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Protein assembly directed by synthetic molecular recognition motifs†

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Tris-functionalized cyanuric acid (TCA) and melamine (TM) selectively recognize each other in aqueous solution with 1 : 1 stoichiometry. We have coupled biotin to TCA and TM to allow pseudo-tetrahedral display of TCA and TM on streptavidin through biotin–ligand binding. Synthetic cyanuric acid/melamine recognition is found to drive selective protein–protein assembly.

Bio-based materials have potential applications as useful structural or functional biomaterials.[1–9] We describe herein non-covalent protein assembly triggered by small molecule recognition at the protein surface. Native protein assemblies exhibit sophisticated function and play crucial roles in biological processes (*e.g.* actins, tubulins and ribosome proteins);[10–14] protein *misassembly* conversely is associated in toxic loss or gain of function, as found in neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease.[15–18] Thus, the function and pathology of native protein assemblies has inspired considerable investigation of both native[19–22] and artificial systems[6,23–25] in an effort to recreate and understand these desirable and undesirable aspects of protein assemblies. Two general strategies of fabricating artificial protein assemblies have been explored: protein-mediated connection[24,25] and multivalent ligand-mediated connection.[23] The ligand-based strategy permits a more straightforward method for tuning the properties of the artificial protein assemblies, as compared to the task of retooling a protein–protein interface;[26–28] we have explored this concept by using the synthetic cyanuric acid and melamine recognition motif to construct an artificial protein–protein assembly interface.

Cyanuric acid (CA) and melamine (M) recognition has been studied extensively in organic solvents and the solid state.[29–37] We have recently reported on the utility of these simple heterocycles as recognition motifs in water. The driving force for assembly in water appears to derive from favorable polar–polar surface area burial along with hydrophobic surface area burial when Tris is used as a scaffold; these effects are likely to be less important in organic solvents where hydrogen-bonding is a strong contributor to recognition. Regardless of the origins and solvent, CA/M recognition is highly selective and is a direct consequence of the complementary hydrogen bond donor–acceptor patterning of each heterocycle. We have previously examined cyanuric acid/melamine recognition anchored at the lipid–water interface with a phospholipid or peptide anchor.[38–40] Assembly at hydrophobic-water interfaces is known to enhance weak hydrogen bonding interactions by shielding H-bond donor–acceptor sites from competitive aqueous solvent. This has been demonstrated with a number of reports on the aqueous-phase assembly of hydrogen bonding amphiphiles. We found that the cyanuric acid/melamine system could be induced to assemble in water without an amphiphilic scaffold; when displayed on the Tris scaffold (Fig. 1), these molecules could bury sufficient surface area on recognition to drive nanoparticle assembly.[41] We were curious whether sterically demanding protein assembly could be mediated by TCA and TM recognition at the protein–protein interface; macromolecular assembly was clearly possible at the lipid–water interface but interfacial stoichiometry was not controlled. Defined protein functionalization may be achieved in a number of ways, but to test the assembly strategy, it was most expedient to couple TCA and TM to biotin, which binds tightly (K_d~10^{-15} M) to streptavidin protein (Scheme 1). Streptavidin is a homotetramer with 4 biotin binding sites and pseudo-tetrahedral protein symmetry.[42–44] Biotin site saturation with TCA–Btn and TM–Btn should place either four TCA or four TM recognition modules at each pseudo-tetrahedral site of streptavidin, thus transforming those ligand sites into protein–protein assembly[42] interfaces (Scheme 1). We first set out to confirm that biotin functionalization of TCA

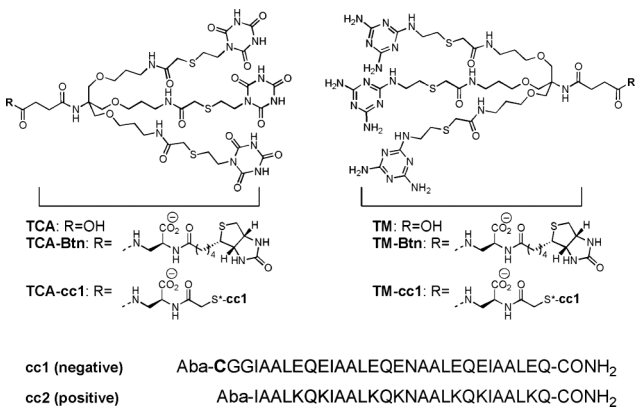

Fig. 1 Synthetic recognition modules used in this study. Peptide sequences for **cc1** and **cc2** are shown using the single letter code. The cysteine residue used to form the thioether bond is shown in bold in **cc1**. Aba is acetamidobenzamide, used as a chromophore to determine concentration.

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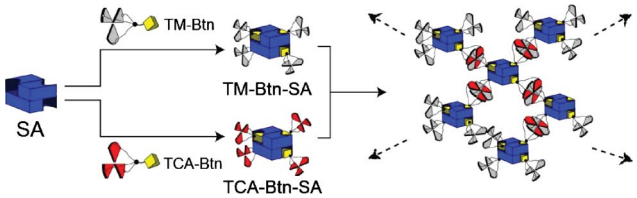

Scheme 1 Illustration of streptavidin (SA, blue) being saturated with TCA–Btn and TM–Btn and formation of protein assembly mediated by TCA/TM recognition.

and TM modules did not affect recognition. Indeed, simple coupling with a diamine resulted in poorly soluble products and it was necessary to use diaminopropionic acid as a linker, which preserved the solubilizing negative charge of the carboxylate group (Fig. 1). The resulting biotin conjugates, TCA–Btn and TM–Btn were readily purified and characterized. Biotin substitution did not affect molecular recognition by TCA and TM modules as judged by isothermal titration calorimetry (ITC), yielding exothermic interaction enthalpies of −29 to −30 kcal mol^{-1} at 25 and 10 °C, respectively, in line with what was observed with other TCA/TM derivatives[41] (Fig. 2). Interestingly, this data fits well to the 1 : 1 heterodimeric binding model, yielding a K_d of 0.59 and 0.070 μM at 25 and 10 °C, respectively. Although we have no direct structural evidence for this complex other than this data fit.

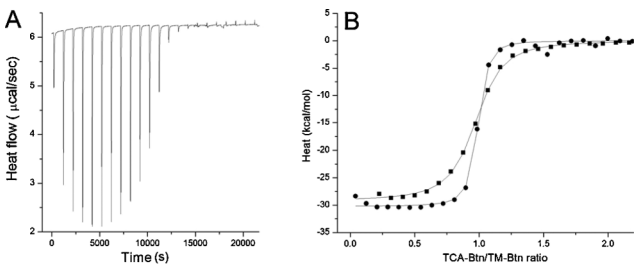

Fig. 2 Isothermal titration calorimetry data for titration of TCA–Btn into a solution of TM–Btn. In (A) raw data taken at 10 °C is shown and integrated data (B) taken at 10 (●) and 25 °C (■). Solid lines represent fits to a 1 : 1 heterodimer binding model.

With this functioning recognition pair in hand, we treated streptavidin separately with either TCA–Btn or TM–Btn in excess to saturate biotin-binding sites with these synthetic recognition modules. Unbound biotin derivatives were purified away from protein by protein precipitation and washing. In isolation, streptavidin-conjugates were soluble protein samples as judged by minimal scattering. However, mixing TCA–Btn–SA with TM–Btn–SA in a 1 : 1 ratio in buffer resulted in rapid formation of large aggregates as detected by DLS. This finding echoes prior results in which mixtures of TCA/TM formed nanoparticles with rigorously maintained 1 : 1 stoichiometry. These protein aggregates could be thermally dissociated in a reversible fashion, though with considerable hysteresis. The melting temperature of the aggregates is slightly higher than room temperature (~30 °C), which is significantly below streptavidin T_m (~75 °C for apostreptavidin, ~110 °C for biotin-saturated streptavidin),[45] so this aggregation is clearly associated with the non-native interaction between the synthetic TCA and TM ligands. On cooling, the system must be cooled to 15 °C before assembly begins. The hysteresis indicates non-equilibrium assembly conditions and suggests that the aggregates formed have a kinetic stability while formation of an "assembly nucleus" from soluble protein requires cooling to 15 °C. These protein aggregates were clearly derived from streptavidin conjugates as indicated by SDS-PAGE (Supporting Information).

We further probed the TCA-Btn-SA/TM-Btn-SA interaction by surface plasmon resonance (SPR) experiments, in which TCA–Btn or TM–Btn was used to surface saturate a streptavidin SPR chip the complementary streptavidin biotin conjugate was flowed over the surface. While control SA protein resulted in no signal, complementary SA conjugate produced strong, concentration-dependent signals consistent with protein–protein association interaction (Fig. 3A); this behavior was similar regardless of which SA conjugate was on the surface and which was in solution. Slow equilibration, indicated by thermal hysteresis in the DLS experiment (Fig. 3A), also prevented direct fitting of kinetic constants; nevertheless, a submicromolar estimate of K_d, was obtained by assumption of a simple binding model and observation of analyte concentration at 50% surface saturation (Fig. 3B). This K_d value is consistent with the apparent K_d of the TCA–Btn/TM–Btn complex in PBS buffer, (if a 1 : 1 complex is assumed) which supports the notion that the aggregation of functionalized SA proteins is mediated by TCA/TM recognition. Thus, it was clear that synthetic recognition modules can induce protein assembly in a reversible way. This was qualitatively underscored by the ability of soluble TCA and TM modules to competitively inhibit protein binding in the SPR experiment (Fig. 3D). Notably, there are two different noncovalent linkages in this assembly; the tighter biotin–streptavidin binding anchors display of a weaker but more reversible synthetic CA/M recognition motif, thus resulting in a supramolecular synthon using a protein building block.

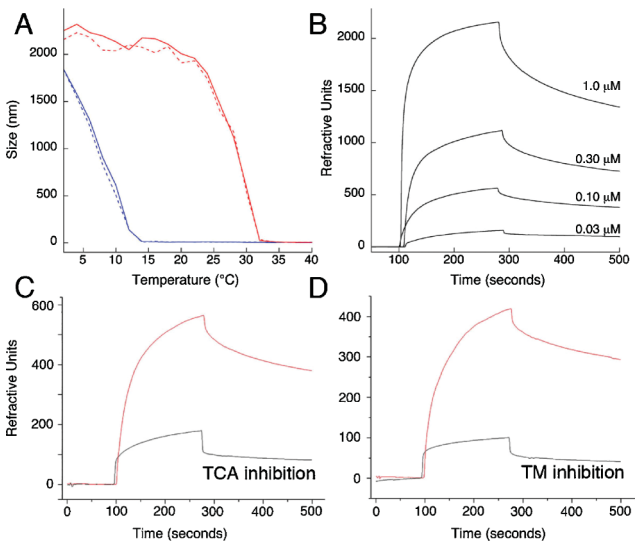

Fig. 3 (A) DLS of a 1 : 1 mixture of TCA–Btn–SA/TM–Btn–SA as a function of temperature, with heating and cooling traces shown in red and blue, respectively. The first heating-cooling cycle is shown in a solid line and the second cycle is shown in a dashed line. (B) SPR signal as function of soluble TM–Btn–SA concentration over a TCA–Btn–streptavidin SPR chip. Inhibition of TM–Btn–SA protein binding to a TCA–Btn–SA chip with TCA is shown in (C), while inhibition of TCA–Btn–SA binding to a TM–Btn–SA chip with TM is shown in (D). A 100-fold excess of soluble ligand (TCA or TM) to protein in solution was used for inhibition.

It is known that streptavidin itself has a propensity to assemble into oligomers beyond the tetramer.[46] We suspect that our recognition modules are working cooperatively with this native oligomerization interface to form a stabilized aggregate. It is possible that partial or complete burial of the TCA/TM complex at this protein–protein interface facilitates assembly. To probe this notion, we synthesized a system with more "exposed" TCA/TM modules by coupling these groups *via* thioether formation with the sulfhydryl sidechain of the N-terminal cysteine of a dimeric coiled-coil peptide (Fig. 4).[47] These peptides were readily synthesized on solid phase, purified, then coupled to TCA/TM chlorides in PBS buffer. While these peptides retained their ability to form dimeric coiled-coils as judged by analytical ultracentrifuge, no higher oligomerization states were observed by AUC under mid to high micromolar conditions (Fig. 4). Thus, it appears that the dimeric coiled coil platform does not mediate assembly, possibly because of insufficient interfacial surface area.

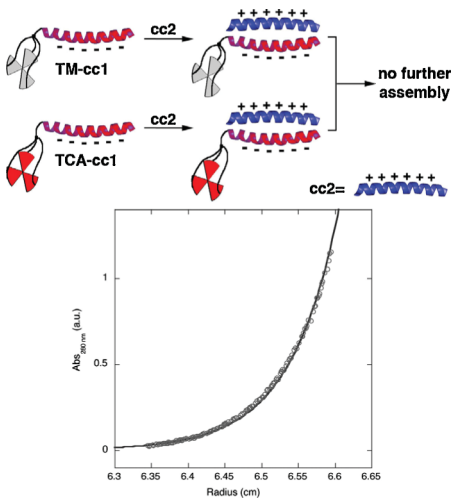

Fig. 4 (Top) Illustration of the formation of peptide heterodimers functionalized with TCA and TM that do not assemble into higher aggregates. (Bottom) Analytical ultracentrifuge data of a mixture of the TCA and TM heterodimers fit to a heterodimer model (solid line).

In summary, we have demonstrated the ability of synthetic recognition groups based on cyanuric acid and melamine recognition to trigger the assembly of streptavidin protein in a reversible manner. We have noncovalently conjugated these recognition modules to streptavidin in a defined manner using the native biotin ligand binding sites through biotin coupling to TCA and TM and shown thermally reversible assembly. Thus, this synthetic recognition motif, which has been shown to be functional in aqueous solution[41] and at the lipid–water interface, also functions at a protein–protein interface. However, our experiments with coiled-coil peptides suggest that an interface that provides sufficient burial of the recognition module is necessary for recognition. This is entirely consistent with our studies on TCA/TM recognition in isolation, and bodes well for future efforts using these synthetic motifs for directing biomolecular interactions in the synthesis of novel biomaterials and functional molecular assemblies.

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