SURFACE ENGINEERING OF IRON OXIDE NANOPARTICLES ISOLATED FROM MAGNETOSPIRILLUM GRYPHISWALDENSE FOR BIOCHEMICAL AND BIOMEDICAL APPLICATIONS

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Abstract:
Superparamagnetic iron oxide nanoparticles with appropriate surface modification can be widely used in various applications including magnetic resonance imaging (MRI) diagnostic contrast agents, anticancer therapy using hyperthermia, magnetic drug targeting, protein and enzyme immobilization, cell labeling and separation or RNA and DNA purification. All these biochemical and biomedical applications require nanoparticles exhibiting a high magnetization and narrow size distribution and possessing non-toxicity and biocompatibility.

As a result of biologically controlled preparation, biogenic magnetite (Fe₃O₄) nanoparticles have properties that make them intrinsically distinct from their synthetic counterparts. Magnetotactic bacteria are microorganisms that are able to biomineralize the membrane-enveloped crystals of magnetite called magnetosomes. Magnetospirillum gryphiswaldense, well laboratory cultured organism, produces cubooctahedral magnetite crystals ranging in size between 20 and 50 nm. The fermentor cultivation under microaerobic conditions, commonly performed in our lab, leads to the sufficiently high cell yield (OD₅65nm ~ 1.5) and to the suitable values of the parameter describing the cell magnetism (cₘₐₜ ~ 1). Magnetosomes are consequently isolated from bacteria by method using a neodymium boron (Nd-B) magnet. In the present work, we coated biogenic magnetite with substances that make them biocompatible, biodegradable, stable, non-toxic and accessible for binding with various active biocomponents depending on particular bioapplication. The natural polymers such as chitosan, N-trimethylchitosan, carboxymethylchitosan or dextran have been used in a coating procedure and the properties of the core-shell systems have been analyzed by TEM, SEM and SQUID magnetic measurements. The magnetite nanoparticles modified by chitosan exhibit the most perfect and complete surface stabilization as evidenced by the narrow and well defined shell. These nanoparticles were successfully tested in the trypsin immobilization for applications in proteomics, where they revealed the superior properties compared to the synthetic counterparts.

1. INTRODUCTION
Techniques based on using magnetisable solid-phase support have found application in numerous biological fields viz. diagnostics, drug targeting, molecular biology, cell isolation and purification, radio immuno assay, immobilization of proteins and enzymes, hyperthermia causing agents for cancer therapy, nucleic acid purification etc [1-3]. While a number of suitable methods have been developed for the synthesis of the magnetic particles of various compositions, for example nano-sizes magnetite particles have been synthesized by coprecipitation of Fe(II) and Fe(III) in alkaline solution, some magnetic bacteria could synthesize more uniform magnetic particles, which consist of magnetite (Fe₃O₄) or greigite (Fe₃S₄) in size and shape compared with artificial magnetite particles.
The increasing effort in this research is reflecting the need for new biomarkers facing the requirements of today’s fast growing biotechnological and pharmaceutical industry [4, 5].

1.1 Magnetotactic bacteria

Magnetotactic bacteria, a special kind of bacteria, were discovered by Blakemore in 1975 [6]. Thus magnetotactic bacteria do not represent a single, defined, taxonomic group. Morphotypes include coccoid to ovoid cells; rods, vibrios, and spirilla of various dimensions; and even multicellular forms. All that have been examined are members of the domain Bacteria and possess cell walls that are characteristic of gram-negative bacteria. [7] These bacteria synthesize intracellular magnetic nano-particles (also called magnetosomes or bacterial magnetic particles (BMPs)), which are enveloped by cytoplasmatic membrane and made of Fe$_3$O$_4$, Fe$_3$S$_4$, Fe$_2$O$_3$ or FeS, etc [8-10]. Several strains of magnetotactic bacteria, including *Magnetospirillum gryphiswaldense* MSR-1, *M. magnetotacticum* MS-1 and *M. magneticum* AMB-1, have been isolated and identified so far [11-13]. A magnetotactic spirillum (strain MSR-1) was isolated from the mud of the entropic river Ryck near Greifswald by Schleifer in 1991. The research of phylogenetic taxonomy demonstrated that MSR-1 is related and belongs to alpha subclass of proteobacteria [14].

1.2 Bacterial magnetic particles; magnetosomes

Single domain bacterial magnetic particles, known as magnetosomes occur in rows 10-20 particles with a defined size of 35-120 nm and are surrounded by a phospholipids’ membrane approximately 2-4 nm in thickness [15]. Each BMP has a single domain of magnetite and are well-dispersed in aqueous solutions because of the enclosing membrane [16]. The magnetite particles are aligned in chains parallel to the cell axis. Each particle possesses a magnetic dipole moment and magnetic interactions between magnetic particles in a chain are oriented parallel to each other along the Earth’s geomagnetic fieldlines and to maintain its position within the boundary of oxic-anoxic zone [17]. This is used by bacteria for navigation, known as magnetotaxis. While magnetotaxis is clearly an important function for magnetosomes, it may not be their only function. Bazylinski and Frankel suggest that the magnetosomes also have unknown physiological function [18].

The molecular mechanism of magnetite biomineralization in bacteria is poorly understood although this process occurs widely in many other organism such as insect [19], birds [20] or migratory fishes [21]. One of the models of the crystallization process have been proposed where ferric iron is reduced on the cell surface, taken into the cytoplasm, transferred into vesicles (magnetosome) and finally oxidized to produce magnetite [22].

The morphology of BMP is varied and species-dependent. Three general morphologies of magnetite have been observed in magnetotactic bacteria using TEM. They include: roughly cuboidal [23]; parallelepipidal [24,25] and tooth-, bullet- or arrowhead-shaped [26, 27]. *M. gryphiswaldense* produces a chain of cubo-octahedral magnetosome particles. The strain has been used as a model organism in a number of studies addressing the physiology and molecular genetics of magnetosome biomineralization and for the development of applications of magnetosomes [13].
2. MAGNETOSOME PRODUCTION

2.1 Culture

Pure cultivation of magnetotactic bacteria is one of the most important biotechnological processes in the application of BMPs. A magnetic bacterium, *Magnetospirillum gryphiswaldense*, capable of growing aerobically has been successfully isolated [14]. In our initial work, laboratory scale cultivation of these bacteria in 10 l fermentor has been done for the production of BMPs from which approximately 1.5 OD565nm or 0.35 g dry weights of BMPs was yielded per litre of culture. MSR-14 was cultured for 35-40 h in the reported medium [13] using 10 l auto-fermentor under low oxygen concentration conditions.

2.2 Collection of cells and purification of magnetosomes

Bacterial magnetite particles are easily separated and purified from disrupted magnetic bacteria by magnetic separation using a magnet. MSR-1 cell cultures were pelleted by centrifugation and disrupted by several passes through a French pressure cell. Bacterial magnetite from disrupted cells was collected magnetically using a neodymium boron (Nd-B) magnet. Collected bacterial magnetite was washed and used for additional modification.

3. SURFACE MODIFICATION OF BMPs

Generally, naked nano-sized particles tend to form agglomerates to reduce the energy associated with the high surface area to volume ratio. For many applications it is crucial to develop protection strategies to chemically stabilize the naked nanoparticles against degradation and agglomeration. These strategies comprise grafting of or coating with organic species, including surfactants or polymers, or coating with an inorganic layer, such as silica or polysaccharides. In many cases the protecting shells not only stabilize the nanoparticles, but can also be used for further functionalization depending on the desired application. We have modified several methods [28-30] to assemble these functional molecules over the BMPs surface using chemical techniques. Coating experiments in our laboratory were carried out with biogenic nanoparticles of magnetite. As biocompatible coating materials chitosan, O-carboxymethyl chitosan (CMC), dextran, N-substituted trimethyl chitosan chloride (TMC) and Tween 20 were used. Before all experiments the
membrane of BMPs was removed and substituted by positive detergent agents Cetyltrimethylammonium chloride (CTAC).

3.2. Examination of purified and modified magnetosomes by TEM

The purified and modified magnetosomes were observed by transmission electron microscopy (TEM, JEOL, Japan).

![Fig. 2 Determination of surface modification of biogenic nanoparticles of magnetite by TEM (magnetite was modified by chitosan (A), dextran (B), N-substituted trimethyl chitosan (C) and TWEEN 20 (D)).](image)

4. USE OF MODIFICATED BMPs AS ENZYME CARRIERS

The use of functional magnetic particles in bioassays facilitates the separation of bound and free analytes by the application of a magnetic field. Magnetic iron oxide nanoparticles are widely used in the development of medical and diagnostic applications such as magnetic resonance imaging (MRI) [33], cell separation [34], drug delivery [35] and hyperthermia [36]. To use these particles for the biotechnological applications, it is important to consider surface modification of magnetic particles with functional molecules such as proteins, antibodies, peptides and DNA. Because of their unique characteristics (narrow-size distribution, a large surface area for reaction, the single magnetic domain size range, etc.) [37] compared to synthetic particles, isolated resp. also modified magnetosome crystals are superior for applications that rely on small amounts of highly functionalized magnetic material with extraordinary magnetic and biochemical characteristics.
The use of BMPs as a stable platform for immobilization of proteins resp. enzymes was tried with trypsin. Trypsin (EC 3.4.21.4) is a serine protease found in the digestive system of many vertebrates, where it hydrolyses proteins [38]. Trypsin predominantly cleaves peptide chains at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. This enzyme has been used widely in various biotechnological processes and it is commonly used in biological research during proteomics experiments to digest proteins into peptides for mass spectrometry analysis, e.g. in-gel digestion.

In our experiments bovine trypsin (BT) was chemically modified with \( \alpha \)-cyclodextrine (ACD-BT) and \( \beta \)-cyclodextrine (BCD-BT) and all were activated by EDC.HCl (\( N \)-(3-dimethylaminopropyl)-\( N \)'-ethylcarbodiimid hydrochloride). Then enzymes were covalently immobilized on surface of biogenic magnetite modified by chitosan. For comparison the activities of free and immobilized enzyme were measured under different conditions. The activity of trypsin was determined with artificial substrate BAPNA (\( N \alpha \)-benzoyl-DL-arginine-4-nitroanilide) spectroscopically (\( \lambda = 405 \text{ nm} \)) (Fig. 3).

![Fig. 3 The schema of trypsin hydrolysis of artificial substrate \( N\alpha \)-benzoyl-DL-arginine-4-nitroanilide.](attachment:image)

All enzymes (free and immobilized forms) were characterized in activities, Michaelis’s constants, temperature stabilities \( T_{50} \) [°C] (temperature, in which the enzyme had loose half of activity in comparison whit room temperature) and time-degradation stabilities (time, after which the enzyme had loose half of its activity) (tab.1).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Free</th>
<th>Immobilized</th>
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<tbody>
<tr>
<td></td>
<td>BT</td>
<td>ACD-BT</td>
</tr>
<tr>
<td>( K_m ) [mM]</td>
<td>2.5</td>
<td>1.7</td>
</tr>
<tr>
<td>( T_{50} ) [°C]</td>
<td>41</td>
<td>55</td>
</tr>
<tr>
<td>( t_{50} ) [h]</td>
<td>0.8</td>
<td>21</td>
</tr>
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(BT - bovine trypsin, ACD-BT - trypsin modified with \( \alpha \)-cyclodextrine and BCD-BT - trypsin modified with \( \beta \)-cyclodextrine) in Michaelis’s constants \( K_m \) [mM], temperature stabilities \( T_{50} \) [°C] and time-degradation stabilities \( t_{50} \) [h]. All characteristics were measured with artificial substrate BAPNA (\( N\alpha \)-benzoyl-DL-arginine-4-nitroanilide) spectroscopically (\( \lambda = 405 \text{ nm} \)).
The positive improve of characteristic by immobilization of trypsin on bacterial nanoparticles of magnetite was determined in all experiments. Additionally we will focus on usage of immobilized enzyme in MALDI-TOF peptide mass fingerprinting.

LITERATURE


