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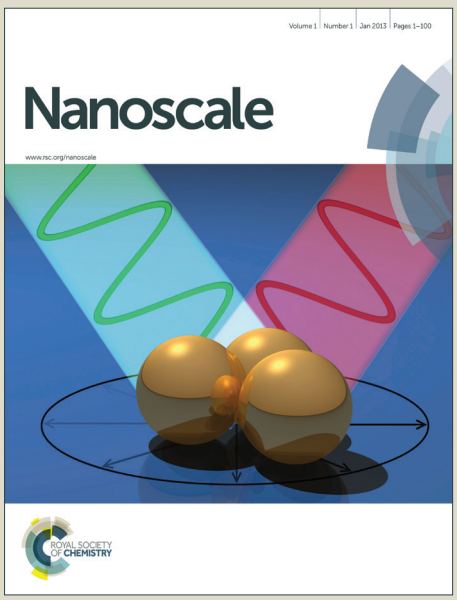

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EDGE ARTICLE

Environmentally Responsive Histidine-Carboxylate Zipper Formation between Proteins and Nanoparticles

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Interfacing synthetic materials with biomacromolecules provides new systems for biological applications. We report the creation of a reversible multivalent supramolecular "zipper" recognition motif between gold nanoparticles and proteins. In this assembly, carboxylate-functionalized nanoparticles interact strongly with oligohistidine tags. This interaction can be tuned through His-tag length, and offers unique binding profiles based on the pH and electrolyte concentration of the medium.

Introduction

Tailoring molecular recognition between synthetic materials and biomolecules provides a versatile strategy for creating bioconjugate systems.[1] A variety of supramolecular approaches have been devised to interface synthetic and biological systems for diverse applications.[2] However, using these systems in physiological environments such as is challenging, where high concentrations of proteins and other biomolecules compete for interaction.

Co-engineering of biomolecules and synthetic materials and provides a strategy for generating high affinity and reversible molecular interactions.[3] Inspiration for this codesign can be obtained from Nature: naturally occurring molecular zippers, including duplex DNA[4] and leucine zippers[5] exhibit robust multivalent reversible interactions in intracellular conditions. Microtubules polymerize and de-polymerize through the formation of specific molecular zippers.[6] This multivalent motif[7] has been used to create synthetic molecular duplexes[8] through non-covalent interactions including electrostatic interactions,[9] hydrogen bonding,[10] π-π interactions,[11] and van der Waals forces to generate zippers.[12]

Multivalency is a key structural perquisite for zipper motifs. Nanomaterials offers molecular scaffolds that can be engineered to present multivalent recognition elements.[13] Gold nanoparticles (AuNPs) provide a particularly versatile platform for biomolecular recognition,[14] and have been interfaced with proteins for a wide variety of applications.[15] The AuNP surface can be readily engineered to feature recognition elements. Additionally, AuNPs can be generated with sizes commensurate to proteins, providing surface complementarity for recognition while maintaining effective biological function.[16]

The metal ion-mediated oligohistidine-nitrilotriacetate recognition motif has been widely employed to capture proteins using nanomaterials.[17] We hypothesized that the oligohistidine cationic tail[18] used in this strategy could be employed as a zipper component for interaction with nanomaterials. In this report, we demonstrate a reversible molecular zipper between His-tagged proteins and carboxylate functionalized AuNPs. This zipper exhibits high affinity binding in physiologically relevant environments, including serum conditions. The system is also environmentally responsive, with binding dictated by solution pH. This new recognition motif presents opportunities for engineering specific molecular interactions between synthetic and biomolecules.

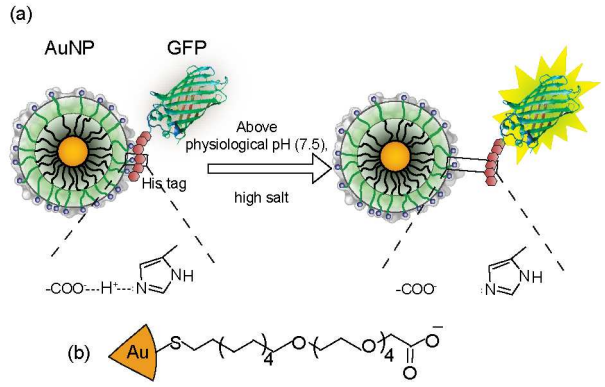

Fig. 1 (a) Zipper formation between AuNP-COOH and N-terminus oligohistidine-tagged GFPs through carboxylate-histidine interaction (b) The chemical structure of 2 nm gold core naoparticle AuNP-COOH.

Results and discussion

The host nanoparticle was provided by AuNPs (2nm core diameter) functionalized with anionic ligands (AuNP-COOH) that can interact with proteins without denaturation.[19] We next explored the interaction of these inherently multivalent carboxylate particles with a family of His-tagged green fluorescent proteins (GFP)[20] (Fig. 1). We cloned and purified three eGFP[21] variants carrying different length of N-terminal His-tags: one His (1xHis-GFP), six His (6xHis-GFP), and twelve His (12xHis-GFP) to determine the required number of interactions, These proteins were all anionic, with predicted pI values of 5.8,

6.1, and 6.5, respectively.

The binding efficiency of AuNP-COOH with the His-tagged GFPs was quantified through fluorescence titration,[22] utilizing the quenching properties of the AuNP.[23] At low ionic strength (5 mM phosphate buffer, PB) AuNP-COOH bound both 12xHis-GFP and 6xHis-GFP with high affinity (Fig. 2a). The binding constant (K_S) values for 12xHis-GFP (K_s= 2.95 ±0.6 x 10^7 M^{-1}) was ~3-fold higher than that of 6xHis-GFP (K_s= 7.8 ±0.38 x 10^6 M^{-1}), indicating that multivalency is crucial for zipper formation. Interestingly, more GFPs bound to each nanoparticle for 12xHis-GFP (n= 11.6 ±0.8) than for 6xHis-GFP (n= 4.7 ±1), potentially due to decreased secondary repulsion between the anionic GFPs.[24] No observable binding was observed with 1xHis-GFP, demonstrating that specific zipper formation was required for interaction.

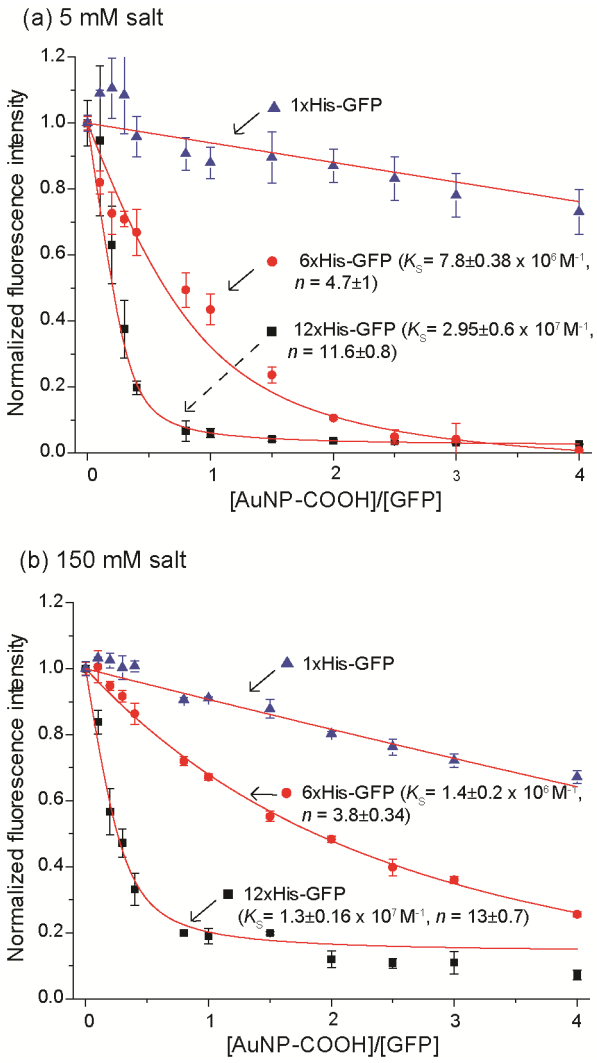

Fig. 2 The interaction of AuNP-COOH with His-tagged GFP variants. Fluorescence (λ_{ex}=475 nm, λ_{em}=510 nm) titrations between nanoparticles and GFPs (100 nM) in (a) 5 mM phosphate buffer (PB), and (b) PBS buffer (150 mM NaCl in 5 mM PB) at pH 7.4. The complex association constant (K_S) and the binding stoichiometry (n) were determined using previously reported method.[21]

The pragmatic use of non-covalent bioconjugates requires high affinity interactions at physiological ionic strength. In previous studies, electrostatic interactions between nanoparticles and proteins were fully disrupted at quite low salt concentrations, typically 10-50 mM salt.[25] In contrast, high binding affinities were observed between AuNP-COOH and both 12xHis-GFP (K_s= 1.3 ±0.16 x 10^7 M^{-1}), and 6xHis-GFP (K_s= 1.4 ±0.2 x 10^6 M^{-1}) in PBS buffer (150 mM NaCl in 5 mM PB, pH 7.4) (Fig. 2b). Notably, a larger n value was observed for 12xHis-GFP, similar to the one at low (5 mM) electrolyte concentration.

Reversible zipper formation at physiologically relevant conditions

One of the key advantages of supramolecular bioconjugates is their ability to respond to environmental changes. pH is an important biological parameter. For example, normal tissues have a pH of 7.4, while tumor tissues have lower pH (~6 to 7).[26] Additionally, pH decreases through the endosomal/lysosomal pathways inside cells, reaching a pH of ~4.8.[27] In our system, the histidine tag in GFPs offers a potentially pH-switchable recognition scaffold. To explore this possibility, we investigated the pH and ionic strength dependent reversibility of the carboxylate-histidine zipper formation. Both 12xHis-GFP and 6xHis-GFP interacted strongly with AuNP-COOH below pH ~7.5 at physiological salt concentration (PBS). Significantly, above pH ~7.5 the carboxylate-histidine zipper disassembled, releasing the GFP from the nanoparticles surface (Fig. 3a and 3b). As expected, 1xHis-GFP did not interact with nanoparticles at any condition (Fig. 3c). Taken together, these studies demonstrated the pH response of the zipper motif.

Reversible zipper formation in serum conditions

In vivo applications including protein and gene delivery require specific and reversible interactions between synthetic carrier materials and the cargo molecules in serum.[28] Serum presents a complex competitive chemical environment featuring a high (~1 mM) concentration of protein,[29] making it challenging to engineer effective recognition motifs. We parametrically investigated the serum concentration and pH dependent reversibility of the carboxylate-histidine zipper. At pH <7.5 and at 10% serum (cell culture condition), 12xHis-GFP exhibited a high affinity binding towards AuNP-COOH (Fig. 4a). Significantly, in 55% serum condition (*in vivo* condition) at pH 7.5 there was substantial binding between AuNP-COOH and 12xHis-GFP (Fig. 4c). While the binding isotherm is complex, considerable binding was observed at high nanomolar concentrations. In contrast, 6xHis-GFP did not bind with AuNP-COOH at any serum condition under investigation (Fig. 4b), indicating that a high degree of multivalency is crucial for carboxylate-histidine zipper formation in complex biological environments.

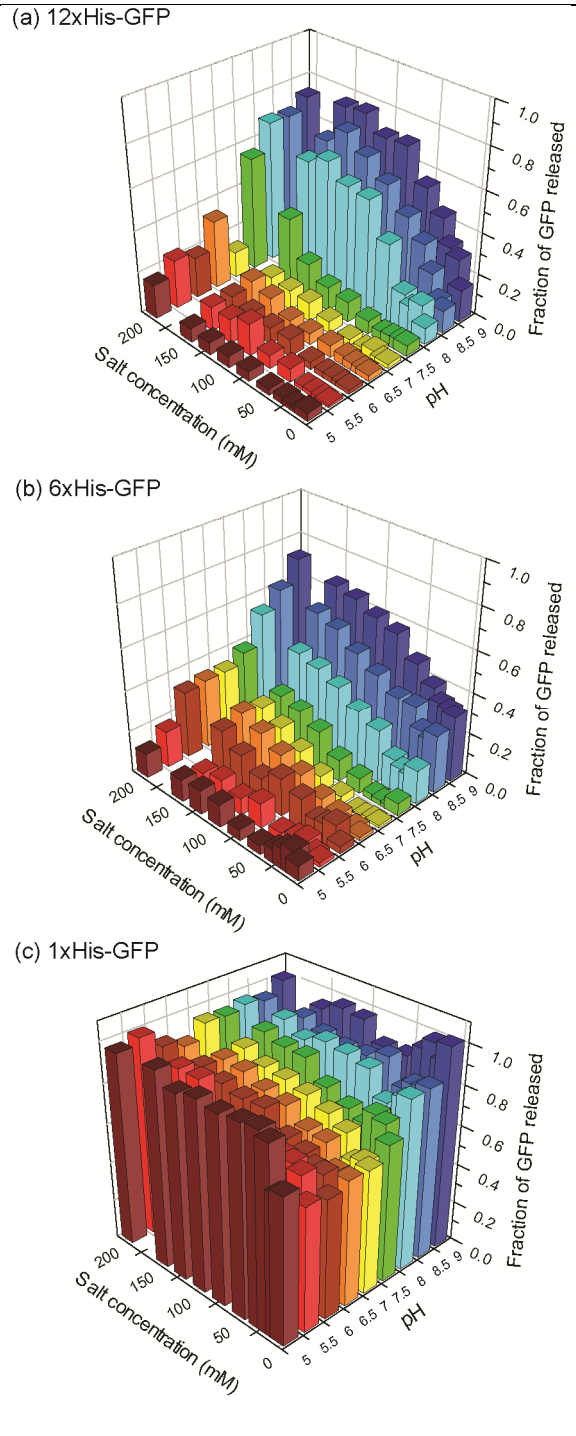

Fig. 3 Responsiveness of the carboxylate-histidine zipper towards pH and salt concentration. Fluorescence titrations between 400 nM of AuNP-COOH and 100 nM of (a) 12xHis-GFP, (b) 6xHis-GFP, and (c) 1xHis-GFP were performed parametrically varied pH and salt (NaCl) concentrations in 5 mM PB. The intensity of GFP released from nanoparticles was normalized against the intensity of free GFP.

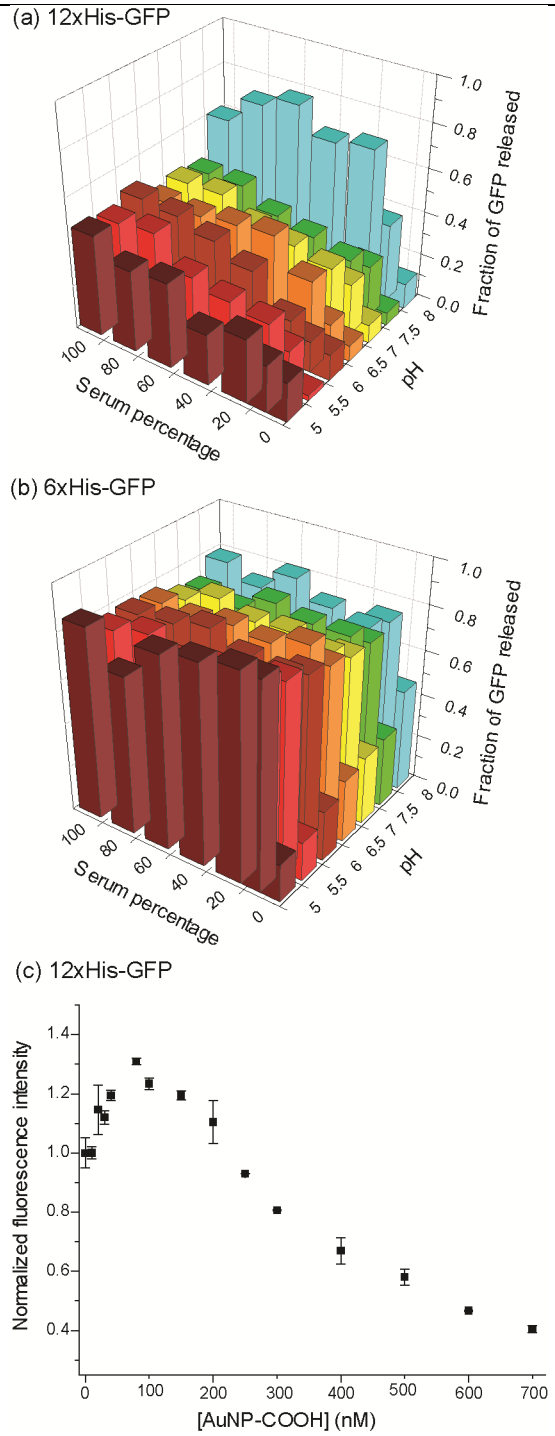

Fig. 4 Reversible carboxylate-histidine zipper formation between AuNP-COOH and (a) 12xHis-GFP, and (b) 6xHis-GFP at serum conditions. 400 nM of AuNP-COOH was titrated against 100 nM of His-tagged GFPs varying the serum percentage and pH at 150 mM salt (1xPBS) concentration. (c) Fluorescence titrations between AuNP-COOH and 12xHis-GFP (100 nM) at 55% serum condition, pH 7.4.

Conclusions

In summary, we have tailored a molecular zipper based on

multivalent carboxylate-histidine interactions through co-engineering of the AuNP surface and proteins. The carboxylate-histidine zipper exhibited high affinity interactions under physiologically relevant conditions that were pH responsive, making these systems attractive starting points for delivery and imaging applications. In a broaer context, these studies demonstrate how co-engineering of biomolecules and nanoparticles can be used to generate bioconjugates with new and useful properties..

Experimental section

Materials and methods

Cloning and over expression of green fluorescent proteins (GFPs)

Genetic engineering manipulation and protein expression were done according to standard protocols. (a) To generate 1xHis-GFP, a constitutive expression vector (pUCCB-ntH6-eGFP) was purchased from Addgene (plasmid id- 32557).[30] For the sake of purification, a 6xHis tag was placed on the N-terminus of 1xHis-GFP, upstream of a thrombin cleavage site. (b) 6xHis-GFP expression vector (pET21-d-GFP) was obtained from Novagen. (c) 12xHis-GFP was generated by incorporating twelve histidines in the N-terminus of GFP. Briefly, using GFP as the template, PCR was performed with the following primers. Subsequently, the PCR product was digested (using *Bam*HI and *Hind*III restriction enzymes) and inserted into pQE80 vector, downstream of nucleotides for six histidine tag to construct pQE80-12xHis-GFP expression vector. Successful cloning was confirmed by DNA sequencing.

Forward primer: 5'- ACGATGGATCCCACCATCACCAT -3'

Reverse primer: 5'- GTGACAAGCTTTTACTTGTACAGCTC -3'

To produce recombinant proteins, plasmids carrying 1xHis-GFP, 6xHis-GFP, or 12xHis-GFP was transformed into *Escherichia coli* BL21(DE3) strain. A transformed colony was picked up to grow small cultures in 50 mL 2xYT media at 37 ^{0}C for overnight. The following day, 15 mL of grown culture was inoculated into one liter 2xYT media and allowed to grow at 37 ^{0}C until OD reaches 0.6. At this point, the protein expression was induced by adding isopropyl-b-D-thiogalactopyranoside (IPTG; 1 mM final concentration) at 25 ^{0}C. After 16 hours of induction, the cells were harvested and the pellets were lysed using a microfluidizer. His-tagged fluorescent proteins were purified from the lysed supernatant using HisPur cobalt columns. The integrity and the purity of native protein were determined by 12% SDS-PAGE gel.

1xHis-GFP was cleaved from its 6xHis tag using thrombin-agarose beads (Thrombin CleanCleave™ Kit, Sigma-Aldrich) as described in the instruction manual. After the cleavage, 1xHis-GFP was passed through a HisPur cobalt column to remove the cleaved 6xHis tag. Further, the residual 6xHis was removed by a 10KD-MWCO (molecular weight cut off) filter.

Synthesis and characterization of nanoparticles

Carboxylate functionalized gold nanoparticles (AuNP-COOH) were synthesized according to a previous report.[31] Briefly, Brust-Schiffrin two-phase synthesis was used to synthesize pentanethiol-coated AuNPs with core diameter ~2 nm.[32] The Murray place-exchange method was followed to obtain AuNP-COOH.[33] The monolayer protected nanoparticles were re-dispersed in water. The excess ligand/pentanethiol were removed by dialysis using a 10,000 MWCO snake-skin membrane. The final concentration was measured by UV spectroscopy at 502 nm. To assess their quality, the nanoparticles were characterized by Zeta potential (surface charge), Dynamic Light Scattering (DLS) (hydrodynamic radius), and Transmission Electron Microscopy (TEM) (core size) as shown in Fig. S1.

Fluorescence titration

Fluorescence titration experiments between nanoparticles and GFPs were carried out as described previously.[34] Briefly, the change of fluorescence intensity of GFPs at 510 nm was measured with an excitation wavelength of 475 nm at various concentrations of nanoparticles from 0 to 400 nM on a Molecular Devices SpectraMax M3 microplate reader (at 25 ^{0}C). Quenching of fluorescence intensity arising from 100 nM GFP was observed with increasing nanoparticle concentration. Nonlinear least-squares curve fitting analysis was carried out to estimate the binding constant (K_S) and association stoichiometry (n, [GFP]/[AuNP-COOH]) using a one site binding model.[21]

For the pH and salt dependent interactions (fluorescence titrations) between nanoparticles and GFPs, the concentration of GFP chosen was 100 nM for each study. The concentrations of AuNP-COOH used for the titrations were 400 nM. The fluorescence intensity for each study was normalized against the intensity of GFP without nanoparticles at their respective pH and salt (NaCl in 5 mM PB) concentration. The titrations were carried out in triplicates, and repeated at least twice with different batches of nanoparticles.

Similar fluorescence titrations were performed for the serum concentration and pH dependent interactions between AuNP-COOH and His-tagged GFPs. Both the nanoparticle (400 nM) and GFP (100 nM) concentrations were kept fixed, varying the serum percentage and pH of the solutions. In a typical experiment, AuNP-COOH/GFP complexes were made first, incubated at dark for 10 minutes, then the required serum amount was added to the complexes, followed by immediate shaking for 30 seconds. Fluorescence reading was taken after 30 minutes of incubation.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Nanoparticle characterizations- i.e. Zeta potential, DLS, and TEM and GFP sequence. See DOI: 10.1039/b000000x/

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