Magnetic Immobilization of Dispersin B with Activity in Degradation of Bacterial Biofilm

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Abstract

Dispersin B (DspB) is a member of glycoside hydrolase family 20 (GH20) and catalyzes degradation of biofilms forming by pathogenic bacteria such as *Staphylococcus aureus*. Magnetoreceptor (MagR) is a magnetic protein that can be used as a fusion partner for functionally immobilizing proteins on magnetic surfaces. In the present study, a recombinant protein DspB-MagR was constructed by fusing MagR to the C-terminus of DspB and expressed in *Escherichia coli*. Magnetic immobilization of purified DspB-MagR on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles was achieved and characterized by means of various techniques including SDS-PAGE, FTIR spectrometry, TGA measurements, zeta potential measurement, and SEM analysis. Stability and activity of immobilized DspB-MagR on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles were analyzed under different conditions such as temperature, pH, and storage time. Removal of biofilms forming by *Staphylococcus aureus* and other medical source bacterial species were achieved by using $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles loading with DspB-MagR. This work promoted potential applications of DspB and similar enzymes for medical purposes.

Introduction

Biofilms are a group of bacteria surrounding by extracellular polymeric matrix (EPS) composed of polysaccharides, lipids and nucleic acids [1]. Bacteria take advantages of biofilms in reducing sensibility to antibiotics. Antibiotic resistance of bacteria in biofilm can be 100 to 1000 folds higher than that of planktonic cells [2]. Biofilm formation on medical devices especially implanting devices is a critical problem in healthcare and has inevitable consequences of serious infections and failure of therapy [3]. Bacterial biofilms were involved in over 65% of bacterial infections [4], which are normally caused by *Streptococcus species*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus*, *Enterococcus* and *Candida spp.* [4]. Although different bacteria can form different types of biofilms, poly-beta(1,6)-N-acetylglucosamine (PNAG) is the major polysaccharide component of EPS to most of pathogenic bacteria [5] such as *Staphylococcus epidermidis*, *Staphylococcus aureus* and *A. actinomycetemcomitans* [6]. Therefore, techniques targeting to PNAG are useful in detaching bacterial biofilms.

Bacterial cells within mature biofilm can be released into external environment and attach on another surface to form new biofilm. The beta-N-acetylglucosamine enzyme Dispersin B (DspB) plays important roles during this process [7]. DspB is a hydrolase of PNAG and has functions in biofilm degradation that promotes resistance to antibiotics such as teicoplanin, rifampicin, clindamycin, gentamicin and ciprofloxacin [8, 9]. Scientists attempted to immobilize DspB on biocompatible surface and carriers, which improved antibiofilm activity of DspB [10–13]. However, these approaches mainly rely on chemical modifications of carries and chemical conjugations between enzymes and carriers, which may raise safety issues for medical applications.

Magnetoreceptor (MagR) is a magnetic protein that can interact with external magnetic fields [14]. MagR was explored as a fusion tag with enzymes for functional immobilization of these enzymes on Fe_3O_4 and $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles [15, 16]. Magnetic interactions between MagR and supports reduced

influences of chemical environment and facilitated activities of enzymes over a broad range of environmental conditions. Therefore, it provides an alternative physical means for immobilizing DspB that has less chemical toxicity.

The aim of this study is to take advantages of MagR as the fusion partner of DspB to facilitate functional immobilization of DspB on magnetic $\text{Fe}_3\text{O}_4@\text{SiO}_2$ particles, which can be used for inhibiting and removing biofilms. Activity of recombinant DspB-MagR in degrading bacterial biofilms was evaluated before and after immobilization. $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles loading with DspB-MagR were characterized by means of SEM, FTIR and TGA. This study opens a new avenue with less chemical toxicity for immobilizing proteins that suit to medical applications.

Materials And Methods

Construction, Expression and Purification of Recombinant DspB-MagR

DNA sequence encoding MagR (GenBank Accession NP573062) and DspB (GenBank Accession AY228511.1) was optimized for prokaryotic expression, synthesized by Bio Translation Lab (China), and cloned into the plasmid pET28a to acquire the expression vector pET28a[DspB-MagR].

Expression and purification of DspB-MagR was based on the pET System Manual (Novagen, German). Briefly, a single colony of *Escherichia coli* BL21(DE3) harbouring pET28a[DspB-MagR] was inoculated into Luria–Bertani (LB) medium containing 0.05 mg ml^{-1} kanamycin and shaking at 37°C overnight. The cell suspension was inoculated into LB medium and followed by shaking at 37°C until an optical density at 600 nm (OD₆₀₀) of 0.5–0.6 was reached. Protein expression was initiated by supplying with 1 mM isopropyl-p-D-thiogalactoside (IPTG) and continuing shaking at 16°C for 24 hours. Cells were harvested by centrifugation at 6,000 g, 4°C for 10 minutes, re-suspended in lysis buffer (50 mM NaH_2PO_4 , 10 mM imidazole, pH 8.0/300 mM NaCl), and lysed by using a high-pressure cell disruptor TSO.75 KW (Constant Systems Ltd., UK). The cell lysate was centrifuged at 12,000 g for 10 minutes to remove cell debris and subjected to Ni-NTA affinity chromatography. The Ni-NTA affinity resin was pre-washed and equilibrated with wash buffer (50 mM NaH_2PO_4 , 20 mM imidazole, 300 mM NaCl, pH 8). Unbound proteins were washed three times with wash buffer. Bound DspB-MagR was eluted with elution buffer (50 mM NaH_2PO_4 , 300 mM imidazole, 300 mM NaCl, pH 8.0).

Magnetic Immobilization of DspB-MagR

$\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles were synthesized based on Hu et al. (2017) [17]. $\text{Fe}(\text{NH}_4)_2\cdot(\text{SO}_4)_2\cdot6\text{H}_2\text{O}$ and $\text{FeCl}_3\cdot6\text{H}_2\text{O}$ were mixed with 50 ml deionized water in oxygen-free environment and heated to 60°C with 200–300 rpm rotational stirring, following by the addition of 45 ml of aqueous ammonia and stirring for 30 minutes. After heating the solution to 80°C and aging for an hour, magnetic Fe_3O_4 nanoparticles were

collected by using a magnet and washed three times with deionized water, and then dried under vacuum overnight. Dried Fe₃O₄ nanoparticles (200 mg) were added in 200 ml 80% (v/v) ethanol and dispersed with ultrasonic operation for 15 minutes, following by the addition of 6 ml concentrated ammonia wax and 0.6 ml TEOS. After vigorous stirring at 40 °C for 12 hours, the resultant Fe₃O₄@SiO₂ nanoparticles were collected by using a magnet and washed three times with ethanol and deionized water, and then dried under vacuum overnight.

To immobilize DspB-MagR on Fe₃O₄@SiO₂ nanoparticles, 1 ml solution containing DspB-MagR was mixed with 10 mg nanoparticles for 30 minutes. The nanoparticles with loaded Dsp-MagR was collected by centrifugation at 3000 g for 60 seconds and washed twice with 1 ml PBS (pH 6.0) to remove unloaded enzyme.

Enzymatic Activity Assay of DspB-MagR

Enzymatic activity assay of DspB-MagR was based on Tan *et al.* (2015) [12] by using 4-nitrophenyl-N-acetyl-beta-D-glucosaminide as the substrate to produce chromogenic p-nitrophenolate. Briefly, 10 µl solution containing DspB-MagR were mixed with 0.2 ml substrate solution (50 mM sodium phosphate buffer containing 100 mM NaCl, 5 mM substrate, pH 6.0) and incubated at 37 °C for 10 minutes. The reaction was terminated by adding with 5 µl 10M NaOH. The produced p-nitrophenolate was monitored by measuring the increase of optical density.

Sensitivity of DspB-MagR to pH was investigated by assaying the enzymatic activity in disodium hydrogen phosphate-citric acid buffer (pH 3–8) and glycine sodium hydroxide buffer (pH 9–10). Sensitivity of DspB-MagR to temperature was investigated by assaying the enzymatic activity in sodium phosphate buffer (pH 6.0) at temperature range from 4 °C to 45 °C. Stability of DspB-MagR over time was evaluated by comparing the initial activity with the residual activity after incubation for various periods.

Assaying the Formation and Degradation of Biofilm

Bacterial species, that is, one standard stain of *Staphylococcus aureus* and three bacterial species from replaced knee implants of patients with infections (Union Hospital, Wuhan, China), as well as bacterial mixtures from medical practice were explored for biofilm formation and degradation. Isolation of bacteria from medical practice were based on the majority and morphology of colonies on streaking plates. The isolated bacteria were characterized by 16s RNA sequencing, which showed that these stains are *Staphylococcus sp.*, *Bacillus Cereus*, and *Pseudomonas Putida*.

For growing biobilms, bacteria were cultured overnight and diluted to 0.05 OD₆₀₀ with LB medium. Aliquots (2 ml) were transferred to a 24-well plate. The plate was incubated at 37 °C for 24 hours and washed three times with LB medium to remove planktonic cells.

For assaying the formation of biofilms, biofilms were stained with 1 ml of 0.1% (w/v) crystal violet solution for 10 to 20 minutes, and washed three times with PBS (pH 7.0). The crystal violet remained by

the biofilm was dissolved by adding 200 μ l 30% (v/v) acetic acid and diluted to 1 ml before measuring optical density at 590 nm.

For assaying biofilm degradation, the formed biofilms were treated with DspB-MagR loaded $\text{Fe}_3\text{O}_4@\text{SiO}_2$ (10 mg/ml) and/or lysozyme (1 mg/ml) for an hour at 37 °C. Then the residual biofilms were assayed as described above. Triplicated measurements were performed for each assay.

Results And Discussion

Construction and Expression of DspB-MagR

The *magR* gene were amplified using a pair of primers containing restriction site of endonuclease XhoI and then inserted at the downstream of *dspB* gene to construct the plasmid pET(DspB-MagR) (Fig. 1, Panel A), with a sequence encoding hexahistidine tag at the C-terminus of the recombinant protein DspB-MagR to facilitate the affinity purification.

Expression of DspB-MagR was induced by supplying IPTG to the culture containing *E. coli* host BL21 harboring the vector pET(DspB-MagR). Bacterial growth was significantly reduced upon the addition of IPTG (Fig. 1, Panel B), implying the involvement of bacterial metabolism in synthesizing the heterogenic DspB-MagR retarded the bacterial growth. Expression of recombinant DspB-MagR was confirmed with SDS-PAGE electrophoresis (Fig. 1, Panel C), which showed that there was overexpression of protein with an apparent molecular weight between 50 and 70 kDa. This protein was assigned to be DspB-MagR with a theoretic molecular weight of 60 kDa.

Fast overexpression of heterogenic protein sometimes leads to formation of inclusion body containing proteins of interests that are not correctly folded. In the present study, IPTG-induced DspB-MagR expression was attempted at two temperatures (that is, 16 °C and 37 °C) to avoid the formation of DspB-MagR inclusion body. It was observed that DspB-MagR expression level at 16 °C was lower than that at 37 °C. However, the soluble version of DspB-MagR at 16 °C was significantly improved (Fig. 1, Panel D). It might be due to that bacterial protein synthesis is slow at low temperature, which provides translated DspB-MagR with more time to fold properly.

Purification and Immobilization of DspB-MagR

Expressed DspB-MagR was purified by using Ni-NTA affinity chromatography and immobilized on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles (Fig. 2, Panel A). The homogeneity of DspB-MagR was over 95% after purification and immobilization as determined by densitometric scanning of Coomassie blue stained SDS-polyacrylamide gels. SEM revealed no significant morphological changes of $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles before and after loading with DspB-MagR (Figure S1, see Supporting Information). FTIR spectrometry of $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles with or without DspB-MagR loading showed that there were characteristic bands of Si-O-Si band at 1095 cm^{-1} and Fe-O band at 596 cm^{-1} , 576 cm^{-1} (Fig. 2, Panel

B). There was a shift of absorbance peak from 1634 cm^{-1} (before protein loading) to 1652 cm^{-1} (after protein loading), which was attributed to influence of the amine of polypeptide and proved the immobilization of DspB-MagR on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles. Immobilization of DspB-MagR on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles was also verified by TGA measurement (Fig. 2, Panel C). There was a weight loss of 3% and 5% for both $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles with or without DspB-MagR loading when raising temperature up to $150\text{ }^\circ\text{C}$, which was due to water evaporation. When raising temperature from $150\text{ }^\circ\text{C}$ to $350\text{ }^\circ\text{C}$, a significant weight loss of 1.25% was observed for $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles loading with DspB-MagR due to the thermal decomposition of protein. Therefore, the loading capacity of $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles to DspB-MagR was estimated as $\sim 1.25\text{ mg DspB-MagR per }100\text{ mg nanoparticles}$. Zeta potential of $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles with or without DspB-MagR loading was measured at different pH (Fig. 2, Panel D). Zeta potential is the electrostatic potential that exists at the shear plane of a particle, which is related to both surface charge and the particle's local environment. It was observed that after loading with DspB-MagR, $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles exhibited higher zeta potential than that of naked $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles. This could be attributed to the ionization of functional NH_2 groups of DspB-MagR protein.

Bioactivity of Immobilized DspB-MagR

Enzymatic activity of DspB-MagR before and after loading on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles was investigated under various conditions. Generally, the activity of enzymes is affected by factors such as pH, temperature, functional time [18]. It was observed that DspB-MagR exhibited similar spectrum of sensitivity to pH before and after loading on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles (Fig. 3, Panel A). Both showed highest activity in pH 6. Therefore, immobilization on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles did not change the pH sensitivity of DspB-MagR.

Sensitivity of DspB-MagR to temperature changed after loading on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ (Fig. 3, Panel B). Before immobilization, DspB-MagR exhibited highest activity at $30\text{ }^\circ\text{C}$. While the immobilized DspB-MagR exhibited highest activity at $37\text{ }^\circ\text{C}$ that is the regular body temperature of human, implying that immobilization of DspB-MagR on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles is suited to medical purposes.

Stability of DspB-MagR over time was investigated before and after loading on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles (Fig. 3, Panel C). There were two phases of activity changes for both enzymes over time after incubation at their optimal temperature ($30\text{ }^\circ\text{C}$ for unloaded DspB-MagR and $37\text{ }^\circ\text{C}$ for immobilized DspB-MagR). In the first two to three hours of incubation, both enzymes suffered a quick decrease and increase of the activity. Although the activity of both enzymes kept decreasing after three hours of incubation, immobilized DspB-MagR showed higher activity of than that of unloaded ones at sampling time, implying that immobilization of DspB-MagR on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles is beneficial for retaining the activity of DspB-MagR over time.

Degradation of Biofilm by Immobilized DspB-MagR

Bioactivity of DspB-MagR before and after loading on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ was assayed for detaching bacterial biofilms (Fig. 4). When testing with the standard strain of *Staphylococcus aureus* (SA), naked $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles did not exhibit any effects on removing biofilm. Unloaded DspB-MagR showed limited function and removed about 10% of biofilm. After loading on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles, activity of DspB-MagR on removing biofilm was significantly improved and detached more than 50% of biofilm.

Activity of DspB-MagR loading on $\text{Fe}_3\text{O}_4@\text{SiO}_2$ nanoparticles was further examined on removing biofilms forming by bacteria samples from clinic source, that is, one mixed bacterial species (MB) and three isolated and characterized species, *Staphylococcus sp.* (SS), *Bacillus Cereus* (BC), and *Pseudomonas Putida* (PP). Although it was not significant, immobilized DspB-MagR showed activity in degrading biofilm forming by mixed bacterial species or *Pseudomonas Putida*. As to *Staphylococcus sp.* or *Bacillus Cereus*, immobilized DspB-MagR exhibited significant activity on detaching biofilm, over 40% (*Bacillus Cereus*) or 60% (*Staphylococcus sp.*) of biofilms were removed.

Conclusion

In this study, a recombinant protein DspB-MagR was constructed by fusing a magnetic protein MagR to the C-terminus of DspB. Functional expression and purification of the resultant DspB-MagR was achieved. The magnetic protein MagR was served as a magnetic partner to facilitate immobilization of DspB-MagR on magnetic nanoparticles. The immobilization enhanced the storage stability of DspB-MagR. The optimum temperature of DspB-MagR was shifted from 30 °C (DspB alone) to 37 °C (DspB-MagR), which provides advantages of DspB in medical practice. The magnetically immobilized DspB-MagR showed significant activity to detach biofilms forming by either standard bacterial strain of *Staphylococcus aureus* or bacterial species *Staphylococcus sp.* and *Bacillus Cereus* from clinic practice. Although it is not significant, the immobilized DspB-MagR showed activity to remove biofilms forming by mixed bacterial species or *Pseudomonas Putida* from clinic practice. These results suggest that DspB-MagR immobilizing on magnetic nanoparticles can be used for preventing and treating medical device-associated infections. Moreover, the complex of DspB-MagR and magnetic nanoparticles can be controlled and distributed under the function of magnetic force, which will further broaden its range of applications.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Author Contribution Conceptualization: Hao Xie, Junhui Guo; Methodology: Zewen Liu, Zisong Zhao; Formal analysis and investigation: Hao Xie, Junhui Guo, Zewen Liu, Zisong Zhao; Writing—original draft preparation: Zewen Liu, Zisong Zhao; Writing—review and editing: Hao Xie, Junhui Guo; Funding acquisition: Hao Xie; Resources: Kai Zeng, Yue Xia, Weihua Xue, Ruoyu Wang; Supervision: Hao Xie, Junhui Guo

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Conflict of Interest The authors declare that they have no conflict of interest.

Availability of Data and Material The data and material were obtained from experimental tests in triplicate and described clearly in the text.

Code Availability Not applicable.

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Figures

Figure 1

Expression of recombinant DspB-MagR. Panel (A) shows expression vector pET(DspB-MagR) harboring *dspB-magR* gene. Panel (B) shows IPTG-induced growth of bacterial cells containing expression vector pET(DspB-MagR). Arrow points to the time point of adding IPTG. Panel (C) shows SDS-PAGE analysis of DspB-MagR expression in IPTG-induced cells. Lane M, molecular weight markers; lane U, lysates of uninduced cells; lane I, lysates of cells upon IPTG induction of 3 hours. Arrow points to the position of putative DspB-MagR. Panel (D) shows SDS-PAGE analysis of supernatants (lanes S16 and S37) and

precipitates (lanes P16 and P37) after lysis and centrifugation of cells upon IPTG-induction at 16 oC or 37 oC. Lane M, molecular weight markers. Arrow points to the position of expressed DspB-MagR.

Figure 2

Immobilization of DspB-MagR and characterization of Fe₃O₄@SiO₂ nanoparticles with loaded DspB-MagR. Panel (A) shows purification and immobilization of DspB-MagR. Lane M, molecular weight markers; Lane 1, lysates of IPTG-induced cells, Lane 2, unbound protein fraction from the affinity column; Lane 3, fraction from wash step to remove weakly bound proteins from the affinity column; Lane 4, the

purified DspB-MagR; Lane 5: DspB-MagR loading on nanoparticles; Lanes 6, unbound protein fraction from nanoparticles with loaded DspB-MagR; Lane 7, fraction from wash step to remove weakly bound proteins from nanoparticles. Panel (B) shows FTIR spectra of Fe₃O₄@SiO₂ nanoparticles before and after loading with DspB-MagR; Panel (C) shows TGA analysis of Fe₃O₄@SiO₂ nanoparticles before and after loading with DspB-MagR; Panel (D) shows zeta potential of Fe₃O₄@SiO₂ nanoparticles before and after loading with DspB-MagR.

Figure 3

Sensitivity of DspB-MagR to temperature, pH, storage time before and after loading on Fe₃O₄@SiO₂ nanoparticles. Panel (A) shows the optimum pH of DspB-MagR before and after loading on Fe₃O₄@SiO₂ nanoparticles. Panel (B) shows the optimum temperature of DspB-MagR before and after loading on Fe₃O₄@SiO₂ nanoparticles. Panel (C) shows storage stability of DspB-MagR before and after loading on Fe₃O₄@SiO₂ nanoparticles. The highest of the enzymatic activity of fresh samples of DspB-MagR before or after loading on Fe₃O₄@SiO₂ nanoparticles was determined as 100%.

Figure 4

Bioactivity of immobilized DspB-MagR on detaching bacterial biofilm. Biofilms forming by bacteria *Staphylococcus aureus* (SA), as well as clinic-sourced mixed bacterial species (MB), *Staphylococcus* sp. (SS), *Bacillus Cereus* (BC), and *Pseudomonas Putida* (PP) were treated with PBS (control), naked Fe3O4@SiO2, free DspB-MagR (fDspB-MagR), immobilized DspB-MagR (iDspB-MagR). Residual biofilm was shown as the percentage to control. Star indicates there is statically difference ($p < 0.05$) between the two groups upon ANOVA analysis.

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