Multifunctional Delivery Nanosystems Formed by Degradable Antibacterial Poly(Aspartic Acid) Derivatives for Infected Skin Defect Therapy

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1. Introduction

Treatment of skin defects resulting from burns, tumors, or excision remains challenging because of complicated pathological conditions.[1–3] The epidermal growth factor (EGF) is crucially involved in tissue healing processes by binding to EGF receptor.[4,5] EGF could stimulate the proliferation and migration of keratinocyte, endothelial cells, and fibroblast and facilitate skin regeneration.[6,7] However, the EGF level has been proved to decrease in wounds.[2] Although the application of exogenous EGF can accelerate wound healing, it is difficult to maintain bioactivities due to proteolytic degradation.[3,8] Thus, functional nucleic acid (NA)-based therapy has been proposed for skin defect therapy.[9–12]

Safe and effective NA vectors are the key issue for achieving successful gene therapy.[13,14] Compared with viral vectors, low host immunogenic nonviral vectors, especially polycation-based vectors, are much more interesting due to their excellent properties of flexible structures, easy preparation and large gene payloads.[15–17] Various polycations have been used as promising carriers.[18–24] However, bacterial infections always occurred in the process of tissue healing, which are seriously harmful for tissue repair. Bacterial infections caused health problems and even death for millions of people worldwide.[25,26] The ideal treatment of infected defect requires both anti-infection and simultaneous tissue healing. Therefore, multifunctional antibacterial NA carriers with high performance are in constant demand for successful clinical gene therapy.

Aminoglycoside-based biopolymers have attracted great attention in recent years.[27] Aminoglycosides possess high binding affinity to RNAs and were exploited for developing gene vectors, such as cationic liposaccharides,[27] glycoconjugated polymers,[28] and polyaminoglycosides of tobramycin, gentamicin, and neomycin.[29] Particularly, tobramycin is a new aminoglycoside antibiotic with a broad antibacterial spectrum.[30] More recently, poly(aspartic acid) (PAsp) derivatives, which were prepared by the ring-opening reaction of β-benzyl-l-aspartate N-carboxy anhydride (BLA-NCA) and the subsequent aminolysis reaction, have been reported as promising gene vectors with excellent degradability and transfection performance.[31,32]

Inspired by these above studies, degradable antibacterial PAsp derivatives (TPT) based on tobramycin conjugated PAsp were proposed as multifunctional delivery nanosystems of plasmid encoding EGF (pEGF) to realize the antibacterial therapy and tissue healing of infected skin defects (Figure 1). TPT contains many glycosidic and amide linkages, which could be degraded in acidic, glycosidase, or lactamase environment.[33,34] The preliminary transfection performance of TPT was investigated in NIH3T3 and L929 fibroblast cells through a series of experiments including cell viability and gene...
transfection efficiency. Meanwhile, the antibacterial activities of TPT against *Pseudomonas aeruginosa* and *Staphylococcus aureus* were also evaluated in vitro. TPT was subsequently verified to effectively deliver pEGF to L929 cells. The in vivo infected skin defect therapy was also investigated with TPT/pEGF nanocomplexes.

### 2. Results and Discussion

#### 2.1. Preparation and Characterization of Branched Polymers

The synthetic route of TPT with antibacterial function was illustrated in Figure 1. TPT was prepared via the combination of ring-opening polymerization of BLA-NCA and subsequent aminolysis reaction. In detail, the amino groups of tobramycin were used to initiate the ring-opening polymerization of BLA-NCA to produce T-PBLA. Then, PBLA side chains were subsequently aminolyzed with tobramycin and ethylenediamine (ED) to produce T-PAsp-T (TPT) with cationic PAsp derivative chains. The length of PAsp chains could be controlled by varying the feed ratio of BLA-NCA. Here, the molar feed ratios [the amino group of tobramycin]: [BLA-NCA] of 1:50 and 1:100 were used to produce the corresponding T-PBLA1 and T-PBLA2. TPT1 and TPT2 were obtained via the aminolysis reaction of corresponding T-PBLA, where the molar feed ratio of BLA unit on T-PBLA, tobramycin, and ED was 1: 1: 20. The molecular weights of TPT1 and TPT2 are 4.5 × 10^3 and 8.7 × 10^3 g mol⁻¹, respectively (Table S1, Supporting Information). The typical 1H nuclear magnetic resonance (1H NMR) spectra of T-PBLA and TPT are shown and analyzed in detail in Figure S1 in the Supporting Information. Based on the 1H NMR analysis, it was calculated that TPT possessed about 50% of tobramycin. The molecular weight of degraded TPT2 (3.1 × 10^3 g mol⁻¹) substantially decreased (Figure S2, Supporting Information), verifying the degradability of TPT. The glycosidic and amide linkages containing in TPT are easy to be degraded in acidic, glycosidase, or lactamase environments, which may endow TPT with excellent degradability in vivo.

The ability of TPT to bind pDNA was characterized by agarose gel electrophoresis, dynamic light scattering (DLS) and atomic force microscopy (AFM). Electronegative pDNA would be partially or completely neutralized by positively charged TPT, resulting in gel retardation. As shown in Figure 2a, TPT2 could completely bind pDNA at the mass ratio of 1.5, while TPT1 completely condensed pDNA at the mass ratio of 2. The better condensability of TPT2 was attributed to its higher molecular weight (Table S1, Supporting Information). After degradation, TPT2 could not condense pDNA until the mass ratio of 2.5, indicating that the degradation of TPT could facilitate the release of pDNA from the complexes.

#### 2.2. Biophysical Characterization of TPT/pDNA Nanocomplexes

The particle size and zeta potential of TPT/pDNA complexes at various mass ratios were shown in Figure 2b. The particle sizes of TPT/pDNA complexes gradually decreased
with the increase of mass ratios. All the TPT/pDNA complexes were in a diameter range of 100–300 nm when the weight ratio reached 10, which can readily undergo endocytosis.[24,29] With the increase of the mass ratio, the zeta potentials of complexes also increased to be around 30 mV. The positive net surface charge will produce good affinity for anionic cell surfaces.

The morphologies of TPT/pDNA complexes at the mass ratio of 10 were imaged by AFM (Figure 2c). TPT2 with higher molecular weight can tightly compact pDNA into smaller spherical nanoparticles comparing with TPT1 with low molecular weight, which is consistent with the results from DLS (Figure 2b). After degradation, TPT2/pDNA complexes demonstrated the irregular topography, because degraded TPT2 did not efficiently condense pDNA, again verifying the degradability of TPT. The unstable nanocomplexes might facilitate the intracellular release of pDNA and enhance gene transfection efficiency.[31,32]

2.3. In Vitro Cell Viability and Transfection Assay

The cell viability of TPT at different mass ratios was assessed via 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assays. As shown in Figure 3a, a dose-dependent tendency was observed for the cell viabilities of both NIH 3T3 and L929 fibroblast cells mediated by TPT. At higher mass ratios, the TPT/pDNA complexes possessed excess polymers, which made slightly higher cytotoxicity. Evidently, TPT demonstrated significantly lower cytotoxicity comparing with the “gold standard” branched polyethylenimine (PEI, 25 kDa) at all mass ratios.[24,29]

The in vitro gene transfection performance of the TPT/pDNA complexes was first assessed using pRL-CMV as a gene reporter in NIH3T3 and L929 cell lines comparing with PEI (at its optimal N/P ratio of 10 which was determined in Figure S3, Supporting Information; Figure 3b). Generally, the transfection efficiency mediated by TPT first increased until the mass ratio of 10 and then decreased slightly with the increase of mass ratios. At lower mass ratios, pDNA cannot be condensed efficiently by TPT, while at higher mass ratios the excess cationic polymers could cause little cytotoxicity and result in the slight decrease of transfection efficiency. As expected, TPT2 with higher molecular weight exhibited higher transfection efficiency than TPT1 at all mass ratios. This may be caused by the smaller particle sizes and higher positive charges of TPT2/pDNA nanocomplexes which benefited the cellular internalization (Figure 2b). Notably, the TPT vectors, especially for TPT2, exhibited much higher transfection efficiencies at most mass ratios than those of gold standard PEI (25 kDa) at its optimal N/P ratio in both cell lines. The low cytotoxicity and degradability of TPT might benefit the excellent transfection performance.

To further confirm the transfection performance of TPT, enhanced green fluorescent protein (EGFP) expression was also performed under fluorescence microscopy using plasmid pEGFP-N1 in the selective L929 cells. Figure 3c and Figure S4 in the Supporting Information show the typical images of visual EGFP gene expressions mediated by TPT1 and TPT2 at the optimal mass ratio of 10, as well as the control PEI at its
optimal N/P ratio of 10. Stronger EGFP fluorescence signals were obtained with TPT2, while limited fluorescence signals were observed with TPT1 and PEI. The quantitative analysis of the transfection efficiency suggests 17% EGFP-positive cells for TPT1 and 29% for TPT2 while the control PEI presents only 15%, which agrees with the above luciferase gene transfection results (Figure 3b).

The cellular uptake of TPT1/pDNA and TPT2/pDNA complexes was subsequently investigated in L929 cells. The fluorescence signals displayed in Figure 3d represent...
the uptake level of complexes, where pDNA was labeled by YOYO-1 green fluorescent dye and nuclei was stained by 4′,6-diamidino-2-phenylindole (DAPI) in blue. It is obvious that TPT2/pDNA exhibited stronger signals than TPT1/pDNA. The percentages of the positive cells quantified by flow cytometry (FCM) were 75.5% for TPT2/pDNA, and 43.1% for TPT1/pDNA, respectively (Figure S5, Supporting Information). The enhanced cellular internalization level of TPT2 was supposed to favor the transfection efficiency.

2.4. In Vitro Delivery of pEGF

The pEGF delivery ability of TPT was evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays in L929 cells compared with that of PEI (Figure 4a). The relative EGF expression levels mediated by TPT and PEI were analyzed relative to the internal reference GAPDH. Generally, with increasing mass ratios, the transfection efficiency mediated by TPT1 and TPT2 first increased and then declined. In most cases, TPT2 exhibited higher transfection efficiency than TPT1. The relative expression level of EGF mediated by TPT was much higher than that mediated by PEI. Western blot assay was conducted to further confirm the expression of pEGF mediated by TPT (Figure 4b,b′). Specially, the expression level of EGF in the TPT2/pEGF group was higher than that of the TPT1/pEGF group due to the advanced transfection property of TPT2. These results were in agreement with those of luciferase and pEGFP-N1 expression (Figure 3b,c).

2.5. In Vitro Wound Healing and Cell Proliferation

The effect of TPT/pEGF nanocomplexes on the migration of L929 fibroblast and HEK 293 epithelial cells was evaluated by a wound scratch assay (Figure 4c,c′, Figure S6a,a′, Supporting Information). The minimal wound healing at 24 and 48 h was observed. However, it is obvious that the cells treated with TPT/pEGF nanocomplexes migrated more quickly than the control. The final wound closure of cells mediated with TPT2/pEGF was about 100% while that of TPT1/pEGF was around 81%. Meanwhile, much denser cells were seen for TPT1/pEGF and TPT2/pEGF compared to the control. Wound healing combined the effects of migration and proliferation. The more proliferation and less migration at early stage might account for the minimal wound healing at 24 and 48 h. As a result of proliferation, the dense cells benefited the obvious migration of TPT/pEGF groups at 72 h. Additionally, transfected L929 cells by TPT2/pEGF grew faster than TPT1/pEGF (Figure 4d). These above results verified the outstanding pEGF delivery ability of TPT2 and the bioactivity of expressed EGF, in according with the results of qRT-PCR and western blot (Figure 4a,b,b′).

![Figure 4](https://www.advancedsciencenews.com/doi/abs/10.1002/adhm.201800088)  
*Figure 4. a) Relative expression of EGF in L929 cells treated with TPT/pEGF at different cases compared with PEI/pEGF (at the optimal N/P ratio of 10). b) Relative EGF expressions in L929 cells determined by western blot analysis after the 48 h treatment with different formula and (b′) quantification of relative expression levels (according to the western blot analysis) of EGF. c) Representative images of cell migration at different periods and (c′) percentage of wound closure representing migration distance at 72 h over originally wounded distance. d) Cell counts of L929 cells at 24, 48, and 72 h after various treatments (mean ±SD, n = 3), *p < 0.05.*
2.6. In Vitro Antibacterial Activities

The antibacterial activities of TPT and TPT with polyvinyl alcohol (PVA) gel against \textit{P. aeruginosa} and \textit{S. aureus} were assessed at different concentrations of TPT. As shown in \textbf{Figure 5a}, the inhibitory efficiency of TPT presented as a concentration-dependent tendency. Both minimum inhibitory concentrations (MICs) of TPT1 and TPT2 were 512 µg mL$^{-1}$ for \textit{P. aeruginosa} and \textit{S. aureus}. Tobramycin plays an important role in antibacterial process. The similar percentage of tobramycin in TPT1 and TPT2 might account for the above phenomenon. In addition, it was noted that the inhibitory efficiencies of TPT and TPT with gel against \textit{P. aeruginosa} were slightly higher than those against \textit{S. aureus}, which might be caused by the different structure between the outer envelopes of Gram-negative (\textit{P. aeruginosa}) and Gram-positive microorganisms (\textit{S. aureus}).[35,36] Obviously, the MIC value of TPT with PVA gel was almost the same with corresponding TPT group, implying that the PVA gel has no obvious influence on the

\textbf{Figure 5.} a) Inhibition efficiency of antibacterial polymers for \textit{P. aeruginosa} and \textit{S. aureus}. b) Inhibition zones against \textit{P. aeruginosa} and \textit{S. aureus} of TPT and TPT with PVA gel in PBS. c) Hemolysis ratio of RBCs treated with TPT (mean ±SD, n = 3). d,e) Images of RBCs treated with TPT, water, and PBS.
antibacterial activities of TPT. The above results indicated the good antibacterial activities of TPT and TPT with PVA gels.

The inhibition zone method was also used to further investigate the antibacterial efficiency of TPT. As shown in Figure 5b, obvious inhibition zones around the samples were observed, indicating the excellent antibacterial activities against P. aeruginosa and S. aureus. TPT samples demonstrated larger inhibition zones in P. aeruginosa compared with S. aureus, which was consistent with the antibacterial activities in solution (Figure 5a). In addition, the diameters of inhibition zones of TPT with PVA groups were similar with corresponding TPT groups, further indicating that the effect of PVA gel on the antibacterial activities was negligible. The results were in agreement with those of antibacterial activities in solution (Figure 5a).

2.7. Hemolysis Assay

Biocompatibility, such as blood compatibility, is important for biomaterials. Thus, the blood compatibility of TPT was assessed by hemolysis assay in this work. The hemolysis ratios of TPT1 and TPT2 at the concentration of 1 mg mL\(^{-1}\) were 0.5% and 1.6%, respectively (Figure 5c). The hemolysis ratio of TPT was significantly lower than that allowed for biomaterials (5%) and that of PEI (about 38%), indicating the excellent blood compatibility of TPT. The supernatants of the red blood cells (RBCs) suspensions are clear or weakly pink after incubated with TPT, which are close to that of phosphate buffered saline (PBS; Figure 5d), implying that no obvious hemolysis happened in the TPT group. In addition, RBCs still maintained biconcave disk shape when incubated with TPT while those incubated with water were fragment (Figure 5e). Those above results revealed the good hemocompatibility of TPT with biocompatible PAsp chains.

2.8. In Vivo Anti-infective and Wound Healing Therapy

Based on the above in vitro antibacterial activity and transfection performance, TPT2 with better transfection performance was selected for in vivo infected skin defect therapy. The animal model of infected skin defect was built using Wistar rats. The representative photographs of the wounds on Day 1–14 were shown in Figure 6a. All the infected groups demonstrated the pyosis on Day 1 indicating the successful establishment of infected skin defect model via smearing the suspension containing S. aureus. After the 14 d treatment, the pyosis almost disappeared in the TPT2, TPT2/pEGF, and TPT2/pEGF+TPT2 groups while the pyosis in the w/o group became much more serious. These results confirmed the good antibacterial ability of TPT2. As expected, the wound healing ratios of the pEGF involved groups, especially the TPT2/pEGF+TPT2 group, were higher compared with other groups due to the promoting wound healing ability of pEGF delivered by TPT (Figure 6a,a’).

In addition, the situations of bacteria around the wounds on Day 7 and Day 14 were determined by counting the bacterial colonies on the Luria-Bertani (LB)-agar plate. As displayed in Figure 6b,b’, the numbers of bacteria grown on the LB-agar plates in the TPT-involved groups obviously decreased compared with that in the w/o group. Because of the additional TPT2, the number of bacteria in the TPT2/pEGF+TPT2 group was less than those in the TPT2 and TPT2/pEGF groups, which resulted in the best wound healing. Additionally, the DNA sequence of the bacteria grown on the LB-agar plate corresponded 99% with S. aureus in National Center for Biotechnology Information (NCBI) database (Figure 6c and Figure S7, Supporting Information), indicating the bacteria on the infected wounds were S. aureus.

After the 7 and 14 d treatments, the wounds of the Wistar rats were harvested for immunohistochemistry analyses and hematoxylin and eosin (H&E) staining (Figure 7). The expression of EGF was detected via immunohistochemistry analyses. On Day 7, compared with the w/o group, reddish-brown areas representing EGF were observed clearly in the TPT2 group. The excellent antibacterial ability of TPT2 could provide clean environment for tissue growth and benefit the production of inherent EGF in cells. Due to the exogenous EGF expression of pEGF mediated by TPT2, the TPT2/pEGF group demonstrated the more obvious reddish-brown areas. Particularly, the TPT2/pEGF +TPT2 group presented the highest EGF level benefiting from the pEGF delivered by TPT2 and the antibacterial ability of free TPT2. The above phenomena were also observed on Day 14, indicating that the combined systems TPT2/pEGF and TPT2 could produce more EGFs, which is good for wound healing.

The representative formation of re-epithelialization tissues on the wounds was observed via H&E staining. A neo-epidermis layer (pointed by red arrow) already formed on Day 7 in the TPT2/pEGF+TPT2 group. However, for the w/o group, not to mention epidermis formation, obvious inflammation (pointed by white circle) could be observed. In addition, on Day 14, many hair follicles (pointed by red arrow) were also observed in the TPT2/pEGF+TPT2 group, while the inflammation resulted from infection still remained in the w/o group. The overgrown of keratinized layer (pointed by green arrow) observed in the w/o group might be caused by the inflammation. These results of H&E staining also confirmed the remarkable in vivo antibacterial ability and simultaneous wound healing mediated by the TPT2/pEGF +TPT2 group.

To evaluate the in vivo cytotoxicity of TPT2, the organs of Wistar rats including heart, liver, spleen, lung, and kidney in the TPT2/pEGF+TPT2 group were dissected for H&E analysis on the 7th and 14th day. As shown in Figure S8 in the Supporting Information, no obvious toxicity was observed in all the organs indicating the safety of TPT2 in vivo. The above in vitro and in vivo results were consistent and verified that the TPT-mediated multifunctional delivery system is promising for infected tissue defect therapy.

3. Conclusions

In summary, degradable antibacterial vectors (TPT) based on tobramycin conjugated PAsp derivatives were successfully prepared via the ring-opening polymerization of BLA-NCA initiated by the amino groups of tobramycin and the subsequent aminolysis reaction with tobramycin. TPT demonstrated...
Figure 6. Photoscopic images a) and ratio a’) of wound healing of rat model for Day 1–14. Photoscopic images b) and bacteria number b’) of bacterial colony-forming units obtained from different tissues of rats treated under various conditions. c) Parts of the 16S rDNA sequencing results of the bacteria (from the infected soft tissues) compared with sequences in NCBI database. The error bars indicate the standard deviation of three measurements, *p < 0.05.
good degradation, low cytotoxicity, and hemocompatibility. TPT also demonstrated good transfection performances and its pEGF delivery ability facilitated proliferation of fibroblast cells. Meanwhile, TPT exhibited excellent antibacterial activities against P. aeruginosa and S. aureus benefiting from the introduction of antibiotic tobramycin. Notably, such TPT-mediated multifunctional delivery nanosystem could simultaneously realize the antibacterial therapy and tissue healing of infected skin defect in vivo. The present work would shed light on the design of next-generation multifunctional delivery systems to treat infected tissue defect.

4. Experimental Section

**Materials**: Tobramycin (98%), triphosgene (>99%), anhydrous dimethyl sulfoxide (DMSO), ED (>98%), branched PEI (Mw ∼25 000 Da), and MTT were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO. Anhydrous 1,4-dioxane L-aspartic acid β-benzyl ester (BLA, >98%) was purchased from Tokyo Chemical Industry Co. Ltd, Japan. The monomer BLA-NCA was prepared as described in the earlier work.[32] Plasmid pRL-CMV encoding Renilla luciferase (Promega Co, Cergy Pontoise, France) and plasmid pEGFP-N1 encoding EGF (BD Biosciences, San Jose, CA) were amplified in Escherichia coli and purified according to the supplier’s protocol (Shanghai Jikai Gene Chemical Technology Co., Ltd, China). The strains of P. aeruginosa (ATCC 27 853) and S. aureus (FRF 1169) were obtained from Promega (Madison, USA).

**Preparation of Branched PBLA with Starting Tobramycin (T-PBLA)**: T-PBLA was prepared via the ring-opening polymerization of BLA-NCA, which was initiated by amino groups of tobramycin. Two molar feed ratios (1/50 and 1:100) of [NH2]:[BLA-NCA] were used in this work. In detail, 9 mg (or 4.5 mg) of tobramycin was dissolved in 2 mL of anhydrous DMSO, and then 0.8 g of BLA-NCA in 5 mL of anhydrous DMSO was added into the solution of tobramycin. The reaction proceeded under a nitrogen atmosphere with stirring for 72 h at 50 °C. Excess acetone was used to precipitate T-PBLA from the mixture, and the crude product was washed with acetone at least twice before vacuum drying.

**Preparation of Cationic T-PAsp-T (TPT)**: TPT was synthesized by the aminolysis reaction of T-PBLA with tobramycin as well as excess ED. 0.5 g of T-PBLA was dissolved in 3 mL of DMSO, and then 1 g of tobramycin in 7 mL of DMSO was added. The mixture was kept stirring under nitrogen atmosphere at 30 °C. After 24 h, 1.5 mL of ED was added, the mixture was kept for another 12 h to complete the aminolysis procedure. At last, the mixture was diluted with deionized water and dialyzed against deionized water with dialysis membrane (molecular weight cut off (MWCO), 3500 Da) for 48 h. The pure product TPT was collected through freeze-drying.

**Preparation of TPT/Plasmid Nanocomplexes**: TPT was beforehand dissolved in sterile deionized water at the concentration of 2 mg mL⁻¹ and stored at 4 °C. TPT/plasmid nanocomplexes were prepared by the weight ratio (w/w) of TPT to plasmids as described in earlier work.[27] Nanocomplexes at various weight ratios were prepared via mixing TPT

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**Figure 7.** Immunohistochemical and H&E staining of the tissues harvested from the wound areas of rats in different groups after 7 and 14 d treatments.
solution and plasmid (pRL-CMV, pEGFP-N1, and pEFG) solution (0.1 mg mL$^{-1}$). These mixtures were incubated at 25 °C for 30 min before use. **Physical Characterization:** The molecular weights and chemical structures of TPTs were characterized by gel permeation chromatography (GPC) and $^1$H NMR spectroscopy. As described in the earlier work, the agarose gel electrophoresis assay was conducted by using a subcell system (Bio-Rad Laboratory, Hercules, CA). The particle sizes and zeta potentials of TPT/plasmid nanocomplexes were measured by a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). The morphologies of nanocomplexes were imaged by an AFM equipped with a Nanoscope IIIa controller (Bruker Dimension Icon). To investigate the biodegradability, TPT was dissolved in PBS solution (pH = 5.8), and the molecular weight of degraded TPT was measured with GPC after being constantly shaken for 24 h in a 37 °C incubator at 100 rpm according to the previous work.

**In Vitro Antibacterial Assay:** The antibacterial activity of TPT against *S. aureus* samples were added. After 24 h incubation at 37 °C, the results were described in the Supporting Information. TPT was added into the 2% RBCs suspension at the concentration of 2 µL of LB medium and the bacterium $\eta$1 of each bacterium ($\eta$sol) was determined from Equation (1):

$$\eta_{\text{sol}} = (A_1 - A_2)/(A_0 - A_1)\times100\%$$

where $A_1$, $A_2$, and $A_0$ are the OD$_{400}$ values of the bacterium culture containing a polymer, LB medium containing a polymer, bacterium culture in LB medium, and LB medium alone, respectively. MIC was determined as the lowest concentration of antibacterial materials when no visible growth of bacterium was observed.

To observe the antibacterial activity of TPT and TPT with PVA visually, the zone of inhibition assay on top of the LB-agar plate were conducted by using oxford cup method as described previously. Briefly, 25 mL of LB-agar medium containing $2 \times 10^5$ cfu mL$^{-1}$ of each bacterium (*P. aeruginosa* and *S. aureus*) was evenly sprayed onto the surface of the LB-agar plate, and then oxford cups were placed on the inoculated agar, and then 100 µL of samples were added. After 24 h incubation at 37 °C, the photographs of the inhibition zones around the oxford cups were captured by using a digital camera and the diameter of inhibition zone was measured.

**Hemolysis Assay:** The hemolysis assay was conducted using RBCs collected from rabbit blood as described previously. The details was described in the Supporting Information. TPT was added into the 2% RBCs suspension at the concentration of 1 mg mL$^{-1}$.

**In Vivo Anti-Infective and Wound Healing Therapy:** Wistar rats (Male, 6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). All animal experiments were approved by Ethical Committee of Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College. 15 of Wistar rats were randomly divided into five groups (three rats in each group): control (no bacterium with PVA gel), w/o (with bacterium and PVA gel but without TPT), TPT2 (with bacterium and PVA gel containing TPT2), TPT2/pEGF (with bacterium and PVA gel containing TPT2/pEGF), and TPT2/pEGF+TPT2 (with bacterium and PVA gel containing TPT2/pEGF+TPT2). To establish the wound infection model, two 1.5 × 1.5 cm$^2$ full-thickness skin wounds were created on the dorsal side skin by excision. Each wound was covered with 100 µL of *S. aureus* suspensions containing $10^8$ cfus and sterile gauze as described in the previous work. The control group was only covered with sterile gauze. After *S. aureus* was incubated on defined skin wounds for 24 h, PVA gel, PVA gel with TPT2, PVA gel with TPT2/pEGF, and PVA gel with TPT2/pEGF and TPT2 were smeared evenly on the wounds every day. Herein, the concentration of TPT2 was 0.5 mg mL$^{-1}$ and the amount of pEGF was determined according to the optimal mass ratio of 10. To evaluate the wound healing, the wounds were photographed and measured every day from Day 1 to Day 14.

In addition, on the 7th and 14th day of therapy, the entire wounds with adjacent normal skin were excised and fixed in 4% paraformaldehyde for immunohistochemical analyses and H&E staining. The infected tissues were hortenized and diluted (1000 times) with normal saline. The amount of the bacteria in the infected tissues of the rats was determined via counting the colonies on LB agar where the diluted homogenates were spread. Meanwhile, the main organs were also dissected and treated via H&E staining to observe the in vivo toxicity.

In addition, the full length of 165 rDNA of single-colony bacteria in the infected tissues was sequenced as described in our previous work. The obtained sequences were compared with those in NCBI database by Basic Local Alignment Search Tool (BLAST).

**Statistical Analysis:** The experiment data were presented as means ± standard deviation, where they were repeated at least three times. The differences between two groups were calculated by using the Student’s $t$-test. A $p < 0.05$ was used as the criterion of statistical significance. All analyses were performed GraphPad Prism (Version 5, GraphPad Software, Inc.)

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.