminus of the construct was a primary amine to facilitate further conjugation reactions. Ac-terminal amidation was performed during the synthesis to stabilize the substrate and prevent additional modification during subsequent chemical reactions. During the amidation the c-terminus is converted to a secondary amine, which is unreactive in EDC coupling reactions [36]. Carboxylated 585 QDs were purchased from Invitrogen Corporation (Carlsbad, CA). Exogenous matrilysin (MMP-7) was purchased from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma Aldrich.

2.2. QD-PA conjugation

The PA construct was conjugated to carboxylated 585 QDs (CdSe with a ZnS coating) by an 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide (EDC) reaction. N-hydroxy succinimide (NHS) was omitted during the conjugation, resulting in unfunctionalized carboxylic acid groups that can be used to conjugate FA to the QD-PA. The PA construct (80 µL, 10 mg/mL feed weight of 0.80 mg) was added to 1.87 mL of 1 µM QDs in 10 mM borate buffer at pH 7.4. To facilitate the coupling, 57 µL of EDC was added and the reaction stirred for 2 h. Molecular weight cutoff filters (100 kDa) were used to remove unreacted PA from the final QD-PA construct. The QD-PA construct was washed three times with 10 mM borate buffer at pH 7.4 to ensure purity [25].

2.3. QD-PA cleavage by MMP-7

The ability of the QD-PA construct to function as a cleavable substrate was investigated using exogenous MMP-7. MMP-7 (20 µL) was added to the QD-PA construct (100 µL, 100 nM) to achieve a final concentration of 100 nM MMP-7 in 10 mM borate buffer fortified with 50 µM $ZnSO_4$ at pH 7.4. The final volume of the reaction was 200 µL and the final concentration of QD-PA was 50 nM. The reaction was incubated for 24 h at 37 °C. Cleaved peptide was removed from the reaction by filtration using a 100 kDa molecular weight cutoff filter. The QD-PA construct was washed three times with 10 mM borate buffer at pH 7.4. Control samples were prepared in the same fashion with MMP-7 omitted [25].

2.4. QD-FA conjugation

QDs were functionalized with folic acid via an EDC reaction. NHS (0.05 M in 10 mM borate buffer, pH 7.4) and EDC (0.05 M in 10 mM borate buffer, pH 7.4) were added to 100 µL of 1 µM carboxylated QDs. The reaction was stirred for 30 min at room temperature. The addition of EDC and NHS to the carboxylated QD forms a highly reactive carboxylate-NHS intermediate that will react with the amine of the folic acid. After 30 min, 100 µL of 125 µM folic acid in 10 mM borate buffer was added. The reaction stirred for 2 h at room temperature. The QD-FA conjugate was purified using 3.5 kDa molecular weight

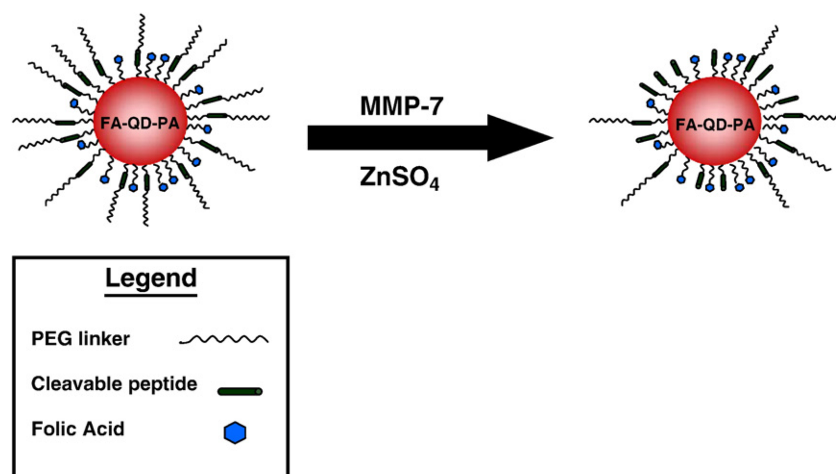

Fig. 1. Schematic of the cleavage of the FA-QD-PA nanoparticle by MMP-7. Upon the addition of MMP-7, the PA construct is cleaved revealing the FA.

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