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 produgs [13–15].

One such substrate, the peptide sequence RPLALWRS, is cleavable by MMP-7 (also known as matrilysin). 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...
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 [30–35]. In a 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 exogenous MMP-7 in vitro.

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.

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 folic acid 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, PEG3400-[Ahx]-RPLALWRS-[Ahx]-PEG5000-K(5-FAM)-NH2, was purchased from AnaSpec Corporation (San Jose, CA). All other reagents were purchased from Sigma Aldrich.

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 functionalized 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].

2.2. QD-PA conjugation

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 ZnSO4 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].

2.3. QD-PA cleavage by MMP-7

The QD-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 functionalized 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. 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
2.5. QD-PA conjugation with FA

An EDC/NHS coupling reaction was used to functionalize the pre-
synthesized QD-PA construct with folic acid. EDC (0.05 M in 10 mM
borate buffer, 25 µL) and 25 µL of 0.05 M NHS in 10 mM borate buffer
was added to 1 mL of 0.15 µM QD-PA conjugate. The reaction was
stirred for 30 min at room temperature followed by addition of 500 µL
of 125 µM (feed weight 27.85 µg) folic acid in 10 mM borate buffer. The
reaction was stirred for 2 h and was purified using 3.5 kDa molecular
weight dialysis kit for 24 h with 3 buffer exchanges.

2.6. FA-QD-PA cleavage by MMP-7

The ability of the FA-QD-PA construct to function as a cleavable
substrate was investigated as for the QD-PA precursor, described
above.

2.7. Fluorescence measurements

Fluorescence measurements were obtained using a Nanodrop
ND3300 fluorometer (Nanodrop Technologies, Wilmington, DE).
Borate buffer (10 mM, pH 7.4) was used as the blank for both the
absorbance and fluorescence. Fluorescence of all QDs was measured at
585 nm using an excitation at 470 ± 10 nm. Enzymatic cleavage of the
QD-PA constructs was determined by measuring changes to the
fluorescence spectra following incubation with MMP-7. The cleaved 5-
FAM was separated from the QD construct using 100kD molecular
weight cutoff filters. The spectra were scaled to normalize QD peak
fluorescence (585nm) among all samples. The peak fluorescence
intensity values at 520 nm and 585 nm were then directly compared
to the spectra of control, uncleaved QD-PA particles to calculate the
extent of PA construct cleavage following MMP-7 treatment.

2.8. Size measurements

Nanoparticle size was determined using dynamic light scattering
(DLS) measurements conducted on a Malvern Nano Series Zetasizer
with a 633 nm laser. The duration of each scan was 60 s and 3 scans
were accumulated. The concentration of all QD samples for DLS
assessment was 25 nM.

2.9. Statistics

One way analysis ANOVA tests were conducted using a \( p \leq 0.001 \)
and a confidence interval of 95% (overall significance level of 0.05),
unless otherwise noted. Student t-tests were conducted with a
confidence interval of 99%.

3. Results and discussion

3.1. QD Functionalization and characterization

A dual ligand QD, functionalized with both the PA construct and
the FA ligand was successfully synthesized and characterized. The PA
construct was conjugated to the QD using an EDC coupling where the
carboxylate group of the QD reacts with the N-terminus of the
peptide. The c-terminus of the PA construct is capped with an amide to
prevent further EDC conjugations, hence preventing aggregation [36].
By omitting NHS in the conjugation of PA to the QD, the EDC reaction is
not as efficient and results in unreacted carboxylate groups [36]. These
carboxylate groups are then available to react with another ligand. An
EDC/NHS coupling reaction was used to functionalize the pre-
synthesized QD-PA construct with folic acid (FA-QD-PA). The NHS
activates the carboxylic acid groups on the QDs, making them highly
reactive toward amine nucleophiles [36]. The unreacted carboxylic
acid groups react with the amine group on folic acid, resulting in the
conjugation of a secondary ligand. For comparison purposes, a QD-PA
and a QD-FA construct were synthesized using an EDC reaction.

Commercially available and chemically defined QDs were used in
this study despite their well known potential for toxicity resulting
from the CdSe constituents in the optical core. This work is focused on
the development of fluorescence beacon that may be used as
research materials in in vitro cell culture systems and animal models.
The ease in sensing nanoconstruct characteristics and the reproduc-
bility that is achieved through the use of commercially available
materials is of considerable value, offsetting the limitations of dual-
ligand nanoconstruct application due to QD core toxicity. The
chemistries described in this work can be translated to the synthesis
of dual-ligand nanoparticles with alternate core chemistries, includ-
ing those with significantly reduced toxicity and potentially appro-
riate for use in humans. For example, nanoscale iron oxides
functionalized as described in this work may be useful as in vivo
reporters of biological activities for humans using magnetic resonance
imaging as the detection tool.

For the FA-QD-PA nanoparticles, the addition of the PA construct was
confirmed by dynamic light scattering (DLS), fluorescence measure-
ments and gel electrophoresis. The nanoparticle construct experienced
a statistically significant (one-way ANOVA) size increase upon the
addition of the PA construct (Table 1). QD size increased from 18.2 ±
0.27 nm for unconjugated carboxylated QDs to 81.5 ± 10.5 nm for QDs
functionalized with the PA construct. The statistically significant size
increase indicates that the PA construct was successfully conjugated to
the QD nanoparticle. Upon the addition of the FA to QD-PA, the size of
the dual ligand construct (85.4 ± 2.3 nm) does not change significantly
(one-way ANOVA), suggesting that FA ligand is shielded from
interrogation by DLS and implying attachment to the QD, not to the
end of the PA construct. Hydrophilic FA ligand conjugated to the end of
the PA construct is likely to remain exposed at the PEG-water interface
and available for DSL interrogation resulting in a detectable and
significant additional size increase following conjugation.

Conjugation of the PA construct containing a 5-FAM label on the
terminal end is confirmed by the emergence of a new fluorescence
emission peak on the QD (Fig. 2A). The 5-FAM optical peak is distinct
from the native QD fluorescence emission at 585. Gel electrophoresis
was used to monitor the change in electrophoretic mobility mediated
by the addition of the PA construct (Fig. 2B). The QD-PA construct (lane 7)
had reduced electrophoretic mobility as compared to unmodified QDs
(lane 5), consistent with successful PA conjugation. These results are in
agreement with the analysis of Smith et al. [25]. The addition of folic
acid was also confirmed using gel electrophoresis (Fig. 2B). The
electrophoretic mobility of the FA-QD-PA construct (lane 8) was
reduced as compared to the unmodified QDs (lane 5), FA-QD (lane 6)
or the QD-PA construct (lane 7). Previously, Derfus et al. attached siRNA
and a targeting peptide on a QD for siRNA delivery using reversible and
non-reversible sulfide bonding [37]. In contrast to this work, we have
functionalized two different ligands on a QD using a carefully titrated set
of sequential covalent EDC reactions. By using the reactive amine and
carboxylate functional groups that are common in synthetic peptides,
proteins, and biomolecules for dual-ligand synthesis, we have devel-
oped a versatile, widely applicable technique for constructing second
generation targeted nanoparticle therapies.

Table 1

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Size (nm)</th>
<th>Standard deviation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxylated QDs</td>
<td>18.2</td>
<td>0.3</td>
</tr>
<tr>
<td>FA-QD-PA</td>
<td>85.4</td>
<td>2.3</td>
</tr>
<tr>
<td>QD-PA</td>
<td>81.9</td>
<td>10.5</td>
</tr>
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</table>
3. FA-QD-PA cleavage by MMP-7

The ability of the FA-QD-PA construct to function as a cleavable substrate was investigated using exogenous MMP-7 [25]. While several studies have shown that MMP-7 is expressed by several tumor phenotypes, the exact concentration in the local area is unknown. Previously, in vitro studies have used concentration of up to 100 nM [25,38,39] while in vivo studies of other MMPs have documented similar concentrations [38, 39]. In this study, MMP-7 concentrations of 100 nM were used to test the FA-QD-PA construct.

Cleavage susceptibility of the FA-QD-PA construct was monitored using the physical separation of the QD from the 5-FAM molecule induced by MMP-7 activity. For comparison purposes, the enzymatic susceptibility of the nanoparticle QD-PA was also investigated. The intensity of the 520 nm peak of the FA-QD-PA conjugate following treatment with MMP-7 was compared to the intensity of the control sample (Fig. 3). 5-FAM fluorescence intensity of the FA-QD-PA construct decreased by 35.2 ± 10.5% after treatment with MMP-7. Loss of fluorescence intensity at 520 nm is consistent with MMP-7 mediated PA cleavage of the QD-PA construct measured here (40.2 ± 2.1%) as well as the unconjugated PA construct (35.6 ± 3.4%) [25]. Furthermore, PA cleavage on the nanoparticles and for the unconjugated PA construct are not significantly different (p < 0.001, one-way ANOVA). PA cleavage from the QD that is also functionalized with folic acid is similar in extent to that measured for the QD-PA construct that lacks FA. These results suggest that the addition of the FA to the nanoparticle does not change the proteolytic susceptibility of the QD bound PA construct.

The molecular design of a dual-ligand nanoconstruct that can be translated to in vivo applications is a balance among multiple functional characteristics. We expect the nanoconstruct described here to be resistant to clearance by the reticuloendothelial system (RES) in vivo. The overall molecular mass of the PA component (primarily controlled by the molecular masses of the proximal and distal PEG) is likely to have significant influence on the cardiovascular persistence of the nanoconstruct. The overall PA length in this work has been selected based on PEGylated nanoparticles with extended cardiovascular half-life described in literature and through preliminary syntheses (results not shown). The distal PEG size on the PA component will presumably modulate the rate of PA cleavage through partial control of enzyme access to the peptide. Transport of the enzyme from the bulk fluid phase to the peptide occurs through the distal PEG and is likely to be influenced by the distal PEG molecular mass. The relative lengths of the PEGs on the PA constituent will control the enzyme from the bulk fluid phase to the peptide occurs through the distal PEG and is likely to be influenced by the distal PEG molecular mass. The relative lengths of the PEGs on the PA constituent will control the enzyme from the bulk fluid phase to the peptide occurs through the distal PEG and is likely to be influenced by the distal PEG molecular mass. The relative lengths of the PEGs on the PA constituent will control the enzyme from the bulk fluid phase to the peptide occurs through the distal PEG and is likely to be influenced by the distal PEG molecular mass. The relative lengths of the PEGs on the PA constituent will control the enzyme from the bulk fluid phase to the peptide occurs through the distal PEG and is likely to be influenced by the distal PEG molecular mass.

4. Conclusion

PA targeting requires the colocalization of the cleavable masking agent and the concealable ligand. In this work, a PA targeted QD has been successfully designed, synthesized and characterized. Using sequential EDC reactions, the PA construct and folic acid were covalently attached to the QD. The addition of folic acid to the QD-PA does not affect the substrate susceptibility to MMP-7. Subsequent work will explore the ability of the NP probe described here to assess...
PA targeting ex vivo and in vivo by quantitative fluorescence measurements and fluorescence imaging. This laboratory has demonstrated the capacity for single-ligand nanoconstructs with similar PA chemistry to be exhaustively cleaved at enzyme concentrations of 100 nM and with significant cleavage in the presence of as little as 5 nM of MMP-7 enzyme [25]. MMP-7 concentrations in vivo have been reported to be at least 100 nM [38,39], suggesting that the current nanoconstruct can be cleaved in vivo. Achieving significant MMP-7 secretion from cultured cells, however, is a challenge. In addition, cell culture media plays a profound role in modulating the capacity for MMP-7 synthesis. While the use of QDs in clinical applications is unlikely due to their potential for unintended cytotoxicity, the strategy for PA targeting described herein could be applied to other nanoparticle cores. Nanoparticle cores with particular practical potential include iron oxides for MR imaging, gold nanoparticles for PET imaging and dendrimers or liposomes for targeted drug delivery.

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References