Denaturation-Resistant Bifunctional Colloidal Superstructures Assembled *via* the Proteinaceous Barnase-Barstar Interface

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Supporting information

Results and discussion

Optimization of the PEG coating parameters for 'Smooth Surface' and non-smooth MPs. In order to render the magnetic particles utilized in this work more colloidally stable and attain the desired stringency of control experiments, we used coating (covalent conjugation) of the particles with PEG derivatives (heterobifunctional carboxy-PEG-amino, 3 and 10 kDa, see *Methods*). The following criteria were used for the choice of PEG derivatives to be conjugated with MPs prior to their functionalization with barnase:

1. Overall efficiency of self-assembly assessed as relative average fluorescence intensity of the resulting assemblies obtained *via* mixing equal amounts of MPs and FPs for each condition tested (typically, 5 µl of 2 g/l suspensions each);
2. Rigor of the blocking controls, i.e., specificity of interactions mediating assembly of the particles.

It appears from the comparison of the results obtained with 'Smooth Surface' and non-smooth MPs that the former satisfy the above criteria when conjugated with PEG 10 kDa, while the latter give better results with PEG 3 kDa (data not shown). Regarding comparative efficiency of self-assembly of the two types of MPs after this optimization step, non-smooth MPs proved superior to 'Smooth Surface' particles, which is confirmed by optical and SEM images (Fig. S1 and S2). Therefore, non-smooth particles were chosen for testing self-assembly of the initial conjugates and disassembly of preliminarily obtained assemblies in extreme conditions.

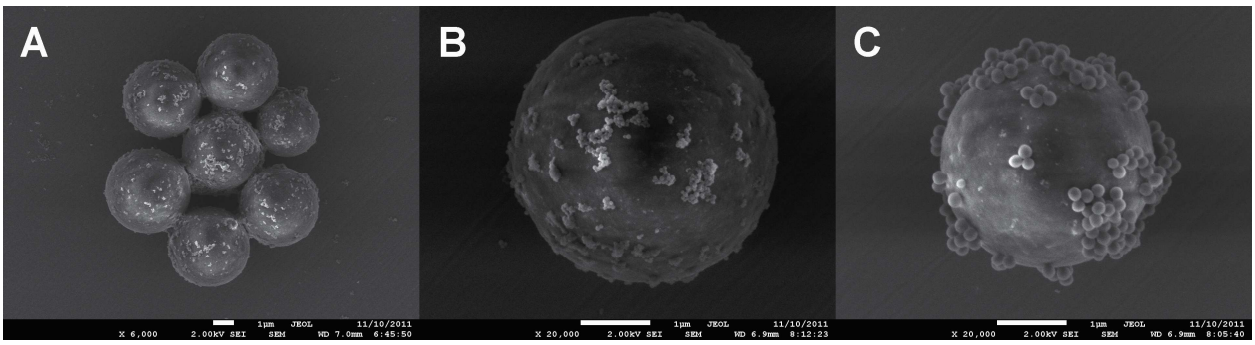

Figure S1. 'Smooth surface' MPs (SSMPs) are not satisfactorily efficient as modules for BBS-mediated self-assembly. (A) A grouping of superstructures assembled from [SSMP 3.4 µm – PEG 3 kDa – Bn] and [Yellow 60 nm – Bs] conjugates. (B) A single assembly from the same sample as in (A). (C) A single superstructure assembled from [SSMP 3.4 µm – PEG 3 kDa – Bn] and [Purple 200 nm – Bs] conjugates. Shown are assemblies obtained by combination of [SSMP 3.4 µm–PEG 3kDa–Bn] and [Yellow 60 nm–Bs] (A, B), and [SSMP 3.4 µm–PEG 3kDa–Bn] and [Purple 200 nm–Bs] conjugates (C).

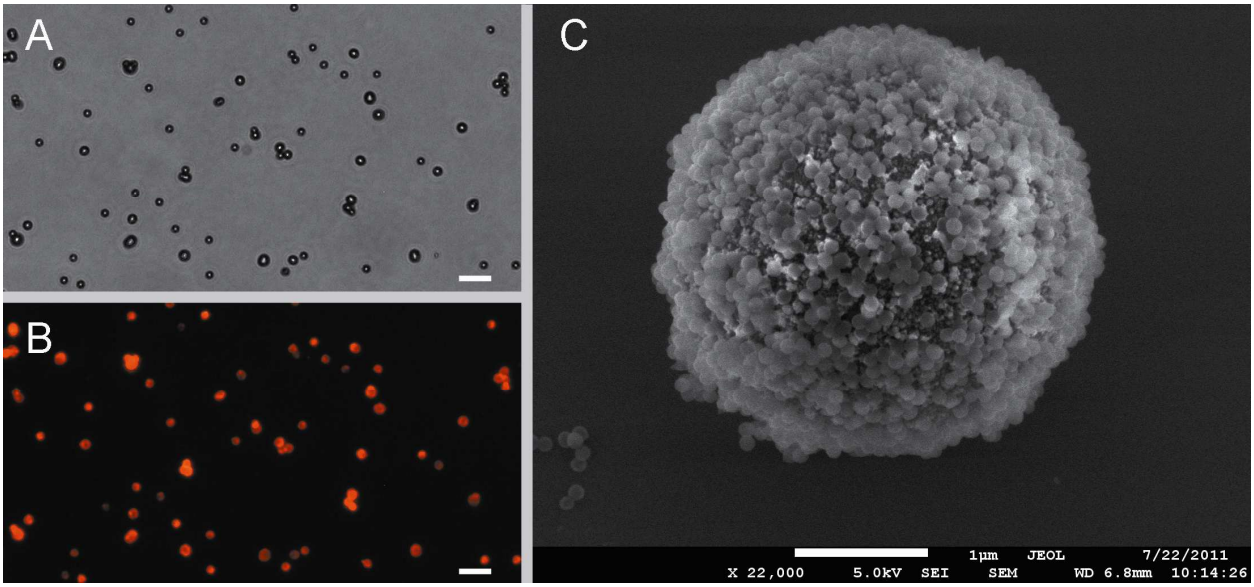

Figure S2. Non-smooth MPs are optimal for the BBS-mediated assembly. Optical images (*A* – bright field, *B* – upon excitation of fluorescence) and a SEM micrograph (*C*) of the assemblies obtained by mixing [MP (non-smooth) 3.3 µm – PEG 10kDa – Bn] and [Pink 53 nm – Bs] conjugates. Note the substantially denser coating of the MP surface with small FPs in comparison with 'Smooth surface' MPs shown in Fig. S1. Scale bar (*A, B*), 10 µm.

Time dependence of the efficiency of self-assembly. To assess the efficiency of self-assembly at different time points, equal volumes of 2 g/l suspensions of MPs and FPs were coincubated with gentle agitation for the corresponding time intervals, followed by termination of the self-assembly process by a magnetic separation procedure. The resulting curve is presented in Fig. S3. Notably, the process of self-assembly is rapid and does not require any additional conditions but agitation; however, even in its absence, particles assemble with similar efficiency, which may be slightly deteriorated because of sedimentation of large 3.3 µm MPs.

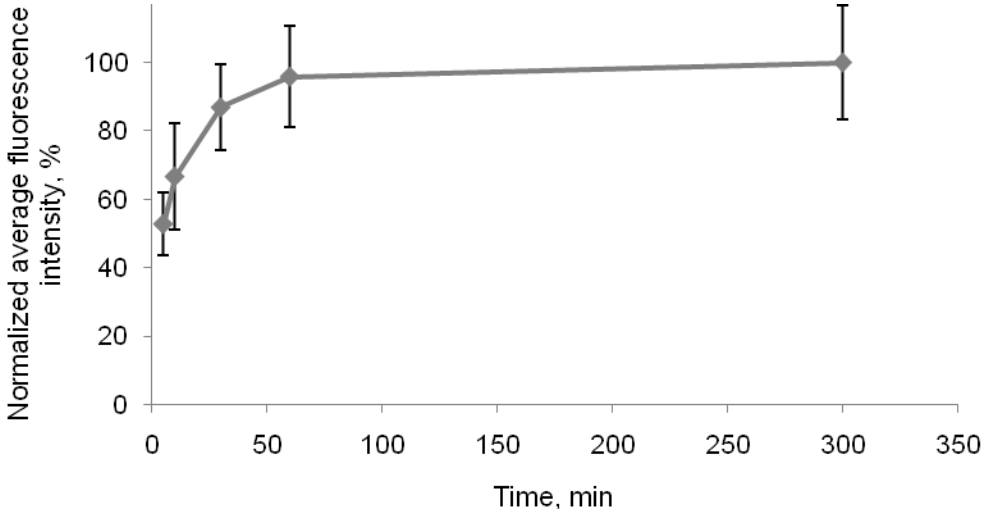

Figure S3. Time dependence of the efficiency of self-assembly.

Variation of parameters of extreme conditions for testing properties of self-assembly and disassembly of the five systems. As described in the main text, for testing of self-assembly and disassembly processes resistance towards denaturing conditions, the most extreme values of denaturing agent concentrations were used. However, in order to attain a more complete picture of the behaviour of the self-assembly systems under experimental conditions, we also tested intermediate concentrations of urea, GdmHCl (1, 2, 4, 6, 8M) and NaCl (1, 2, 3, 4, 5M). 504 samples were examined in total. Apparently, if the self-assembly systems proved unusually durable in the most extreme conditions, they are also well resistant to milder conditions, which we observed. Fig. S4 illustrates the results obtained with the lowest used concentrations of the denaturing agents. Comparison with the most severe conditions shows that the behaviour of four of the systems, with the exception of the GAb • bioIgG system, is relatively independent of the denaturing agent concentration, within the limits of experimental error. That is well observed in the case of GdmHCl and urea. However, the GAb • bioIgG system proved very susceptible to as small concentrations of denaturants as 2M, though in the case of urea, one can note concentration-dependent behaviour of this system, i.e., 2M urea affects integrity of the corresponding assemblies less severely. In addition, the pH-resistance of the assemblies is similar at pH 1.5 and pH 2.9 – all of the systems are remarkably durable, except the GAb • bioIgG system and, to a less extent, the protein A • rabbit IgG system.

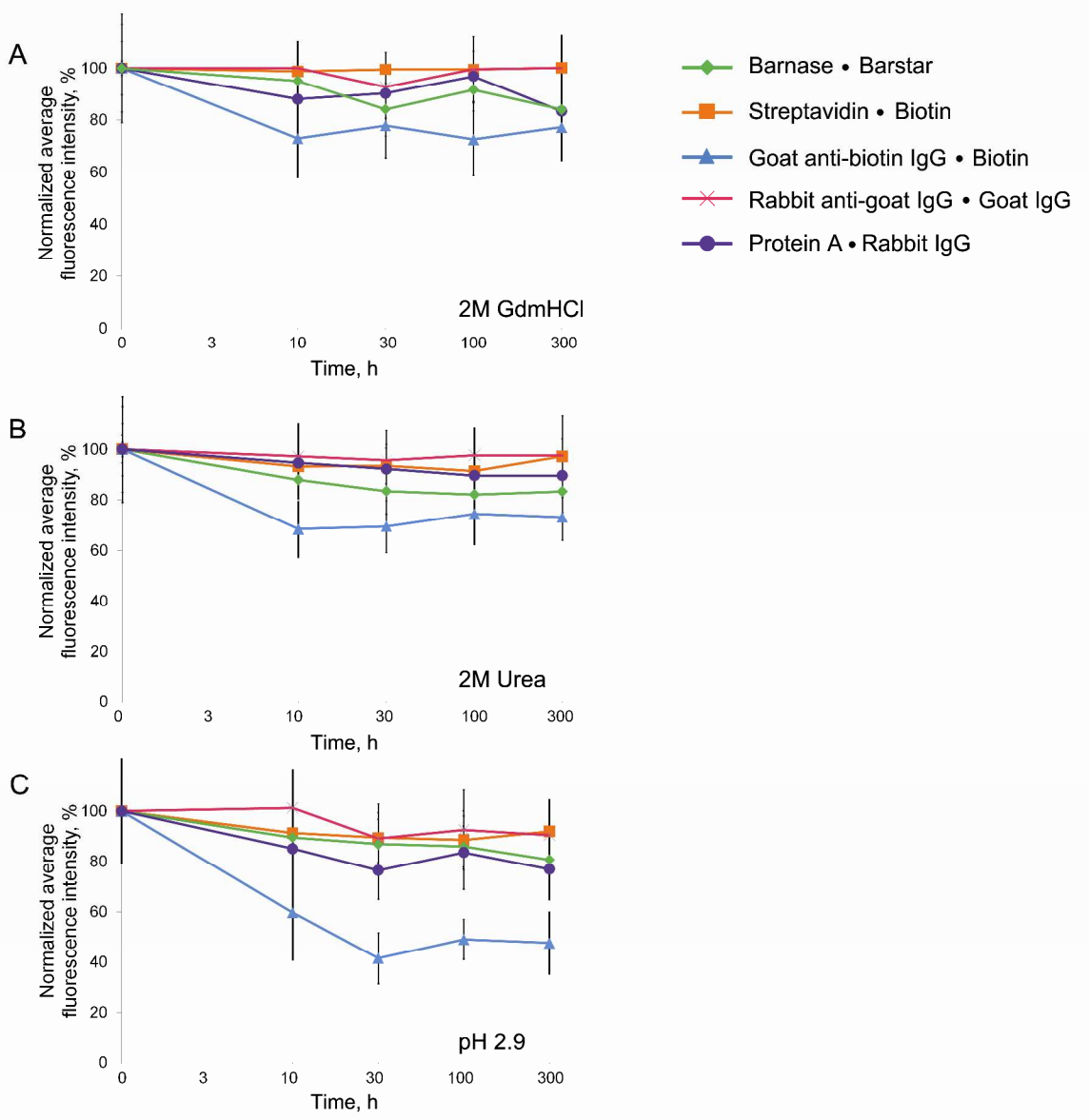

Figure S4. Summary of disassembly dynamics for the five self-assembly systems in relatively mild denaturing conditions: (A) in 2M GdmHCl, (B) – in 2 M urea, and (C) – at pH 2.9 (in citrate-phosphate buffer).

Thermostability of the assemblies. Thermal denaturation is one of the most studied ways of protein denaturation, thus, one would naturally test stability of the assemblies at physiologically high temperatures. We tested thermostability of the structures, preassembled in optimal conditions, at 60–80°C for time intervals (~2 h) that are more than sufficient to denature molecular species. The results of the corresponding experiments are presented in Fig. S5. As can be seen from the diagram, the five self-assembly systems behave similarly as a whole demonstrating high resistance towards destruction in such harsh conditions. GAb • bioIgG

system appears to be inferior in its thermostability to the rest of the self-assembly systems, as in most of the cases discussed above.

It should be noted, for comparison, that the melting temperature of the barnase–barstar complex is 75°C at pH 6.8 (ref. 1). Streptavidin • biotin complex is known to dissociate at 70°C (ref. 2). Thus, the thermostability data also confirms the suggested assumption regarding the superior stability of hybrid biomolecule-particulate colloidal constructs as compared to molecular species engaged in such structures.

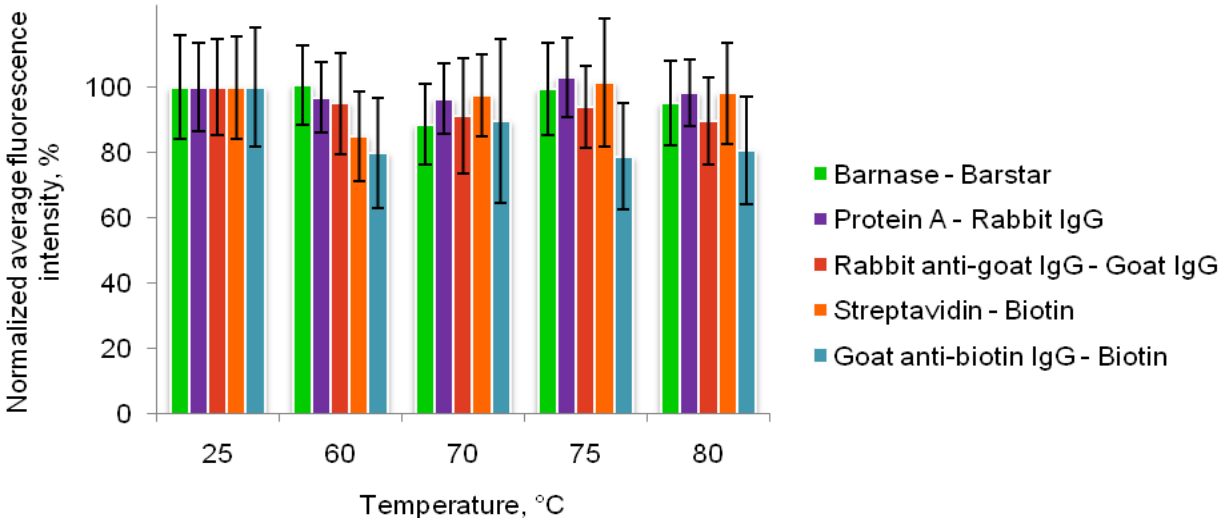

Figure S5. Thermostability of the structures preassembled in optimal conditions.

Additional discussion regarding resistance of the assemblies towards severe denaturing conditions.

The major idea we would like to emphasize in the article is that there are combinations of particle types and biomolecular complementary pairs (including biomolecules as fragile as proteins) resulting in nanoparticle assemblies that demonstrate unexpected stability under severe chemical conditions introduced by denaturing agents, salt or low pH. Apparently, both specific biomolecular and Van der Waals and other interparticle forces (the latter being 'turned on' by the former) contribute to the observed behavior of the systems. If the forces responsible for maintenance of integrity of the assemblies in these conditions were mediated only by non-specific interactions, then one would notice no difference in behavior of the systems tested in terms of their stability under extreme conditions. However, such a difference does exist, and is consistently exhibited by the Goat anti-biotin antibody • Biotin system in all conditions of disassembly experiments.

Considering the problem of nature of nanoparticle interactions, one would admit that Van der Waals forces between particles *per se* can contribute to a large extent to the overall energy of

interactions, being 'arguably the most ubiquitous form of nanoscale interaction'.[3] The computational approaches for estimation of Van der Waals forces include Hamaker discrete and integral approximations,[4] continuum Dzyaloshinskii–Lifshitz–Pitaevskii theory (macroscopic theory of Van der Waals forces),[5,6] and the discrete coupled-dipole method.[7,8] It should be noted that these theories have been elaborated using mostly simple monodisperse and homogeneous particles as models for calculation. While the protein-assisted self-assembly systems studied in the present work are characterized by non-uniformity of particle coating with protein molecules as well as hydrophobic-hydrophilic 'patchiness' of the proteins themselves, it is in principle possible to apply the mentioned theories for estimation of the interaction energies in these systems. However, the estimates may be fairly approximate taking into account the complex and multicomponent nature of the self-assembly systems.

Apart from the mentioned chemical aspect of stability of the assemblies, the question of their resistance to conditions of extreme *mechanical* stress remains open. Further investigation is needed to test if under these conditions specific biomolecular interactions contribute significantly to the assembly stability as compared to other interparticle forces (Van der Waals, electrostatic, *etc.*), what is the relation between them, and how one can design the self-assembly modules to ensure specific and robust assembly. This question can be addressed using highly precise and sophisticated techniques, many of which employ complex dynamometric devices, special negative feedback systems, *e.g.*, Atomic Force Microscopy (AFM) (ref. 9 and references therein). It is plausible to use these techniques (*e.g.*, AFM[10]) to measure the interaction energy between the particles within the assembly; however, here we again face issues of non-uniformity of the stoichiometry of the particles in the assemblies as well as of number of pairs of linking entities (*i.e.*, protein–protein or protein–small ligand pairs in the present work) between the particles within a given pair. That considerably complicates and increases requirements to the methodology of measurements.

Moreover, one can consider an important aspect of the discussed measurements. Recently, a previously unappreciated dependence of the forces measured by AFM on the timescale of measurements has been reported[10]: at least at single-molecule level, it has been shown that using rapid nanomechanical interfaces at the microsecond timescale one can measure rupture forces in the streptavidin–biotin complex up to 750 pN (ref. 10), a value predicted by molecular dynamics simulations and not reported before in the studies using alternative experimental approaches (*e.g.*, ref. 11). Thus, the resistance of the assemblies under the conditions of mechanical stress will also be dependent upon the timescale of specific applications they are intended to be employed in. That is, on the one hand, it could be of interest to probe assemblies for resistance to rupture under rapidly applied extreme mechanical stress conditions. However, a prolonged action of a more moderate external mechanical force, *e.g.*, constant flow, may not have the same effect. Hence, the applications themselves determine the way the *mechanical* 'ultimate strength limit' of the system (as opposed to the *chemical* one studied in the present work) can be measured.

From the standpoint of colloidal stability, it should be noted that because self-assembly is not simple aggregation of the particles, colloidal stability of individual particles is of paramount

importance. Here, the concept of 'structural-mechanical barrier' (SMB) introduced by Rehbinder,[12] seems to be the most relevant for discussion of the colloidal behavior of the systems under study. SMB refers to 'a strong factor of stabilization of colloidal systems due to the formation of interfacial adsorption layers of low and high molecular weight surfactants that lyophilize surfaces'.[9] These surfactants are able to form three dimensional gel-like structural layer at the interface, but are not necessarily highly surface-active with respect to a given interface. Examples of such 'protective colloids' include proteins, carbohydrates (*e.g.*, cellulose derivatives like carboxymethylcellulose), *etc.*, that is, (macro)molecules with complex structures possessing regions of higher and lower hydrophilicity within the same molecule.[9] Adsorption of these macromolecules to the surface of the particles seems to be an essentially irreversible process (either physisorption or chemisorption) in the cases where the energy gain of sticking of the monomers exceeds kT.[13] In the hybrid self-assembly systems studied herein, proteins act as protective colloids ensuring high colloidal stability of the particles. Although the 3-μm magnetic particles used in this work lack sedimentation stability simply because of their large size, they do not aggregate (upon conjugation with PEG and proteins). Neither do the smaller 200-nm fluorescent particles (and their conjugates with corresponding proteins) that demonstrate resistance not only to aggregation but also to sedimentation for at least one week after preparation. High colloidal stability of the modules allows specific self-assembly of the particles into individual structures (cf. Figure 2) and not just plain aggregation or coagulation of the particles.

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