General, high-affinity approach for the synthesis of fluorophore appended protein nanoparticle assemblies†

Marinella G. Sandros, De Gao, Cagil Gokdemir and David E. Benson*

Received (in Cambridge, MA, USA) 31st January 2005, Accepted 5th April 2005
First published as an Advance Article on the web 21st April 2005
DOI: 10.1039/b501315a

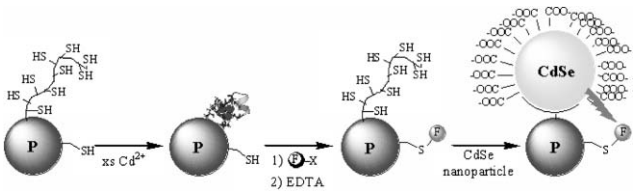
Metallothionein fusion proteins allow for site-specific, orthogonal functionalization of proteins to a variety of nanoparticles.

Functionalized protein appended nanoparticles are an exciting new class of hybrid materials, due to their use as fluorescence and electrochemical biosensors.[1,2] The chemistry used to attach proteins to semiconductor or metal nanoparticles is a critical component that controls the function of the protein–nanoparticle assembly.[2] Covalent attachment of proteins to nanoparticles with short protein–nanoparticle separations increases stability[3] and electronic/photonic communication.[2] A number of covalent attachment chemistries have been developed; such as cysteine coordination,[4–6] poly-histidine peptide fusions,[7] phosphoprotein,[8] phage selected peptides,[9–12] and multimeric-protein templated nanoparticle assemblies.[13,14] For affinity reasons, poly-cysteine fusion proteins are ideal for generating protein attached Au, CdSe, and CdSe/ZnS core-shell nanoparticles,[4–6] however only the non-site-specific amine or carboxylate reactive groups can be used for orthogonal cofactor–protein attachment chemistries.[2] While poly-histidine peptide fusion proteins have been successfully used to achieve site-selective, orthogonal, covalent attachment of cofactors and nanoparticles to proteins,[7] a strategy that allows for the high affinity protein–nanoparticle binding that is provided by poly-cysteine fusion proteins, while also providing orthogonal site-selective thiol modification chemistry, would provide more robust functional protein–nanoparticle assemblies. This communication demonstrates a method that forms site-selective, orthogonal attachment of both a fluorophore and a nanoparticle to a protein using a polycysteine domain derived from metallothionein. A mutant of a maltose binding protein–metallothionein fusion protein (MBP-MT) with a solvent accessible cysteine is generated and modified with Bodipy 577/618 maleimide fluorophore. Energy transfer from either CdSe, CdSe/ZnS core-shell or Au nanoparticles to the MBP-MT attached Bodipy 577–618 fluorophore is used to demonstrate binding and determine binding affinities.

The metallothionein fusion protein strategy is based on the reversible Cd^{2+} ion protection of metallothionein cysteines. One-third of the residues in metallothioneins are cysteines, which coordinate soft metal ions and effectively form the interior of these small proteins.[15] Removal of soft metal ions coordinated to a metallothionein domain provides a protein with a high affinity for nanoparticles with chemically soft metal ions on the surface. Thus, in the presence of Cd^{2+} ions, metallothionein cysteines can be protected, leaving any additional solvent exposed cysteines available for modification by thiol reactive compounds (Scheme 1). Therefore, a protein of interest containing a surface cysteine mutation and a metallothionein domain will allow nanoparticle adhesion and thiol reactive compound attachment in a site-selective fashion. In principle various soluble proteins can be used, making the method modular.

To demonstrate the viability of this strategy we have generated a bacterial expression plasmid that codes for a metallothionein fusion protein with a solvent accessible cysteine. The α-domain of *Nototheniа coriiceps* metallothionein[16] was fused to the carboxy-terminus of maltose binding protein through a Gly-Ser linker (MBP-MT, supplementary information). The bacterial expression vector also allows for rapid genesis of other metallothionein fusion proteins. The low thermal stability of this metallothionein domain[17] facilitates a high affinity protein–nanoparticle interaction, due to the large change in free energy of protein folding between the apo and Cd^{2+} ion bound forms.[17] The N282C mutation (predicted to provide a solvent accessible cysteine) of MBP-MT plasmid DNA was generated by standard recombinant DNA technology, expressed and purified by standard methods, and characterized by MALDI.

N282C MBP-MT exhibited Cd^{2+} ion selective protection of the metallothionein domain cysteines. Dithionitrobenzoate reaction kinetics[18] indicated one free thiol per protein in the presence of excess Cd^{2+} or Zn^{2+} ions and twelve free thiols per protein in the absence of metal ions. Pretreatment with excess Cd^{2+} ions for 30 minutes along with the addition of a 1.5-fold molar excess of 4,4-difluoro-3,5-bis(4-methoxyphenyl)-8-(4-maleimidylphenyl)-4-bora-3a,4a-diaza-s-indacene (Bodipy 577/618 maleimide, Molecular Probes, 575.12 *m*/*z*, λ_{max} = 575 nm) yielded a peak in the MALDI spectrum (Bruker Q-TOF) 574.0 ± 2.2 *m*/*z* larger than observed for unreacted N282C MBP-MT, consistent with Bodipy attachment. This procedure yielded a protein with 0.95 ± 0.05 equivalents of Bodipy 577/618, judging by UV-visible



Scheme 1 Orthogonal modification strategy for fluorophore appended protein–nanoparticle assemblies: P, surface cysteine mutant of a metallothionein fusion protein; F, thiol reactive fluorophore; CdSe, water-soluble nanoparticle.

† Electronic supplementary information (ESI) available: experimental details, nucleotide sequences, binding affinity determinations for Bodipy N282C MBP-MT binding to CdSe, CdSe/ZnS core-shell, and Au nanoparticles, and transmission electron micrographs. See http://www.rsc.org/suppdata/cc/b5/b501315a/

*dbenson@chem.wayne.edu

absorbance and fluorescence emission spectra. Reactions with wild-type MBP-MT and Bodipy 577/618 did not alter the MALDI determined mass, alter the UV-visible absorbance spectrum, or show any Bodipy 577/618 based fluorescence emission with 363 nm excitation. The number of Bodipy 577/618 maleimide attachment sites was determined by HPLC analysis of chymotrypsin or trypsin digested samples. A single peak in the 575 nm absorbance chromatograms (supplementary data) was observed regardless of whether trypsin or chymotrypsin was used. The absence of more than one Bodipy 577/618 related chromatogram peak argues for a single attachment site of the Bodipy 577/618 maleimide to N282C MBP-MT. The chromatography data, along with the lack of Bodipy 577/618 maleimide labeling of wild-type MBP-MT, suggest that Cys 282 is the single site for Bodipy 577/618 maleimide modification. Therefore, Cd^{2+} ion selective protection of metallothionein cysteines allows for the site-selective placement of thiol reactive compounds without modifying the nanoparticle adhesion domain.

Metal-free MBP-MT and derivatives were found to bind water soluble CdSe and CdSe/ZnS core-shell nanoparticles. TOPO capped CdSe nanoparticles were prepared by literature methods,[19–21] while CdSe/ZnS nanoparticles were purchased from Evident Technologies (Hops Yellow). Water-soluble derivatives of these nanoparticles were prepared by a procedure similar to the synthesis of lipoic acid capped CdSe/ZnS core-shell nanoparticles[22] using 16-thiolhexadecanoate (THDA). Reaction (24 h, room temperature) of wild-type MBP-MT, or unmodified N282C MBP-MT, with 3.0–3.5 nm diameter THDA capped CdSe nanoparticles yielded an increase in CdSe fluorescence emission intensity (Fig. 1A). Transmission electron micrographs showed no alteration in CdSe nanoparticle topology upon MBP-MT binding (supplementary material). No change in fluorescence emission intensity was observed for reactions between MBP-MT and THDA capped CdSe/ZnS core-shell nanoparticles. Therefore, the increased CdSe emission intensity is attributed to a perturbation of CdSe surface states, which might arise from the decrease in solvent dielectric upon MBP-MT binding. For Bodipy 577/618-N282C MBP-MT incubated with CdSe or CdSe/ZnS core-shell nanoparticles, energy transfer from the nanoparticle ($\lambda_{em} \sim 560$ nm, decreased intensity) to the MBP-MT attached Bodipy 577/618 ($\lambda_{em} \sim 620$ nm, increased intensity) was observed (Fig. 1B & 1C). Due to the increase of emission intensity upon MBP-MT binding

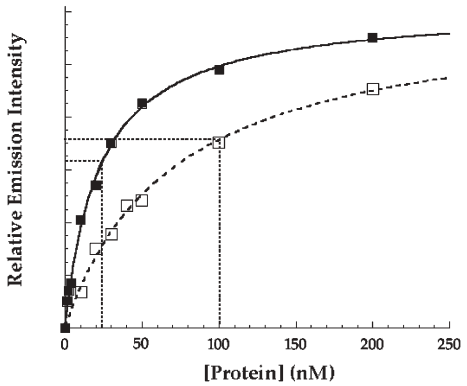


Fig. 2 Determination of MBP-MT (filled squares) and MBP-His_5 (open squares) affinity for 3.0–3.5 nm diameter THDA capped CdSe nanoparticles. Data were fit to equation 1; line, MBP-MT, $K_A = 4.5 \cdot 10^7\ M^{-1}$, $R = 0.998$; dashed line, MBP-His_5, $K_A = 1.1 \cdot 10^7\ M^{-1}$, $R = 0.997$. Graphical estimates of the dissociation constants (dotted lines) for titrations, where MBP-His_5 has a 1.4-fold larger increase in CdSe emission intensity than MBP-MT.

to CdSe nanoparticles, energy transfer in the Bodipy 577/618-N282C MBP-MT THDA capped CdSe nanoparticle sample (dashed line, Fig. 1B) was evaluated in comparison to an N282C MBP-MT THDA capped CdSe nanoparticle sample with no Bodipy 577/618 (solid line, Fig. 1B). If N282C MBP-MT or the CdSe nanoparticles (dotted line, Fig. 1B and C) were omitted from either of these systems, no emission at 620 nm from Bodipy 577/618 was observed. Based on these observations, and those from a previously reported system,[23] energy transfer is presumed to be the dominant quenching mechanism for the 560 nm intensity. The 620 nm emission band in the presence of Bodipy N282C MBP-MT is presumed to be from the resonant emission of Bodipy 577/618 from both the CdSe and CdSe/ZnS core-shell nanoparticles. A difference in the energy transfer efficiency between the CdSe and CdSe/ZnS core-shell nanoparticles is noted and discussed in the supplementary information. These MBP-MT alterations of nanoparticle fluorescence properties confirm that the metallothionein domain of MBP-MT confers binding of the protein to THDA capped CdSe and CdSe/ZnS core-shell nanoparticles.

These metallothionein fusion proteins were found to have a higher affinity for CdSe nanoparticles than the previously reported

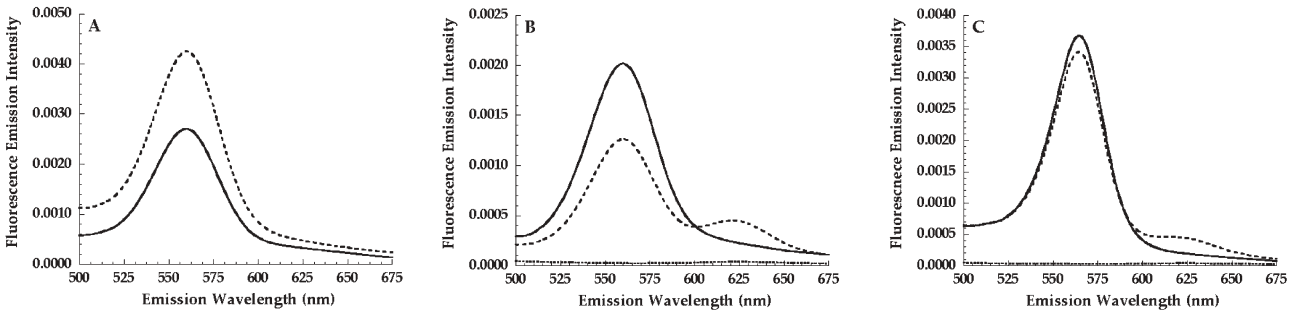


Fig. 1 Fluorescence emission spectra ($\lambda_{ex} = 363$ nm) of MBP-MT bound CdSe nanoparticles: A) 3.0–3.5 nm CdSe nanoparticles, without (solid line) and with MBP-MT attached (dotted line); B) N282C MBP-MT attached 3.0–3.5 nm CdSe nanoparticles, without (solid line), with Bodipy 577/618 attached (dotted line), and N282C MBP-MT with Bodipy 577/618 and no CdSe nanoparticles attached (dotted line); C) MBP-MT attached 3.0–3.5 nm CdSe/ZnS core-shell nanoparticles, without (solid line), with Bodipy 577/618 attached (dotted line) , and N282C MBP-MT with Bodipy 577/618 and no CdSe nanoparticles attached (dotted line).

MBP–polyhistidine fusion proteins (Fig. 2). The association constant (K_A) of MBP-MT for THDA capped CdSe nanoparticles was determined by fitting changes in the fluorescence emission intensity (I_{obs}) to equation 1[24] (Fig. 2):

$$I_{obs} = \frac{(I_f - I_0)\bullet[\text{Protein}]}{[\text{Protein}] + {}^1/_{K_A}} + I_0 \quad (1)$$

Equation 1 assumes the CdSe nanoparticle concentration is no more than one half of the dissociation constant and fits to this equation suggesting one MBP-MT is bound per CdSe nanoparticle. This assumption is different from the multiple non-interacting proteins per nanoparticle model in the analysis of a maltose binding protein–pentahistidine fusion protein (MBP-His_5)[7] binding to lipoic acetate capped CdSe/ZnS core-shell nanoparticles.[23] The single binding site model[24] is justified by the same affinity being determined for MBP-MT binding to a variety of THDA capped CdSe nanoparticle concentrations, judged by extinction coefficients of the band gap absorbance.[25] The association constant determined for MBP-MT binding to THDA capped CdSe nanoparticles was $4.7 \pm 0.7 \cdot 10^7$ M^{-1} (K_D = 21 nM). A similar analysis was applied to MBP-His_5 binding to THDA capped CdSe nanoparticles, consistent with an association constant of $1.1 \pm 0.4 \cdot 10^7$ M^{-1} (K_D = 90 nM). Therefore, MBP-MT does bind more tightly to THDA capped CdSe nanoparticles than MBP-His_5.

Metallothionein fusion proteins demonstrated a high binding affinity for a variety of nanoparticles. The affinity for Bodipy-N282C MBP-MT binding to THDA capped CdSe nanoparticles was found to be $1.3 \cdot 10^7$ M^{-1} (K_D = 80 nM) from fitting the ratio of emission intensity at 620 nm (Bodipy 577/618) to 560 nm (CdSe). The weaker affinity of Bodipy-N282C MBP-MT relative to unlabeled MBP-MT for THDA capped CdSe nanoparticles is possibly due to Bodipy aggregation (supplementary information). For Bodipy-N282C MBP-MT binding to THDA capped CdSe/ZnS core-shell nanoparticles, a two-fold decrease in affinity ($K_A = 6.3 \pm 0.3 \cdot 10^6$ M^{-1}; K_D = 160 nM) was found using the same analysis method. Due to the insolubility of THDA capped Au nanoparticles, borate-capped Au nanoparticles were synthesized and Bodipy-N282C MBP-MT binding was observed *via* energy transfer quenching of Bodipy 577/618. Titration of Bodipy-N282C MBP-MT to 2.0–4.0 nm diameter Au nanoparticles yielded an association constant of $3.1 \pm 0.3 \cdot 10^7$ M^{-1} (K_D = 32 nM), derived by plotting the $[\text{MBP-MT}]_{free}$ *vs.* [MBP-MT-Au]/$[\text{MBP-MT}]_{free}$. The extrapolated affinities show the presumed ordering of affinities (Au > CdSe > CdSe/ZnS core-shell). These results indicate that metallothionein fusion proteins have a high affinity for a variety of nanoparticles with soft surface metal ions and conform to hard/soft acid–base theory.

Here we report a method to engineer proteins that can be site-specifically functionalized with thiol reactive compounds and nanoparticles, using metallothionein fusion domains. The conformational preferences of cysteines from the metallothionein domain provide reversible protection from maleimide modification and allow for selective surface cysteine functionalization. Energy transfer from CdSe and CdSe/ZnS core-shell nanoparticles to Bodipy modified N282C MBP-MT demonstrates direct MBP-MT adhesion to these nanoparticles. MBP-MT was found to have a higher affinity for CdSe nanoparticles than MBP-His_5. Finally, the metallothionein domain of MBP-MT confers a high affinity for nanoparticles with chemically soft metal ions on the surface. Therefore, metallothionein fusion proteins provide a general, high affinity method for generating protein–nanoparticle assemblies with site-specific, orthogonal thiol reactive molecules.

We thank Wayne State University for financial support.

Marinella G. Sandros, De Gao, Cagil Gokdemir and David E. Benson*
Department of Chemistry, Wayne State University, Detroit, MI, 48202, USA. E-mail: dbenson@chem.wayne.edu

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