Barnase-barstar module as a platform for targeted delivery of imaging and therapeutic nanoconstructs

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Standard procedures for design of targeted imaging and therapeutic compounds are based on an attachment of recognizing molecules to visualizing agents or drugs. In frame of this approach the fully genetically encoded anti-receptor antibody-photosesitizer [1] and immunoRNase [2] were constructed. A fluorescent protein, Killer Red, and a ribonuclease barnase were used as toxic principles. They were fused to the single-chain scFv-fragment of anti-HER2/neu antibody 4D5 that recognizes the extracellular domain of cancer marker HER2. The both bifunctional fusion proteins demonstrated specific cytotoxic effect on HER2-positive human carcinoma cells.

A novel strategy for design of heterostructures based on the ribonuclease barnase and its inhibitor, barstar, was suggested [3, 4]. The barnase and barstar are small, stable, very soluble, resistant to proteases proteins. The complex between them is extremely tight with a $K_d \sim 10^{-14}$ M.

The N- and C-terminal parts of both proteins are localized outside of the barnase·barstar interface and are therefore accessible for fusion with targeting, visualizing or toxic compounds. The fusions thus constructed, anticancer miniantibodies-barnase (scFv-barnase or scFv-dibarnase) and scFv-barstar, then form extremely stable dimers (or trimers) due to the practically irreversible pairing of the ligands through their native interface. It was shown that the constructed oligomers provide effective delivery of radioisotope to the xenografted HER2/neu overexpressing human adenocarcinoma SKOV3 in mice. The barnase fusion with fluorescent protein EGFP was used for visualization of cancer cells by assembling two fusion proteins, EGFP-barnase and anti-HER2/neu scFv-barstar. The efficient killing of cancer was achieved when instead of fluorescent protein the toxic agent, pseudomonas exotoxin A, was linked to barstar and assembled with anticancer scFv-barnase. The suggested strategy is applicable to virtually any proteins that can be functionally attached to the barstar and barnase molecules. It seems particularly well suited to the production of heterooligomeric constructs because the extremely specific barnase·barstar interaction eliminates reliably the mispairing problems. The important advantage of barnase·barstar over the majority of other dimerization modules is that their interaction ratio is precisely 1:1, and neither of the partners is aggregation prone.

To describe the “Protein-assisted NanoAssembler” method, we would like to introduce the following glossary: individual single-functional particles are *conjugated* with one of the proteins (barnase or barstar) to form *modules*; modules are *assembled* together to form *superstructures*. Since the link between the modules is mediated by proteins and is very strong or quazicovalent,

we say that the assembly is specific or *protein-assisted* to distinguish from ordered packing or ordinary agglomeration.

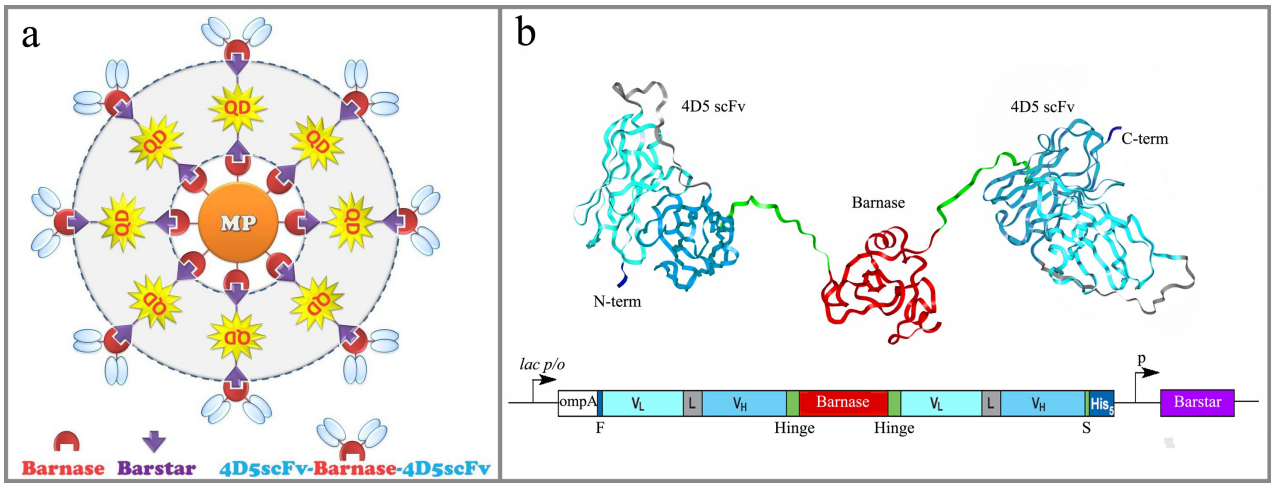

Fig. 1**. Conceptual design of trifunctional superstructures using barnase and barstar non-covalent binding system (BBS).**
(a) Layer-by-layer assembly: [magnetic particles(MP)-barnase] module as a core, then a layer of [quantum dots (QD)-barstar] modules, and, finally, a layer of [4D5scFv-barnase] modules. (b) Construction of the recognition module: molecular model (ribbon representation, PDB 1fve and 1bnr) of the 4D5scFv-barnase-4D5scFv (above) and gene construct encoding the recombinant protein. The 4D5 scFv-barnase-4D5 scFv-His_5 construct starts with N-terminal short FLAG tag (F, dark blue) followed by 4D5 scFv in VL-linker-VH orientation (VL, turquoise; linker, grey; VH, cyan), 16-amino-acid hinge linker (green); barnase (red), hinge linker and 4D5 scFv. The construct terminates in His_5-tag (dark blue) attached via a short spacer (S) with the sequence Gly-Ala-Pro (green) to the C-terminus of scFv-barnase-scFv fusion protein. The fusion gene is under control of the *lac* promoter and the ompA signal peptide is used to direct the secretion of the recombinant proteins to the *E. coli* periplasmic space. Barstar coexpression controlled by its own constitutive promoter (p) is required to suppress the cytotoxicity of barnase fusions. *lac* p/o, *lac* promoter/operator.

The presented concept was tested with the use of scFv antibody fragments that were recombinantly fused to barstar and barnase (**Fig. 1**), while inorganic nanoparticles - colloid gold, semiconductor nanocrystals [5], magnetic particles [6] - were linked to partner proteins, barnase and barstar, respectively. A high efficiency and specificity of the tumor cells labeling were achieved in all listed cases. The assembly has demonstrated that the bond between these proteins is strong enough to hold macroscopic (5 nm - 1μm) particles together. The properties of the assembled superstructures can be programmed on demand by linking different agents designated for specific goals (**Fig. 2**).

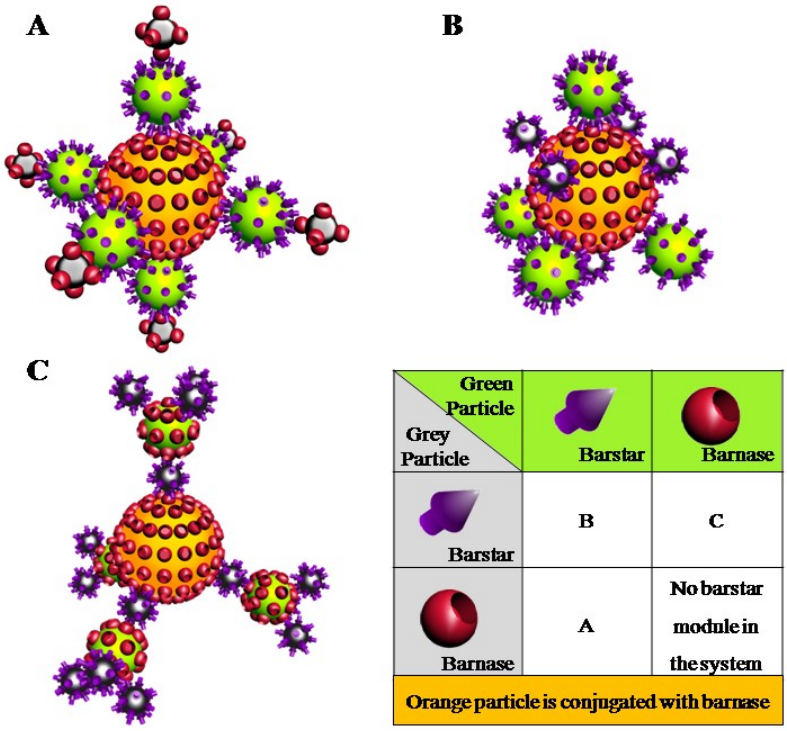

Fig. 2. **Possible variants for assembling of three particles into a superstructure by the "Protein-assisted NanoAssembler" method**. Correspondence of different combinations of BBS proteins conjugated with the green and grey particles and the structure number (*A, B, C*) is shown in the table. Barnase is chosen as the protein conjugated with the core orange particle.

This universal platform provides a straight-forward technology to design a multifunctional nanoheterostructures "when the whole is greater than the sum of the parts".

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3. *S.M. Deyev et al.* Design of multivalent complexes using the barnase·barstar module. Nat. Biotechnol. **21**, 1486-1492 (2003).
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