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The Method for Protein Display on the Surface of Bacterial Magnetic Nanoparticles

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In this study, we present a comprehensive approach to the design and development of magnetosomes with antibodies immobilized on their surface by integration in membrane *in vitro*. Designed fusion proteins Mbb and Mistbb consisted of anchor proteins and BB-domains of *Staphylococcus aureus* protein A as IgG-binding region were used for development of IgG-binding magnetosomes. The magnetosome membrane protein MamC and membrane protein of *Bacillus subtilis* Mistic were selected as anchor proteins. Using Response Surface Methodology (RSM), the high level of fusion proteins integration into bacterial nanoparticles membrane was achieved. IgG-binding magnetosomes obtained through this strategy could serve as multifunctional platform for displaying various types of antibodies. Such systems could be applied as theranostic agents.

Keywords: Magnetosomes, Antibody immobilization, Protein display, Mistic, AFM.

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1. INTRODUCTION

The systems consisting of antibodies conjugated to the surface of magnetic nanoparticles (MNPs) are being increasingly used in the diagnostics and therapy. Their efficiency for the detection of cancer cells, the magnetic separation of stem cells, magnetic immunoassay and as a carrier for targeted delivery of drugs have been previously demonstrated in the numerous studies [1]. Despite the fact that artificial MNPs are commonly used in these applications, chemically synthesized magnetic crystals have several disadvantages, e.g. size and shape heterogeneity [2]. In recent years the magnetosomes were proposed as an alternative to synthetic MNPs. Magnetosomes are intracellular magnetic crystals produced by magnetotactic bacteria and also referred to as bacterial magnetic nanoparticles (BMPs) (Fig. 1).

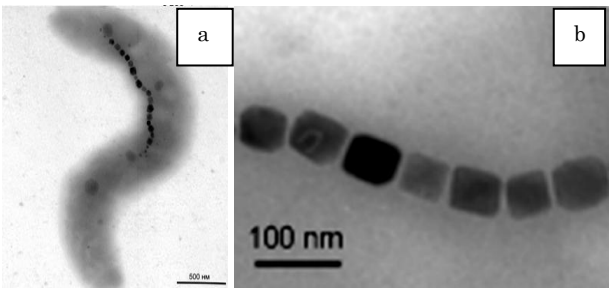

Fig. 1 –Magnetospirillum sp. SO-1 (a), magnetosome chain (b)

The advantages of magnetosomes in comparison with artificial MNPs are: i) uniform species-specific size (45-60 nm) and shape; ii) crystal is coated with a lipoprotein membrane, that makes BMPs easily dispersed in aqueous suspension and provides an opportunity to modify a surface by methods of genetic engineering; iii) low cytotoxicity [3].

In this study, we present a comprehensive approach to the design and development of BMPs with antibodies immobilized on their surface by integration in membrane *in vitro*. For this purpose, we designed the fusion proteins Mbb and Mistbb consisted of anchor protein and BB-domains of *Staphylococcus aureus* protein A as IgG-binding region. MamC and Mistic were selected as anchor proteins.

2. MATERIALS AND METHODS

2.1 Cultivation of Magnetotactic Bacteria

The medium for *Magnetospirillum* sp. SO-1 consisted of (per liter of medium): 1 ml mineral solution, 0.7 g KH2PO4, 0.5 g sodium succinate, 0.1 g yeast extract, 0.35 g NaNO3, 10 ml 0.01 M ferric citrate, 0.05 g sodium thioglycolate. The pH was adjusted to 6.75 with NaOH. The cells were cultivated at 28 °C under microaerobic conditions in 15l fermentor for 3-4 days.

2.2 Magnetosomes Isolation and Purification

The stationary phase cells were centrifuged at 10000g for 10 min at 4 °C, resuspended in 20 mM HEPES buffer, pH = 7.4, contained 4 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by sonication (Sonopuls, Bandelin). Magnetosomes were magnetically isolated from disrupted cell fractions using a neodymium-boron (Nd-B) magnet and washed 15 times with 20 mM HEPES buffer, pH = 7.4. The washed magnetosomes were suspended in the same buffer and stored at + 4° C.

2.3 Expression and Purification of Fusion Proteins Mbb and Mistbb

Expression, purification and IgG-binding activity assays of fusion protein Mbb were reported previously [4]. Gene *mistic* was obtained by PCR from genomic DNA of *Bacillus subtilis* (NdeI-MisF 5'-AGAGGAGATAT**CATATG**GGCTTT-3' and BamHI-MisR 5'-CAGAACC**GGATCC**TTCTTTTTTCTC-3'). *Nde*I and *BamH*1 digested *mistic* PCR product was cloned into vector pET23a(+)/mbb where it substituted *mamC*

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in genetic construct *mbb*. XL1 Blue E. coli cells were transformed by the pET23a(+)/mistbb recombinant plasmids and other steps of Mistbb expression and purification were carried out as for Mbb.

2.4 Optimization of Fusion Proteins Insertion into Magnetosome Membrane

The optimization of Mbb and Mistbb insertion into magnetosome membrane was carried out according to Response Surface Methodology (RSM). The following factors that have a significant impact on the integration of fusion proteins in the magnetosome membrane have been selected after series of pilot experiments: pH, NaCl, together with the influence of mechanical impact mode on suspension (vortexing or sonication). The respective levels with the encoded levels for the factors are listed in Table 1. The resulted calculated data were obtained using Minitab 15.0. 10 μg of magnetosomes and 50 μg of each fusion protein were used in each optimization experiments. The volume of reaction mixture was 1 ml.

Table 1 – The level of variables for BBD

Variables	Code	Level		
		-1	0	1
pH	A	5.0	8.0	11.0
NaCl (mM)	B	0	250	500
Sonication (s)/Vortex (s)	C	5	30	55

2.5 Atomic Force Microscopy

Experiments were performed on NTEGRA Prima microscope (NT-MDT, Russia). AFM was used for visualization, size measurements and magnetic force measurements of biogenic magnetic nanoparticles. Semicontact scanning mode was used in experiments. 10 μl of solutions was incubated on freshly cleaved mica for 1 min before rinsing thoroughly with milliQ water and drying in a dry air stream. Samples were scanned immediately after drying at room temperature and ambient humidity with no special treatment.

2.6 MALDI-TOF Analysis of Membrane

MALDI-TOF-MS (Bruker REFLEX III, Germany) equipped with a 337-nm nitrogen laser was used to analyze the molecular weights of proteins. Alpha-cyano-hydroxycinnamic acid (CHCA) was used as matrix. Samples were extracted, applied onto the target and allowed to air dry. Subsequently, 0.5 μL of CHCA in acetonitrile (ACN)/0.1 % trifluoroacetic acid (TFA) (1:2, v/v) solution was applied to the dried sample and again allowed to dry. MALDI-TOF-MS spectra were acquired in positive-ion, with acceleration potential of 20 kV and linear modes with external calibration. The spectra were calibrated using the protein Standard I (5.000 to 20.0000 kDa range) and II (20.000 to 70.0000 kDa range) from Bruker-Daltonics..

2.7 ELISA

IgG-binding activity of Mbb and Mistbb was compared by ELISA. Human insulin (1 mg) was preadsorbed in the wells of an ELISA plate overnight at +4 °C. The residual sorption was blocked by a 1.5% solution of BSA in a PBS-Tween buffer (PBS, 0.05% Tween 20) for 1 h. Monoclonal mouse antibodies (0.1 μg) against human insulin (Imtek, Russia) were added to the wells and incubated for 1 h at room temperature. The wells were washed four times with PBS-Tween buffer, and then the fusion proteins were added at the specified dilution and incubated for 1 h. After a similar washing procedure, the plates were incubated with 0.1 μg of mouse antibodies against the histidine tag (Imtek, Russia) for 1 h and detection was performed using a hydrogen peroxide/horseradish peroxidase detection system with TMB (Sigma, United States) as a chromogenic substrate. A polypeptide carrying a histidine tag at the C-terminus was used as a negative control.

2.8 Magnetic ELISA

IgG-binding activity of modified magnetosomes was tested by ELISA. 10 μg of modified magnetosome was incubated for 1 h at room temperature with 100 μl of rabbit antibodies (100 μg/ml) labeled with horseradish peroxidase (Imtek, Russia). After that magnetosomes were separated on magnetic stand (Promega, USA) and washed 5 times with 100 μl PBS-Tween buffer to wash out from unbound antibodies. After washing, magnetosomes were incubated with a chromogenic substrate TMB for 1 min, the reaction was terminated 50 μl 1 M HCl. When checking the optimal parameters predicted by the mathematical model.

3. RESULTS AND DISCUSSION

Below we demonstrate the application of Mistic as anchor protein for integration into magnetosome membrane. Being the fusion proteins expressed and purified, the IgG-binding activity of Mistbb and Mbb was compared. As it was shown by ELISA, Mistbb and Mbb exhibited the similar IgG-binding activities. Thus both proteins were selected for further experiments.

The factors influencing the process of fusion proteins integration have been identified in the series of pilot experiments: pH, NaCl concentration and method of mechanical impact. Protein composition of modified and intact magnetosome membrane was performed by MALDI-TOF-MS to detect the insertion of fusion proteins in magnetosome membrane (Fig. 2).

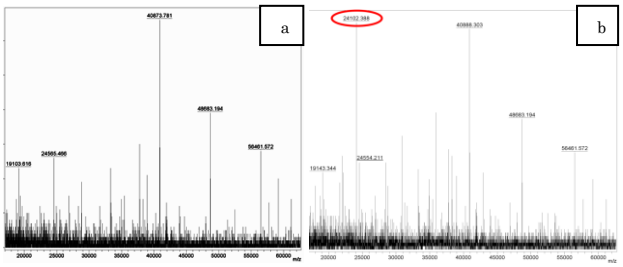

Fig. 2 - Proteomic profile magnetosome membrane before modification (a), after modification (b)

Response Surface Methodology (RSM) was used for optimization of fusion proteins integration into magnetosome membrane. This methodology is an effective tool for prediction of optimal process condition for a multivar-

iable systems [5]. Box-Behnken design (BBD) was used to determine the optimum levels of the three variables selected for each fusion protein. To determine the level of Mbb and Mistbb IgG-binding activity exhibiting after insertion into magnetosome membrane the magnetic ELISA was performed. Experimental design and magnetic ELISA results are compared in Table 2.

Table 2 - BBD along with OD450 (ELISA) production as response

Run	Variable Level			**Mbb** ELISA OD_{450}		**Mistbb** ELISA OD_{450}	
	A	B	C	Sonication	Vortex	Sonication	Vortex
1	0	-1	-1	0,598	0,236	0,708	**0,994**
2	1	-1	0	0,184	0,861	0,319	0,129
3	0	-1	1	**1,258**	**1,058**	**1,132**	0,779
4	-1	0	1	0,465	0,465	0,258	0,232
5	0	0	0	0,681	0,652	0,441	0,297
6	0	0	0	0,469	0,674	0,548	0,134
7	-1	0	-1	0,652	0,469	0,841	**1,066**
8	-1	-1	0	0,442	0,168	0,258	0,634
9	0	0	0	**0,931**	0,64	0,473	0,155
10	1	0	1	0,352	0,598	0,265	0,793
11	0	1	1	0,861	0,352	0,347	0,14
12	1	1	0	**0,974**	0,442	0,206	0,808
13	-1	1	0	0,636	**0,931**	0,493	0,471
14	1	0	-1	0,84	0,184	0,203	0,817
15	0	1	-1	0,168	0,681	**0,982**	0,265

The highest OD_{450} values were obtained when integration was performed under parameters predicted by the RSM (Fig. 3).

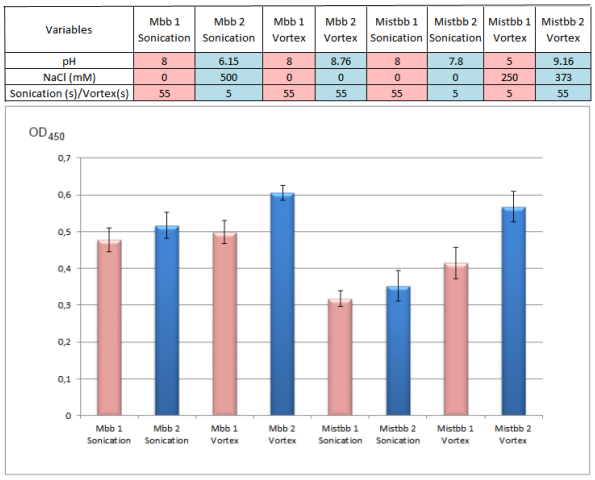

Fig. 3 - Magnetic immunoassay results

The morphology and size of modified magnetosome was analyzed by atomic force microscopy. The size of intact magnetosome was 50-60 nm, and the size of antibody was about 25 nm (Fig. 4a-b).

After integration of hybrid proteins in the membrane followed by magnetosome incubation with antibodies, an increase of particle size to 85-95 nm was detected, thus indicating on the antibody binding by fusion proteins immobilized on the surface of bacterial nanoparticles. It should be noted that unmodified magnetosome resized and their morphology did not occur after incubation with the antibodies. This conclusion is based on the fact of the absence of non-specific adsorption of antibodies.

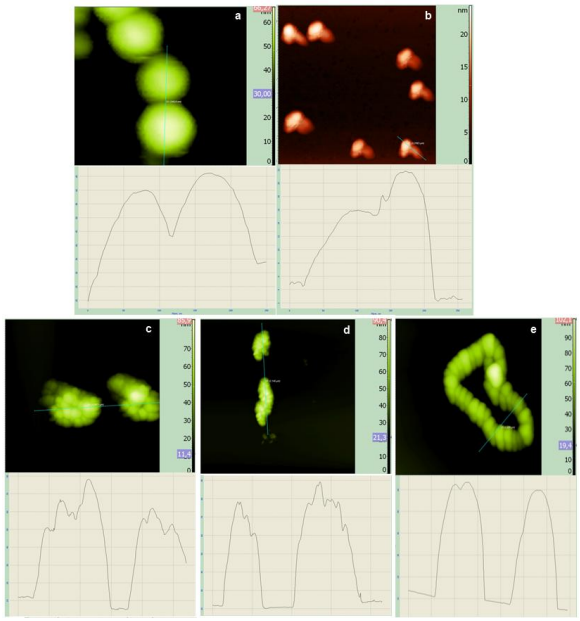

Fig. 4 – AFM data for bacterial magnetic nanoparticles before modification (a), polyclonal rabbit IgG (b), magnetosomes after insertion of IgG-binding fusion protein Mbb (c), after insertion of IgG-binding fusion protein Mistbb (d), unmodified magnetosomes incubated with antibodies (e)

4. CONCLUSIONS

In this study the optimized method for design of IgG-binding magnetosomes by insertion of fusion protein with IgG-binding activity into magnetosome membrane was proposed. One of the benefits of magnetosomes is that their native membrane could perform a platform for the protein display. Another essential advantage is flexibility or universality of IgG-binding magnetosomes, since modified BMPs could be labeled with different antibodies depending on diagnostic purposes.

It was shown that not only magnetosome membrane proteins but also Mistic can be effectively used as an anchor molecule for integration into BMPs membrane.

IgG-binding magnetosomes are applicable for various diagnostics *in vitro*. Moreover modified magnetosomes could be used for *in vivo* applications, e.g. imaging of tumors by MRI. BMPs modified with fluorescent labeled antibodies will expand the possibilities of visualization of biological samples. It was reported previously that magnetosomes are extremely effective in hyperthermal tumor therapy. Thus IgG-binding BMPs has a great potential as “theranostic” agent.

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