Expression and functional evaluation of *Mytilus galloprovincialis* foot protein type 5 (Mgfp-5), the recombinant mussel adhesive protein

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Mussel contains a variety of adhesion proteins, among which, *Mytilus galloprovincialis* foot protein type 5 (Mgfp-5) is one of the major proteins required for substrate adhesion. The labor-intensive nature and insufficiency of the extraction process have frequently resulted in very little purified recombinant Mgfp-5. These prompt technologies such as chemical synthesis and genetic engineering are employed to overcome these limitations. In this study, successful expression and purification of the recombinant Mgfp-5 using *Escherichia coli* BL21 (DE3) and affinity chromatography were reported. Production yield of 12.25% and purity of 96.92%, respectively were observed. The 3,4-dihydroxyphenylalanine (DOPA) content (9.60 pmol/g) and the adhesion (1 116 nN) in modified recombinant Mgfp-5 were 9.32 times and 1.6 times as great as those in the unmodified recombinant Mgfp-5, respectively. Recombinant Mgfp-5 at a concentration of 9.6 mg/L had little cytotoxicity on mouse L-929 fibroblast cells, which was toxic at first in cytotoxicity test, and a concentration of not more than 20 μg/mL would not lead to hemolysis of rabbit erythrocytes. In this case, recombinant Mgfp-5 is biosecure, providing the foundation for Mgfp-5 manufacturing as well as the development of clinical biological adhesive.

**Key words:** Mgfp-5, 3,4-dihydroxyphenylalanine (DOPA), adhesion, cytotoxicity, hemolysis.

**INTRODUCTION**

Marine mussel secretes a specific adhesive protein by its byssus to survive an aqueous environment. This was first observed in the 1980s by researchers and is called *Mytilus* adhesive protein (MAPs) or *Mytilus* foot protein (Mfps) (Waite and Tanzer, 1981). MAPs are one of the potential resources in the field of biotechnological applications for its strong adhesion, flexibility, water resistance and biodegradability, among others (Dove and Sheridan, 1986). MAPs can strongly adhere to surface of various materials under wet conditions, such as glass, plastic, metal, wood, and even polytetrafluoroethylene. Moreover, they can effectively bind to biological tissues or organs, and as a result, are applied in dentistry, dermatology, orthopedics and ophthalmology without noticeable toxicity and immunogenicity (Wu et al., 2014; Waite, 2002).

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Mussel byssus can be divided into proximal thread and distal plate, which secretes 6 types of proteins (Mfp-1 to Mfp-6) and three kinds of precollagens (preCol), namely, preCol-D, preCol-P and preCol-NG MAPs (Rego et al., 2016). These MAPs are rich in unusual amino acid 3,4-dihydroxyphenylalanine (DOPA) that can be catalyzed by polyphenol oxidase, which is central to the cross-linking reactions of cohesive curing and adhesive surface bonding (Silverman and Roberto, 2007). It has been proved that DOPA content is correlated with the adhesive strength of MAPs (Yu et al., 1999). Mfp-5 is located in the byssus plate where the DOPA content is approximately 30% (Waite, 2011). Hence, it directly plays a major role in adhesion (Waite, 2011).

Becton, Dickinson (BD Bioscience) companies have extracted the Mfp-1 and Mfp-2 mixtures (Cell-Tak™) which have been applied in biological adhesive products using the natural method. However, only one (1) mg of the protein can be obtained from about 10 000 mussels by natural extraction. Inefficiency and high cost of such natural extraction process has greatly restricted the application of MAPs. On this basis, chemical synthesis and genetic engineering have been widely applied, attempting to solve the above-mentioned difficulties (Gim et al., 2008; Platko et al., 2008). In this study, the recombinant Mytilus galloprovincialis foot protein type 5 (Mgfp-5) was successfully expressed in Escherichia coli BL21 (DE3) and purified, and the cytotoxicity of protein should be further tested in order to examine whether the recombinant Mgfp-5 could reach the safety standard or not.

The current study focused on the issue of, how recombinant Mgfp-5 could be expressed in E. coli and purified by nickel (Ni²⁺) column affinity chromatography. Subsequently, biological adhesion of recombinant Mgfp-5 was measured by glass coating and atomic force microscopy (AFM). Meanwhile, biological safety was analyzed by cell cytotoxicity test (Cell Counting Kit and CCK 8 qiangen method) and hemolysis test, in order to lay a good foundation for the development and application of sources of biological adhesive.

MATERIALS AND METHODS

Expression of recombinant Mgfp-5 protein

The pUC57 containing the Mgfp-5 gene (constructed and preserved in our laboratory) was constructed according to the sequence of cDNA Mgfp-5 (Gen Bank: AYS21220.1) gene. The plasmid pET28a-mgfp was constructed for Mgfp-5 gene and pET-28a vector using the following primers: F (Nde I cleavage site and protecting bases): 5'-GGAAATTCATATGAGTTCTGAAGAAT, and R (EcoRI cleavage site protecting bases): 5' CGGAATTCCTAACTGCTACACCT. PCR amplification program: 94°C pre-denaturation 5 min; 94°C denaturation 30 s; 55°C annealing 30 s; 72°C extension 45 s, cycle 34 times, 72°C extension 10 min were used. The purified amplified product and pET-28a vector were digested with Nde I and EcoRI, respectively, which were ligated with T4-DNA ligase and then transformed into E. coli BL21 on LB plates containing 50 mg/L Kan at 37°C overnight culture.

Monoclonal E. coli BL21 lacking the Mgfp-5 gene and E. coli BL21-Mgfp with the Mgfp-5 gene were cultured overnight in Luria Broth (LB, contained 50 Kan) followed by centrifugation at 200 r/min and 37°C, respectively. Plasmid pET28a-mgfp was extracted from E. coli strains, and PCR DNA products were separated on 0.8% (w/v) agarose gel.

Bacteria were propagated and cultured at a proportion of 1:100. Subsequently, bacterial cultures were induced by isopropyl-β-D-thiogalactoside (IPTG, 1.0 mmol/L) at OD600 = 0.8. 4 h later, 1.0 mL bacteria liquid was centrifuged at 4°C and 12 000 r/min for 10 min, and cells were collected. Expression of recombinant Mgfp-5 was analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of recombinant Mgfp-5 protein

E. coli BL21-Mgfp were cultured using batch bioreactor (Sartorius). Subsequently, recombinant Mgfp-5 was purified using Ni²⁺ affinity purification under natural or denaturing conditions, containing 8 mol/L urea as previously described (Lv et al., 2016). Following this, western blotting using His tagged was carried out.

The content of the protein was measured using a BCA Protein Assay Kit (Reducing Agent Compatible (Thermo Fisher Scientific, China) according to the manufacturer’s instructions, with bovine serum albumin as the standard. The purity from the purified protein was evaluated by gradation analysis, while the pure protein was freeze-dried and stored at -80°C.

Modification of proteins

Purified protein was dissolved in phosphate buffered saline (PBS) (NaCl 0.1 mmol/L, KCl 3 mmol/L, Na₂HPO₄·12H₂O 10 mmol/L, KH₂PO₄ 2 mmol/L) containing 10 mmol/L ascorbic acid and 20 mmol/L sodium borate (0.1 mol, pH 7). In order to modify tyrosine residues to DOPA, the solution was irregularly oscillated for 2 h at room temperature after adding 10 μL/mL tyrosinase. Recombinant Mgfp-5 was collected by ultrafiltration and dialysis in 5% acetic acid. Bovine serum albumin (BSA) was used as the negative control and Cell-Tak™ as the positive control.

DOPA content analysis

DOPA content was measured by nitroblue tetrazolium and potassium glycinate (NBT/glycinate, 0.24 mmol/L NBT in 2 mol/L potassium glycinate, pH 10) staining assay. The DOPA standard (1 μg/mL) was dispensed (0, 2, 4, 8, 16 and 20 μL) and added into six labeled wells in a 96-cell well format. The final volume of each well was adjusted to 20 μL with Milli-Q water and NBT/Glycinate (180 μL) was added.

All reagents were pipetted into tubes immersed in an ice-water bath. This reaction started by incubating in water bath at 25°C in the dark. An hour later, optical density (OD) was measured at a wave length of 530 nm. DOPA quantity of samples was determined from the standard curve and Milli-Q water was used as a negative control.

Adhesion analysis

Protein samples (10 μL of 1.00 mg/mL) were added into the glass surface and incubated under a humid environment at 25°C for 12 h. The surfaces were dried using natural drying and each was washed thoroughly with Milli-Q water for 2 h with shaking. Protein coating
spots were visualized using Coomassie brilliant blue staining. Images of protein spots were captured using a scanner and were analyzed by PDQuest software according to the protocols provided by Bio-Rad. BSA was used as a negative control.

AFM cantilevers were modified in accordance with techniques adopted by Ducker et al. (1991). The spring constant of AFM (SPM-9500J3) was 5 N/m. A lass sphere (Duke Scientific) of 5 μm in diameter was attached to the tip of cantilever using an epoxy resin (Vantico) under microscope, and the modified cantilever was cured at room temperature for 24 h. The modified AFM cantilevers were mounted onto cells and allowed to contact with 2 μL sample solutions (1.0 mg/mL) on glass slides for 20 min; this allow proteins to adsorbed on the glass bead.

After 10 min of contact, a force-distance curve was obtained by separating the modified cantilever from the glass surface. The pulling velocity for the force-distance curve was measured as 1 mm/s. Recombinant Mgfp-5 was dissolved in PBS and the final concentration was 1 mg/mL, which adhered to spearhead (polytetrafluoroethylene) and centrifugal tube cover (propathene) with different sizes or weights. Macroporous adhesion conditions of protein were observed.

Cytotoxicity test

According to the rule of MAP wound dressing in the use of the human body (usage amount of the 2% human body area less than 1 mL per day), the amount of MAP was 3.0 μg/cm² (Liu et al., 2013). In this study, the mass concentration of Mgfp-5 should be 9.6 mg/mL on the base of 96 well culture plate (Per well area is 0.32 cm² and medium is 0.1 mL per well). L-929 cells cultured in the Dulbecco’s modification of Eagle’s medium Dulbecco (DMEM) supplemented with 10% fetal bovine serum (100 μL) were added to the 96-well cell plate and cultured to cell adherence in a CO₂ incubator at 37°C.

After replacing fresh broth, protein samples (100 μL of 9.6 mg/L) were added in every well. Cell morphology was evaluated 2 days later using inverted phase contrast microscope. Subsequently, CCK-8 (Rainbio) (10 μL) was added into each well and the absorbance value (A) was recorded at 450 nm after 4 h. Relative growth rate (RGR) was calculated using:

\[ \text{RGR} \% = \frac{\text{average absorbance value} \times 100\%}{\text{average absorbance value of blank control}} \]

Cytotoxicity of recombinant Mgfp-5 was evaluated according to ANSI/AAMI/ISO 10993-4:2002/A1:2006 standard, the test sample was considered as hemolytic if the rate of hemolysis was >5%. Finally, the hemolytic ratio was expressed as percentage and was calculated as follows:

\[ \text{Hemolysis ratio} \% = \frac{A_{\text{sample}} - A_{\text{negative}}}{A_{\text{positive}} - A_{\text{negative}}} \times 100\% \]

All assays were repeated at least three times to ensure the reproducibility.

RESULTS

Expression of recombinant Mgfp-5 protein

The PCR assay results are indicated in Figure 1A; these showed amplification of the specific 260-bp band of Mgfp-5 gene fused with hexahistidine affinity ligand, which is clearly absent with the negative control. Recombinant Mgfp-5 was successfully expressed with the apparent molecular weight on SDS-PAGE gel, being greater (about 15 KD) than the predicted one (about 11.4 KD) (Figure 1B) which might be attributed to the higher isoelectric point (9.8) of recombinant Mgfp-5 as well as the combination with SDS (Hwang et al., 2010).

Purification and modification of proteins

Recombinant Mgfp-5 under natural or denaturing conditions (containing 8 mol/L urea) was analyzed. SDS-PAGE analysis indicated that recombinant Mgfp-5 was successfully expressed using IPTG induction and was purified by Ni²⁺ column chromatography (Figure 1C). In addition, yield of dissociated recombinant Mgfp-5 under denaturing condition was much higher than that under natural conditions.

Recombinant Mgfp-5 (37.00±2.55 mg) was purified from 1 L denaturing lysis buffer containing 302.00±16.55 mg of total protein, with the efficiency of about 12.25% and the purity reaching 96.92%. The analysis also showed that some recombinant Mgfp-5 exist as a dimer.
Western blot analyses confirmed that the purified protein was indeed a recombinant Mgfp-5 (Figure 1D).

DOPA content and function analysis

In order to assess the association between DOPA content and protein adhesion, DOPA content was measured. We performed a comparative study with BSA as a negative control and Cell-Tak™ as a positive control. Cell-Tak™ is a commercially available naturally extracted Mytilus edulis adhesive-protein mixture of fp-1 and fp-2 that contains DOPA residues in 5% acetic acid buffer. Recombinant Mgfp-5 and Cell-Tak™ was modified, and DOPA content of the modified recombinant Mgfp-5 (9.6 pmol/g) was discovered to be 9.32, 2.35 and 1.58 times higher than that of the unmodified recombinant Mgfp-5 (1.03 pmol/g), unmodified Cell-Tak™ (4.09 pmol/g), and modified Cell-Tak™ (6.07 pmol/g), respectively (Figure 2A). These findings suggested that part of tyrosine residues in recombinant Mgfp-5 had been oxidized to DOPA.

A simple coating assay was performed to test the adhesive ability of recombinant Mgfp-5. As shown in Figure 2B, recombinant Mgfp-5 and Cell-Tak™ could still adhere to the glass surface, while BSA was almost cleared and cleaned after each surface was washed thoroughly with Milli-Q water. The lighter spot color produced indicated that the adhesive intensity of modified recombinant Mgfp-5 was twice as strong as that of unmodified recombinant Mgfp-5, quantified by PDQest software (Figure 2C). This result demonstrated that modified recombinant Mgfp-5 contributed to the improvement of adhesive ability of the recombinant Mgfp-5.

AFM was selected to further investigate the adhesive ability of the recombinant Mgfp-5. As shown in Figure 2D, adhesive force of modified recombinant Mgfp-5 (~1116.67 nN) was much higher than that of unmodified recombinant Mgfp-5 (~19.67 nN) and Cell-Tak™ (698.33 nN). However, it was interesting that adhesive force of modified Cell-Tak™ showed a descending trend, which might be related to the fact that commercial Cell-Tak™ had already contained enough DOPA, while the intermolecular cross-link of DOPA through oxidizing would affect its adhesive ability, leading to reduced adhesive force.

Adhesion used for laboratory plastic consumables, including spearhead and centrifugal tube cover of different sizes or weights was tested (Figure 2E). It was discovered that modified Mgfp-5 and unmodified Cell-Tak™ could easily and completely adhere to these items within 10 min. It took 30 min for the modified Cell-Tak™ to complete cross-linking for adhesion. More seriously, adhesion of unmodified recombinant Mgfp-5 took about 12 h or even longer to fall off.

Cytotoxicity test

L-929 cells were incubated in a CO₂ incubator at 37°C and monitored after 2 days. As shown in Figure 3, L-929 cells of blank control group (a) attached well on the bottom of the plate. The online cells were clear with diamond or flattened spindle shape. Cells in negative control (b) and test groups (d) kept normal growth condition with no cytolysis observed, but cell density was slightly lower than that of the blank control group.

The cellular structure in a positive control group (c) was damaged. A large number of black spots were observed in the field of view, indicative of cell death occurring in nearly all cells. CCK-8 assay was used to measure the RGR and cytotoxicity (Table 2). RGR of positive control DMSO was 12.11±0.10% and the cytotoxicity of DMSO was in grade 4. RGR of test group with recombinant Mgfp-5 (RGR_Mgfp = 90.36±0.19%) was close to that of positive control (RGR_PBS = 93.12±0.07%). The cytotoxicity of recombinant Mgfp-5 was in grade 1 according to Table 1, which means that recombinant Mgfp-5 (9.6 mg/L) has a very good cytocompatibility.
Figure 2. Adhesion ability of the recombinant Mgfp-5, Cell-Tak™ (with or without tyrosinase modification) and BSA. BSA was used as a negative control. (A) DOPA quantity assay; (B) Coating ability assay: 10 μL of 1.00 mg/mL sample was added into the glass surface and incubated under a humid environment at 25°C for 12 h; (C) PDQuest analysis of protein spots on glass: Images of protein spots were captured by a scanner and were analyzed by PDQuest software using uncoated region as control; (D) measurement of adhesion forces of the recombinant Mgfp-5 using modified AFM analysis; (E) macroscopic adhesion phenomenon: adhesion of laboratory plastic consumables using modified Mgfp-5. Each value and error bar represents the mean of triplicate samples and its standard deviation.

- 1-5 represent BSA, unmodified recombinant Cell-Tak™, modified recombinant Cell-Tak™, unmodified recombinant Mgfp-5, modified recombinant Mgfp-5, respectively.

Hemolysis test

Figure 4 displayed the experimental image of hemolysis test with different concentrations and incubation times which was accompanied by a clear sediment and colorless transparent supernatant in groups 1, 2, 3 and negative controls (Sterile 0.9% sodium chloride solution) with hemolysis-free, while positive controls and groups 4 showed a little sediment and bright red supernatant with apparent hemolysis (Table 3).

Table 4 shows that the maximum concentration of Mgfp-5 at 20 μg/mL did not lead to a severe hemolysis within 3 h incubation by hemolysis ratio. Hemolysis ratio of recombinant Mgfp-5 with 20 μg/mL for 3 h was 2.74% (Table 4). According to ANSI/AAMI/ISO 10993-4:2002/A1:2006, the upper limited value of hemolysis index was 5%. Thus, the recombinant Mgfp-5 with 20 μg/mL has no hemolysis.

DISCUSSION

An impediment to further understand the unique adhesion and mechanism of MAPs is the lack of sufficient and pure protein. Large quantities of MAPs are needed to perform research and development for commercial adhesives. Existing approaches for natural extraction of Mgfp-5 are associated with several disadvantages, including low yield, being easily solidified and difficult to purify (Strausberg and Link, 1990), which might have prompted researchers to turn to genetic engineering for protein Mgfp-5 extraction.

Prokaryotic genetic engineering was one of the preferred techniques for example recombinant Mgfp-5 from M. galloprovincialis as reported by Hwang et al. (), which was expressed in E. coli and only micro test of related function was conducted subsequently, rather than its biosafety (Hwang et al., 2004). Recombinant Mgfp-5
Figure 3. L-929 cells morphology in different culture condition. 100 μL protein solution was added in every well on the base of the concentration of 9.6 mg/L. (a) Cell culture medium DEME; (b) PBS buffer; (c) DMSO; (d) recombinant Mgfp-5.

Table 2. L-929 cells toxicity in different culture condition.

<table>
<thead>
<tr>
<th>Group</th>
<th>RGR (%)</th>
<th>Cytotoxicity grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>100.0±0.00</td>
<td>0</td>
</tr>
<tr>
<td>b</td>
<td>93.12±0.07</td>
<td>1</td>
</tr>
<tr>
<td>c</td>
<td>12.11±0.10</td>
<td>4</td>
</tr>
<tr>
<td>d</td>
<td>90.36±0.19</td>
<td>1</td>
</tr>
</tbody>
</table>

10 μL CCK-8 was added into each well and the absorbance value at 450 nm after 4 h was recorded. Data represent the mean values ± SE (n = 3). (a) Cell culture medium DEME; b, PBS buffer; c, DMSO; d, recombinant Mgfp-5.

was also expressed in *E. coli* after optimizing the conditions for both expression and purification as shown in previous research. This result showed 16% yield and a better purity of 96% (Lv et al., 2016). Under such conditions, the bioreactor was used to produce recombinant Mgfp-5 in quantities (302.00±16.55). The yield of the recombinant Mgfp-5 was increased from 8.3 to 12.25% when 8 mol /L urea was added into the lysis buffer.

Mussel foot has a good adhesion in the water environment. The reason is that the DOPA in the protein has an extremely important role, which is the binding of DOPA to the substrates and the internal cross-linking of the proteins. All these proteins contain DOPA, formed by post-translational modification of tyrosine and have high isoelectric points (IEP) which differ vastly in sequences. Since DOPA can be oxidized and transformed to quinones and thus catalyze 1,2-benzenediols redox cycling at an alkaline pH, in the presence of potassium glycinate as a reductant, the released superoxide reduces NBT to formazan, therefore, allows a specific staining of DOPA-rich proteins (Paz et al., 1988). Then, modified approach of NBT/glycinate staining for the quantitative analysis of DOPA in recombinant Mgfp-5 is used.

Among the MAPs, the protein Mfp-5 had a small molecular-weight which contained a lot of DOPA (about 30%) (Hwang et al., 2004). Recombinant Mgfp-5 in *E. coli* also contained the majority of tyrosine residues, which could be modified to DOPA using tyrosinase (Hwang et al., 2010). However, excessive oxidation of DOPA is
resulted in self-ligation, rendering reduced protein adhesion. Researchers selected sodium borate and ascorbic acid to maintain the stability and protein adhesion of DOPA (Kan et al., 2014; Tatehata et al., 2000). In this study, sodium borate and ascorbic acid were added to protect DOPA from self-ligation during modification process. It was found in this study that DOPA content of modified recombinant Mgfp-5 (9.6 pmol/g) was about 9.32 times higher than that of unmodified recombinant Mgfp-5 which led to significantly improved adhesion. At the same time, it could also be observed that adhesion of commercial Cell-Tak™ decreased is accompanied by the increase in DOPA content possibly because it typically comes with enough DOPA. DOPA content would be increased while adhesion would be decreased once DOPA was further modified.

Atomic force microscopy was applied in detecting the Surface texture of various materials by atomic-level imaging, through which the surface friction, adhesion force and hardness could be determined. Based on this analysis, the adhesion force of modified recombinant Mgfp-5 was better than that observed in commercialized Cell-Tak™, which might be due to the combination of Mfp-1 with Mfp-2, as well as the lower content of DOPA than that of Mfp-5 chosen in the commercial Cell-Tak™. Therefore, both the content of DOPA and the adhesion force of commercialized Cell-Tak™ were inferior to those

### Table 3. Hemolysis of recombinant Mgfp-5 with different concentrations and incubation times.

<table>
<thead>
<tr>
<th>Time (h) groups</th>
<th>CK⁻</th>
<th>CK⁺</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*“+”, completely hemolysis; “-”, hemolysis-free “±”; part of the hemolysis, 1-4, represent recombinant Mgfp-5 solution of 5, 10, 20 and 25 μg/mL, respectively.*

### Table 4. Hemolysis ratio of Mgfp-5 solution with different concentrations for 3 h incubation.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CK⁻</th>
<th>CK⁺</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.058</td>
<td>0.901</td>
<td>0.066</td>
<td>0.071</td>
<td>0.082</td>
<td>0.121</td>
</tr>
<tr>
<td>Haemolysis ratio (%)</td>
<td>-</td>
<td>-</td>
<td>0.91</td>
<td>1.49</td>
<td>2.74</td>
<td>7.20</td>
</tr>
</tbody>
</table>

CK⁻, Sterile 0.9% sodium chloride solution; CK⁺, sterile water; 1-4 represent recombinant Mgfp-5 solution of 5, 10, 20 and 25 μg/mL, respectively.
of recombinant Mgfp-5 that had been modified by

Tyrosinase.

Implant materials must have good biocompatibility

because they will directly contact with tissues and cells

after implanted into body. As a new biomaterial for

adhesion, it is important to evaluate its biosafety from

experimental study to clinical application. The common

method is to implant the test material into the body of an

animal. However, implantation in vivo is limited by the

long experimental period, complicated operation process,

complex body environment and parameter control,

among others (Wang et al., 2012; Lee et al., 2016). In

contrast, experiments conducted in vitro are relatively

simple and their reproducibility can easily be controlled

(Li et al., 2015).

In ANSI/AAMI/ISO 10993-5:1999 standards, the
cytotoxicity test is generally accepted as the first chosen

item on account of convenience, fastness, high sensitivity

and saving animal, etc. L929 cells are the first and most

widely used cell line in cytotoxic test. L929 cells have the

merit of stable passage, fast multiply, low condition for

culture in vitro, being used for many materials cytotoxic

test. The CCK-8 assay is sensitive to detect toxicity of

materials and consistent with the toxicity experiment

results in animals, which is considered to be a good

method in evaluating the cytotoxicity of medical material

in vitro (Li et al., 2016). According to the rule of MAP

wound dressing in the use of the human body (Usage

amount of the 2% human body area less than 1 mL per
day), the amount of MAP was 3.0 μg/cm² (Liu et al.,

2013). Therefore, the mass concentration of Mgfp-5

should be 9.6 mg/L on the base of 96 well culture plates.

In the current study, the RGR of recombinant Mgfp-5 at

a concentration of 9.6 mg/L is above 90% and cytotoxicity

is grade 1 at 2 days. Therefore, the recombinant Mgfp-5

is consistent with medical standard of biomaterial. The L-

929 cells morphology cultured in recombinant Mgfp-5 had

no significant difference as compared to the control and

is well attached at the bottom of the culture plate, which

demonstrated that recombinant Mgfp-5 had no apparent

cytotoxicity and could promote cell proliferation without

affecting their normal function.

The hemolysis test is based on the degree of
erthrolysis and hemoglobin dissociation while the

material contacts RBC in vitro. ISO indicate that medical

biomaterials which will be applied to the body and to

biological tissue ought to be evaluated by the hemolysis


is a phenomenon of hemoglobin release which results in

erythrolysis. Besides the endogenic hemolytic factors

such as abnormality of red blood membranes and

hemoglobin, there are some kinds of extrinsic factors

such as physic agent on material surface, which can lead to

cytotoxicity or may result in machinery damage to

RBC. Generally, if hemolysis activity is observed in the

hemolysis test, the material shows toxic.

In this study, the fresh rabbit blood was added into the
test negative and positive control groups. The results

show that the hemolytic ratio of recombinant Mgfp-5

when the concentration was 20 μg/mL is 2.7%, which is

lower than the national and international standards of 5%.


suggests that protein with less than 20 μg/mL do not

harm the RBC.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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performed the entire experiments. Yujing Zhang was

responsible for protein purification and data analyses,

Wenying Gao performed the adhesion force and toxicity

testing and Yingjuan Wang supervised the research. All

authors have read and approved the final manuscript.

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