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Research articles

Self-assembling nanoparticles biofunctionalized with magnetite-binding protein for the targeted delivery to HER2/neu overexpressing cancer cells

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ABSTRACT

Surface modification of nanoparticles with various biologically active molecules makes it possible to realize the huge biomedical potential of various nanoobjects. Functionally active particles are usually obtained through stabilization by polymers and subsequent chemical conjugation with biomolecules, such as, antibodies or aptamers. This entails a number of problems, such as non-oriented conjugation, low coupling yield and the inability to easily vary the attached components on demand. To solve this problem, we developed a novel method of magnetite nanoparticles stabilization with simultaneous modification by functionally active protein – Barstar, for subsequent self-assembly of the necessary components with these particles through the interaction of high-affinity protein pair Barnase * Barstar ($K_{aff} = 10^{14}\ M^{-1}$). Namely, we developed a biocompatible Bs-C-Mms6 fusion protein containing the C-terminal part of the Mms6 (magnetite-binding protein of magnetotactic bacteria) and Barstar (an inhibitor of bacterial ribonuclease Barnase). We obtained stable in PBS magnetite nanoparticles modified with Bs-C-Mms6. These particles can be used for self-assembly with any type of Barnase-containing molecules. To demonstrate the effectiveness of this approach for the development of targeted nanoparticles, we performed a self-assembly of these particles with a fusion protein of Barnase and DARPin9.29, namely DARPin9.29-Bn. DARPin9.29 recognizes the extracellular domain of clinically important HER2/neu oncomarker. We have shown that such particles selectively bind this oncomarker and can be used to detect HER2/neu-positive cancer cells for diagnostic purposes.

1. Introduction

The nanobiotechnologies advances have a significant impact on the development of modern methods of therapy and diagnosis of socially significant diseases. Targeted delivery – the concept of delivery of certain drugs and diagnostic compounds to the specific tissue sites within the living organism, is one of the key points in the development and progress of personalized medicine. Now significant attention of researchers is being directed toward one certain area of personalized medicine, namely, theranostics, which implies the combination of diagnostic and therapeutic approaches on a single platform. It seems promising to use different multifunctional supramolecular systems based on nanoparticles for theranostic applications, because different nanostructures, due to their physico-chemical properties, possess a number of properties that are impossible to obtain using different components individually. This comprehensive effect on the tumor tissue allows realizing the principle that the whole is greater than the sum of its constituent parts [1–5].

However, such constructions transition to clinical practice is often hampered with complicated chemical conjugation of functional components (antibodies, toxins, etc.) to nanostructures. Moreover, biocompatibility and stability under physiological conditions of most of them is severe problem during the development of functionally active structures for cancer theranostics [6–8].

Here we propose the novel facile method of nanoparticle biomodification with functional molecules for biomedical applications. This method is based on the development of functionally active nanoparticles during the processes of synthesis and stabilization without using any chemical conjugation. Namely, we designed new recombinant fusion protein of Barstar and C-terminal part of Mms6, Bs-C-

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Mms6, for magnetic nanoparticles simultaneous synthesis and modification without using any toxic polymer during nanoparticle production. The first component of this protein, Mms6, is a protein of magnetotactic bacteria that orient in the Earth's magnetic field due to specialized organelles called magnetosomes which contain membrane-enveloped single crystalline magnetite. Mms6 (especially its C-terminal part) plays a crucial role in magnetite crystals biomineralization and in magnetosomes formation in *Magnetospirillum magneticum* (strain AMB-1) [9–13]. The second component of this protein, namely, Barstar, is a natural inhibitor of ribonuclease Barnase. Barnase and Barstar are small, extremely stable and hydrophilic proteins, in which both N- and C-terms are accessible for genetic fusion, and these proteins possess extremely fast kinetics ($k_{on} = 10^8\,M^{-1}\cdot s^{-1}$) and high affinity of binding ($K_{aff} = 10^{14}\,M^{-1}$) [14–16]. Thus, these proteins form the universal platform for self-assembly of nanoparticles with any kind of biological compound using Barnase * Barstar interaction. On this platform, nanoparticle modified with Barstar during the synthesis process, and attached component is fused with Barnase and then added and just mixed with nanoparticle to get a functional nanostructure.

To demonstrate the capabilities of the developed method, we designed nanoparticles capable of selective targeting HER2/neu overexpressing cancer cells using DARPin scaffold protein as HER2/neu recognizing molecule on the nanoparticle surface. HER2/neu is overexpressed in 20–30% cases of human breast cancers and correlates with poor prognosis and high metastatic tumor potential [17,18]. We showed that DARPin-modified nanoparticles with the help of the developed approach capable of efficiently targeting and labeling HER2/neu-positive cells in comparison with HER2/neu-negative ones.

2. Materials and methods

2.1. Design of plasmid pET22_Bs-C-Mms6

The plasmid pET22_Bs-mRFP (previously obtained in Molecular Immunology Laboratory of IBCh RAS), containing the Barstar and mRFP genes, and the sequence encoding the hinge-like heavy chain part of human immunoglobulin G3, and in which the RFP gene is flanked with the restriction endonucleases *Nco*I and *Hind*III recognition sites, was used to create the genetic construct encoding the Bs-C-Mms6 protein. Cloning of the sequence encoding the C-terminal portion of the Mms6 protein (hereinafter, C-Mms6) of *Magnetospirillum magneticum* AMB-1 (YMKSRDIESAQSDEEVELRDALA) was performed at the *Nco*I and *Hind*III restriction sites. Because of the small size of this sequence (69 bp), it was decided to amplify not from genomic DNA, but using overlapping primers (5′-TAGACCCATGGGGTATATGAAAAGC CGTGAT ATTGAAAGCGCACAGAGCGATGAAGAAGTTG-3′ and 5′-AATGCAAGC TTATCATGCCAGTGCATCACGCAGTTCAACTTCTTCATCGCTCTGTG-3′) to complete them to double-stranded DNA, used further as an insert. The correctness of the obtained plasmid pET22_Bs-C-Mms6 was confirmed by restriction mapping and sequencing. In the resulting construct, the gene encoding the Bs-C-Mms6 target protein was under the control of the strong RNA polymerase promoter of bacteriophage T7 and the lac operator.

2.2. Isolation and purification of Bs-C-Mms6

For the isolation of the recombinant Bs-C-Mms6 protein, competent *E. coli* cells, strain BL21 (DE3), were transformed with plasmid pET22_Bs-C-Mms6. The cells were cultured in LB medium (with ampicillin 100 μg/mL) with vigorous stirring at 37 °C until OD reached 0.8 and then was induced and 1 mM IPTG. After the induction, *E. coli* were grown for 10 h at 37 °C. After this, the cells were cooled and centrifuged at 6000*g* for 10 min at 4 °C. The cell pellet was resuspended in buffer I (5 mM Tris-HCl, 40 mM K_2HPO_4, 500 mM NaCl pH 8.0), the cells were disrupted in an ice bath using an ultrasonic disintegrator. Cell debris was removed by centrifugation at 50,000*g* for 60 min. The pH of the supernatant was adjusted to 8.0 and filtered through a 0.22 μm membrane. The filtrate was applied to a Ni^{2+}-NTA column (GE Healthcare). The column was washed with buffer I with 15 mM imidazole and 0.2% Triton X-100, the protein was eluted with buffer I with 250 mM imidazole.

2.3. Cell lines and incubation conditions

SK-BR-3 and CHO cell lines were cultured in DMEM medium supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine at 37 °C in a humidified atmosphere with 5% CO_2. Cells were passaged 2–3 times a week when reaching 80–90% of the monolayer. The removal of cells from the plastic surface was carried out with 2 mM EDTA, without the use of trypsin (to prevent the enzymatic cleavage of receptors from the cell surface). Cell lines were maintained in culture for no more than two months, after which the culture was updated from the collection of frozen cells.

2.4. MTT assay

The cytotoxicity of proteins and nanoparticles was investigated with an MTT test. Cells were seeded on a 96-well plate at $7.5{\cdot}10^3$ cells per well in 200 μL DMEM with 10% FBS. After culturing the cells at 37 °C in a CO_2 incubator overnight, the medium was removed and medium (negative control) or medium containing the test particles (or proteins) at different concentrations in a volume of 200 μL per well were sterilely added and cells were incubated for 24 h. The medium was then removed; the cells were washed 1 time with medium. Next, 100 μL of MTT solution (0.5 g/L in DMEM) were then added to the wells, incubated for 1 h at 37 °C in an atmosphere with 5% CO_2. At the end of this time, the MTT solution was removed and 100 μL of DMSO was added to the wells, the plate was shaken until the formazan crystals dissolved completely. The optical density of each well was measured using Infinite M1000 Pro (Tecan, Austria) microplate reader at a wavelength of $\lambda = 540$ nm.

2.5. Quantitative analysis of magnetic particles binding to cells

The quantification of binding of magnetic nanoparticles to cells was carried out using the previously developed MPQ-cytometry method [2]. A suspension of cells labeled with nanoparticles in a volume of 30 μL was placed into the instrument measuring zone in a plastic tube of 3 mm in diameter. The excitation of nanoparticles in the measuring coil of the detector was carried out at frequencies $f_1 = 87$ kHz and $f_2 = 702$ Hz with amplitudes $H_2 = 64 \pm 6$ Oe, $H_1 = 33 \pm 3$ Oe, respectively, the response was detected at the combinatorial frequency $f_1 + 2f_2$.

2.6. Cell labeling with DARPin9.29-Bn * Bs-C-Mms6-FITC structures

The cells harvested from the surface of the culture plastic were washed with PBS, resuspended in 500 μL of PBS with 1% BSA, at a concentration of 10^6 cells/mL and cooled to +4 °C. Then, DARPin9.29-Bn was added to the cells to get a final concentration of 10 μg/mL and incubated for 15 min at +4 °C. The cells were then washed twice with PBS with 1% BSA, resuspended in 500 μL of PBS with 1% BSA, and Bs-C-Mms6 labeled with FITC (Bs-C-Mms6-FITC) was added to the cells. The cells were incubated for 15 min at +4 °C and washed twice with PBS with 1% BSA, and finally resuspended in 200 μL of PBS with 1% BSA for flow cytometry analysis.

2.7. Flow cytometry

A flow cytometer BD Accuri C6 (Becton Dickinson) was used to analyze the cells in the FL1 fluorescence channel (488 nm excitation, 533/30 nm emission). 10,000 cells were analyzed in each sample. As a

control (autofluorescence of cells), cell samples prepared without the addition of proteins were used. The data obtained was analyzed using the CFlow Plus and FlowJo programs.

2.8. FITC conjugation

FITC-labeled DARPin9.29-Bn and Bs-C-Mms6 proteins were prepared as follows: 10 μL of FITC at 6 g/L in DMSO was added to 100 μL DARPin9.29-Bn at 2 g/L or to 100 μL Bs-C-Mms6 at 1 g/L in PBS and incubated for 8 h at RT with subsequent purification using Zeba Spin Desalting Columns, 7 K MWCO.

2.9. Fluorescence spectroscopy

The 15 μg of nanoparticles incubated with fluorescently labeled proteins were placed into 96-well plate and analyzed with Infinite M1000 Pro microplate reader in bottom fluorescence mode at excitation and emission wavelengths of 490 nm and 525 nm, respectively, at 400 Hz in flash frequency mode with gain of 190.

3. Results

It was previously shown by NMR spectroscopy, that the DEEVE sequence of the C-terminal part of the Mms6 protein of *Magnetospirillum magneticum* AMB-1 (hereinafter, C-Mms6 – YMKSRDIESAQSDEEVELR-DALA) exhibits the high affinity for Fe^{2+} and Fe^{3+} ions and is most actively involved in magnetosome nucleation [19].

Thereby, to obtain functionally active magnetite nanoparticles, we designed a fusion protein consisting from the C-terminal part protein of Mms6 from *Magnetospirillum magneticum* AMB-1 and Barstar (hereinafter the fusion protein is designated Bs-C-Mms6). The scheme of genetic construct pET22_Bs-C-Mms6 encoding the Bs-C-Mms6 protein (15.6 kDa) is shown in Fig. 1A. In this construct, the gene encoding the Bs-C-Mms6 target protein is under the control of the strong T7 RNA polymerase promoter and the lac operator. As-synthesized plasmid *pET22_Bs-C-Mms6* was used to express the fusion protein in *E. coli* [BL21(DE3) strain] (Fig. 1B).

The retention of the functional activity of the Barstar within the composition of this fusion protein was studied by its ability to inhibit the activity of bacterial ribonuclease Barnase. Using the method of acid-insoluble RNA precipitate [20], it was shown that the obtained Bs-C-Mms6 protein possesses RNase inhibiting activity (Fig. 1C) and, accordingly, can be effectively used for the self-assembly of nanostructures based on the Barnase*Barstar interaction. From the point of view of the biocompatibility of the developing nanoparticles, it is worth noting the fact that the Bs-C-Mms6 protein does not exhibit cytotoxicity in a wide range of tested concentrations (0.07 μM–10 μM). This fact was confirmed with the MTT test on cell lines of various origins, namely, on human breast adenocarcinoma SK-BR-3 and on Chinese hamster ovary CHO cells (Fig. 1D).

Next, in order to obtain the magnetic core of functionally active nanoparticles, we synthesized magnetite nanoparticles in alkali conditions by coprecipitation of $FeCl_2$ и $FeCl_3$ similar to described by us earlier [1,2]. The TEM microphotograph of these particles is presented in Fig. 1E, the picture of electron microdiffraction is presented in Fig. 1D. These particles represent spherical single crystals of magnetite, the size of single crystal obtained from the microphotographs was found to be 10.9 ± 1.9 nm. However, the method of dynamic light scattering shows the diameter of these particles to be 79.9 ± 31.8 nm. This difference between the TEM physical size of monocrystalline nanoparticles and DLS size is due to the measurement of nanoparticles in dry and solvated state, respectively. Solvent molecules (water), associated with hydrophilic MPs lead to the increase of observed diameter.

As synthesized magnetite nanoparticles (MPs) were stabilized with the obtained fusion protein Bs-C-Mms6. MPs were incubated with this protein at different concentrations in MilliQ water for 8 h with triple washing from non-bound protein with centrifugation. Bovine serum albumin (BSA) and Barstar (Bs) proteins were used for control experiments. It was shown that after washing from non-bound proteins, the hydrodynamic size of the nanoparticles incubated with both the target protein and the control proteins (BSA and Bs) changed significantly in comparison with unmodified nanoparticles (see nanoparticle size distributions in Fig. 1G-I and Table 1 in [21]). However, only nanoparticles stabilized with Bs-C-Mms6 protein retained aggregative and sedimentation stability in phosphate buffered solution (PBS) for a long period of time (2 months, no further observations were made). The use of control proteins, namely, BSA and Bs in the whole range of tested concentrations, did not result in the stabilization of the nanoparticles in the PBS: the particles precipitated in PBS within a few minutes.

To stabilize MPs with Bs-C-Mms6 protein, a protein concentration of 5 μM was chosen as the minimum one required for stabilization in PBS. MPs incubated with Bs-C-Mms6, BSA and Bs, in PBS are shown in Fig. 2A. The hydrodynamic size of the nanoparticles stabilized by Bs-C-Mms6 (MP * Bs-C-Mms6) in PBS was found to be 137 ± 38 nm.

Similarly to the Bs-C-Mms6 protein, MP*Bs-C-Mms6 nanoparticles did not exhibit cellular cytotoxicity: after the incubation of cells with MP * Bs-C-Mms6 in the concentration range of 0.3–50 μg/mL for 24 h, the cell survival rate was found to be 100 ± 1.3% (Fig. 2B).

In order to demonstrate the effectiveness of the developed platform for nanoparticle biomodification, we designed magnetic nanoparticles capable of selective binding to HER2/neu oncomarker on the surface of cancer cells. To this end, we used the “lego”–approach with the protein adaptor system Barnase * Barstar and HER2/neu-recognizing scaffold protein DARPin9.29 [22]. Nanostructures were obtained as schematically illustrated in Fig. 2C. The principle of biomodification is in stabilization of magnetite particles (MP) with magnetite-binding protein Bs-C-Mms6 and subsequent self-assembly of the MP * Bs-C-Mms6 with the fusion protein DARPin9.29-Bn containing recognizing module DARPin9.29 and one component of the adaptor system, namely, Barnase.

Using the described approach, we obtained nanostructures MP * Bs-C-Mms6 * DARPin9.29-Bn. First of all, we tested the specificity of these nanostructures in a cell-free system *in vitro*. For this purpose, MP * Bs-C-Mms6 (as well as MP * BSA for control experiments), were incubated with 0.03, 0.3 and 3 μM of DARPin9.29-Bn labeled with fluorescein isothiocyanate (DARPin9.29-Bn-FITC) in MilliQ water with 0.05% Tween-20 with subsequent washing from non-bound protein by triple centrifugation. Next, the relative fluorescence intensity was measured at wavelengths corresponding to the excitation and emission of FITC (490 and 525 nm, respectively). The data presented on Fig. 2D confirm the specificity of protein DARPin9.29-Bn-FITC interaction with nanostructures MP * Bs-C-Mms6.

Further, the specificity of the DARPin9.29-Bn protein was investigated in terms of interaction with the HER2/neu receptor on the cell surface. To this end, cells overexpressing the HER2/neu receptor, namely breast adenocarcinoma SK-BR-3 and HER2/neu negative cells, namely CHO, were labeled with DARPin9.29-Bn-FITC protein and examined with flow cytometry. Flow cytometry histograms presented in Fig. 2E prove the high specificity of DARPin9.29-Bn-FITC interaction with HER2/neu + SK-BR-3 cells.

Next, we verified the self-assembly of protein components DARPin9.29-Bn and Bs-C-Mms6 on the surface of cancer cells. To this aim, two-step assembly of these proteins was realized: cells were incubated with DARPin9.29-Bn and with Bs-C-Mms6-FITC with washing steps from non-bound components during all intermediate stages. The data presented in Fig. 2F show a high selectivity of assembly of these components only on the surface of HER2/neu positive cells SK-BR-3.

The designed self-assembled structures based on magnetite nanoparticles MP * Bs-C-Mms6 * DARPin9.29-Bn were used for targeted delivery to SK-BR-3 cells. These nanostructures (as well as MP * Bs-C-Mms6) were incubated with SK-BR-3 and CHO cells, washed from unbound nanostructures, and the number of cell-bound nanostructures

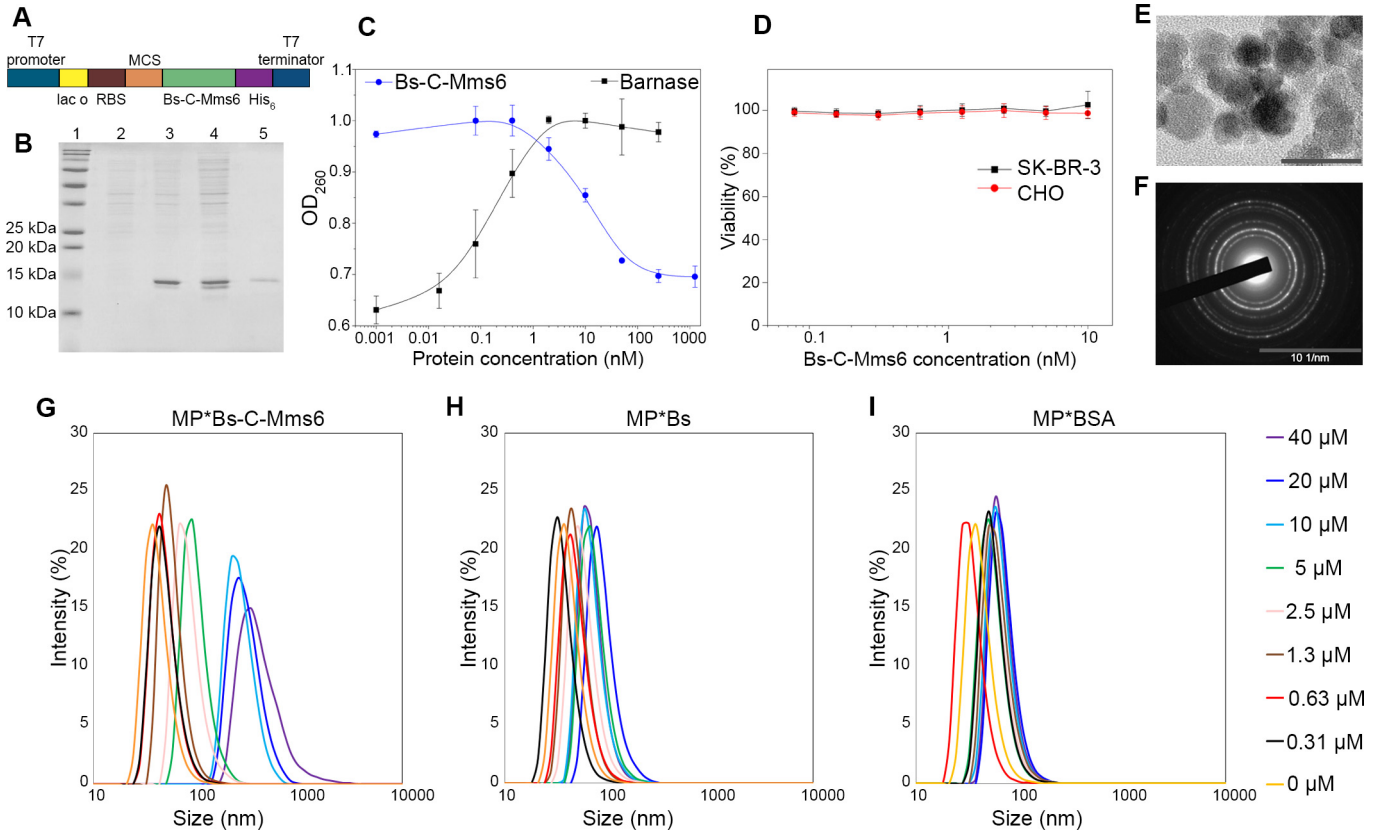

Fig. 1. (A) The genetic construction of the Bs-C-Mms6 protein. The gene of Bs-C-Mms6 protein is under the control of T7 promoter followed with lac operator (lac o), ribosome binding site (RBS) and multiple cloning site (MCS). (B) SDS-PAGE of Bs-C-Mms6. 1 – protein marker, 2 – sample before induction, 3 – sample after induction with 1 mM IPTG, 4 – fraction applied to the Ni^{2+}–NTA column, 5 – purified protein Bs-C-Mms6. (C) Enzymatic activity of Barnase and inhibition of Barnase activity with Bs-C-Mms6. The optical density, corresponding to the concentration of free mononucleotides and proportional to the RNase activity of the tested samples, was measured at λ = 260 nm (OD_{260}) and is presented in dependence from the concentrations of tested proteins. Error bars are SD (n = 3 for each data point). (D) MTT assay of cell viability after incubation with Bs-C-Mms6 protein for 48 h. Error bars are SD (n = 3 for each data point). (E) TEM image of iron oxide magnetic nanoparticles. Scale bar, 20 nm. (F) Electron diffraction pattern of magnetic nanoparticles. (G) Size distribution of magnetite nanoparticles incubated with Bs-C-Mms6 in different concentrations. (H) Size distribution of magnetite nanoparticles incubated with Bs in different concentrations. (I) Size distribution of magnetite nanoparticles incubated with BSA in different concentrations.

was determined by the previously developed MPQ-cytometry method [2]. It was shown that the nanostructures MP*Bs-C-Mms6*DARPin29.9-Bn specifically interact with SK-BR-3 cells: the number of particles bound to SK-BR-3 cells was 59.14 ± 3.03 pg/cell, with cells CHO was 18 ± 4 pg/cell (Fig. 2G). SK-BR-3 cells were also visualized by scanning electron microscopy with the microscope Zeiss EVO LS10 (Zeiss) before and after labeling with MP*Bs-C-Mms6*DARPin29.9-Bn nanostructures at an accelerating voltage of 20 kV (with EP 70 Pa, BSD) with lanthanoid contrasting for the direct visualization of magnetite nanoparticles bound to cells without any fluorescence labeling (Fig. 2H and I).

4. Discussion

It is known that the synthesis of magnetosomes in magnetotactic bacteria is preceded by the formation of membrane vesicles (or membrane invaginations that do not lose contact with the plasma membrane [23]), the lipid component of which contains lipopolysaccharides and different lipids [24]. Protein components of magnetosomes can be fractionated in dependence on their localization within magnetosomes. Components anchored in the membrane are usually removed with 7 M urea together with 2 M thiourea and 4% CHAPS. However, after such extreme treatment some small proteins are still bound to the surface of magnetite of magnetosome. These proteins are Mms5, Mms6, Mms7, Mms13 in magnetotactic bacteria AMB-1, and they can be removed from the surface only after triple boiling in 1% SDS [11]. One of these proteins, namely, Mms6 is most extensively studied. Mms6 (6.5 kDa) is an amphiphilic protein with a hydrophobic N-terminal part anchored in the membrane and a hydrophilic C-terminus of ~30 amino acid residues forming the α-helix. These proteins are characterized by the presence of a long repetition from alternating Leu and Gly residues [11,25]. The C-terminal part contains mainly acidic (Asp, Glu), and hydroxyl-containing (Ser, Thr) amino acids. Most probably, these amino acids participate in chelation of iron and catalyze the nucleation of Fe_3O_4 crystals in bacteria. It was previously shown that Mms6 is capable of magnetite biomineralization *in vitro* and formation of crystalline nanoparticles with narrow size distribution very similar to the structure inside living bacteria [11]. This feature offers great opportunities to improve the quality of chemically synthesized magnetic nanoparticles and their use for biomedical applications.

The proposed here method of nanoparticles synthesis and stabilization with the use of the fusion protein of the C-terminal part of Mms6 and Bs, allows not only to design stable in physiological conditions protein-coated nanoparticles, but already at the stage of synthesis to get a functionally active component in the composition of the particles, namely, Barstar, which is used for further biomodification of nanoparticles.

The Barnase*Barstar protein pair is a universal “protein glue”, which allows to create self-assembling structures with functional biomolecules. Moreover, we have previously shown that under extreme conditions (such as 8 M GdmHCl, 5 M NaCl, or pH 2), the interaction of Barnase*Barstar is still retained and is not inferior in strength to

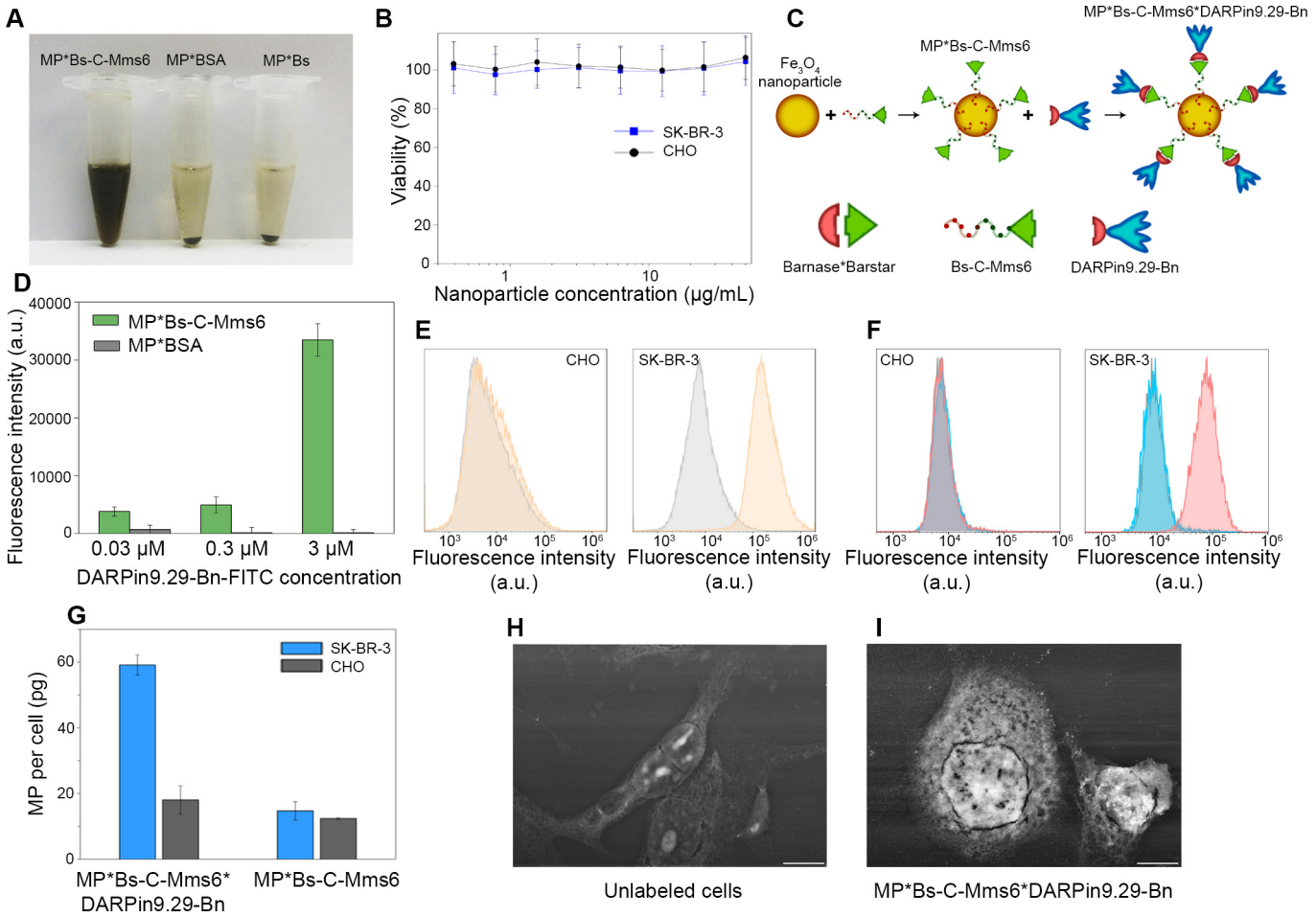

Fig. 2. (A) Magnetite nanoparticles modified with Bs-C-Mms6 (MP*Bs-C-Mms6) stability in phosphate buffered saline, pH 7.4, in contrast to MP*BSA and MP*Bs. (B) MTT assay of cell viability after incubation with MP*Bs-C-Mms6 protein for 48 h. Error bars are SD (n = 3 for each data point). (C) The schematic illustration of uncoated magnetite nanoparticle decoration with targeting molecules. (D) Functional activity test of nanoparticles modified with Bs (and with BSA as control). MP*Bs-C-Mms6 and MP*BSA were incubated with DARPin9.29-Bn-FITC and processed with fluorescence spectroscopy method (excitation/emission 490/525 nm). Error bars are SD (n = 3 for each data point). (E) Flow cytometry assay of cells labeled with DARPin9.29-Bn-FITC. Gray histograms – cells' autofluorescence, orange histograms – cells labeled with DARPin9.29-Bn-FITC (excitation laser – 488 nm, emission filter 525/30 nm). (F) Flow cytometry assay of SK-BR-3 (HER2/neu+) and CHO (HER2/neu–) cells labeled with DARPin9.29-Bn and Bs-C-Mms6-FITC. Gray – cells' autofluorescence, blue – cells labeled with Bs-Mms6-FITC only, red – cells labeled with DARPin9.29-Bn and Bs-C-Mms6-FITC (excitation laser – 488 nm, emission filter 525/30 nm). (G) MPQ-cytometry assay of SK-BR-3 (HER2/neu+) and CHO (HER2/neu–) cells labeled with MP*Bs-C-Mms6*DARPin9.29-Bn nanostructures in contrast to non-targeted MP*Bs-C-Mms6 nanostructures. Error bars are SD (n = 3 for each data point). Scanning electron microscopy (Zeiss EVO LS10) of unmodified SK-BR-3 cells (H) and SK-BR-3 cells labeled with MP*Bs-C-Mms6*DARPin9.29-Bn (I). Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

covalent bonds [26]. And at the same time, with the use of standard genetic engineering methods, it is possible to obtain nanoparticles modified with high density by oriented and active biomolecules. Thus, the problems of steric hindrance arising when using standard methods of chemical conjugation are solved.

We have also shown that neither the fusion protein Bs-C-Mms6 itself nor the Bs-C-Mms6-stabilized nanoparticles have a cytotoxic effect on cells of various origins, which is an extremely important property from the point of view of using the developed designs for therapy and diagnostics in a living organism.

In this paper, to demonstrate the effectiveness of the developed method, we showed the possibility of designing nanoparticles for the detection of HER2/neu positive human breast cells. HER2/neu is a clinically significant oncomarker that is overexpressed in 20–30% of breast cancer cases and correlates with a decrease in overall patient survival, resistance to chemotherapy and a high metastatic tumor potential. HER2/neu early and accurate diagnosis is of great importance for the development of proper approaches to therapy.

For targeted delivery of nanoparticles to HER2/neu-positive cells, we used the DARPin9.29 molecule. DARPins (Designed Ankyrin Repeat Proteins) are a relatively new class of recognizing scaffold proteins of a non-immunoglobulin nature. Typically, DARPins consist of 4–6 ankyrin repeats that form the recognition domain and are resistant to acidic pH, heat and other severe conditions. The absence of cysteines in the structure, the ease of biotechnological production in *E. coli* and good water solubility make them an ideal agent for the creating of targeted supramolecular structures for therapy and diagnostics.

The use of targeted DARPins within the composition of magnetic nanoparticles stabilized with protein Bs-C-Mms6 opens up wide possibilities for the creation of new generation agents for theranostics.

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