Direct synthesis of substrate-independent nanoparticles for antibacterial application

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Abstract

In the present study, we designed a substrate-independent antimicrobial nanoparticle (SNP) via self-assembly of poly (acrylic acid) (PAA) and poly (diallyl dimethyl ammonium chloride) (PDDA). The prepared nanoparticle, with a cube structure, as revealed by scanning electron microscope (SEM), maintained structural integrity even after extensive washing. In addition, the SNP was endowed with substrate-independent adhesive affinity to various surfaces by carbodiimide reaction owing to the carboxyl group of PAA. The results of blood compatibility and cytocompatibility demonstrated that the SNP had a limited effect on blood coagulation and cell proliferation. The results of antibacterial tests indicated that the SNP exhibited significant inhibition ability for both gram-negative and gram-positive bacteria, reducing cell amount by 97.2% and 98.2% within 24 h for \textit{Escherichia coli} and \textit{Staphylococcus aureus}, respectively. The hemocompatibility and cytocompatibility were attributed to the introduction of carboxyl group, and the significantly antimicrobial property was ascribed to the introduction of PDDA. Furthermore, the SNP maintained outstanding long-term antimicrobial property. In general, it was believed that the designed SNP probably showed potential for applications in various biological and clinical fields.

1. Introduction

Despite the considerable improvement of sterilization technology in biological field, the inhibition of bacterial proliferation still faces critical challenges \cite{1}. The bacterial infection may occur on implanted devices after long-term usage, which can lead to lethal syndrome to patients \cite{2}. Current antimicrobial strategies mainly focus on either incorporating direct, on-contact antibiotics, such as gentamicin and kanamycin, or development of silver ion compounds. Antibiotics show excellent anti-infective properties, and silver as well as its compounds have long been used, in one form or another, as antimicrobial agents owing to the broad-spectrum antimicrobial properties \cite{3}. Recently, with the rapid development of nanotechnology, nanomaterials, particularly nanoparticles, have generated significant impact in fields related to medical biology. Nanoparticles which have prominent specific surface area and high reactivity, exhibit unique physical, chemical, and biological properties \cite{4}. Thus, combining antibiotics with nanomaterials and engineering silver into nanoparticles have been increasingly investigated. However, the inherent disadvantages of these strategies were revealed after intended use. For example, antibiotics may lead to erythema, Achilles tendon lesions, renal dysfunction, and other side effects \cite{5}. Although the risk of silver nanoparticles is still subject to debate, mounting studies have concluded that silver nanoparticles also produced toxic effect in human cells during sterilization process \cite{6,7}. Furthermore, the major limitation of the strategies mentioned above is the failure in constructing sustained antibacterial surface, since the antibiotics and silver nanoparticles may be released to the environment from usage \cite{8,9}. Therefore, it is still a bottleneck to develop a surface with biocompatibility and long-term antibacterial property.

To address the problems, we presented a facile route to preparation of substrate-independent antimicrobial nanoparticle (SNP) through self-assembly of poly (acrylic acid) (PAA) and poly (diallyl dimethyl ammonium...
chloride) (PDDA). PAA is a kind of typical anticoagulant polymer rich in carboxyl groups, whereas PDDA, an ordinary and water soluble cationic polyelectrolyte, exhibits quaternary ammonium moiety as pendant groups in its chemical structure and displays outstanding antimicrobial activity \cite{10, 11}. It was reported that the antimicrobial activity of free PDDA is higher than the one observed for PDDA in nanoparticles. However, it is difficult to use PDDA directly as an antimicrobial material because of its water solubility \cite{12}. In this study, we immobilized PDDA on the surface of nanoparticles, hence the excellent antibacterial property of PDDA was maintained. After assembly with PAA, the prepared nanoparticles would be endowed with substrate-independent adhesive affinity to various solid surfaces owing to the carboxyl groups. To construct nanoparticles, PAA solution was added into PDDA solution by ultrasonic-assisted drop method. Subsequently, the chemical composition, morphology, water contact angle, blood compatibility, cytotoxicity, and bactericidal ability for the SNPs were systematically investigated.

2. Experimental

The details on experimental procedures are provided in Supporting Information.

2.1. Materials

Poly (acrylic acid) (PAA), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were obtained from Aladdin (China), Ti-6Al-4V alloy (TC4) were obtained from PanZhu (China), 3, 4-Dihydroxyphenethyamine (DA-HCl), poly (diallyl dimethyl ammonium chloride) (PDDA) and N-hydroxysuccinimide (NHS) were obtained from Aladdin (China). Peptone, yeast extract, and agar were obtained from Hopebio (China). Escherichia coli and Staphylococcus aureus were obtained from Bianzhen (China). All the other chemicals (analytical grade) were obtained from Kelong (China), and were used without further purification.

2.2. Synthesis of SNPs

50 mg PAA and 50 mg PDDA were dissolved in 100 ml DI water, respectively. Then, the PAA solution was added into the PDDA solution drop by drop, followed by ultrasonic dispersion (West sw-1012s bath ultrasonic machine, 600 W, 28 kHz) for 0.5 h. The particles were separated by centrifugation, and the solid products were washed with distilled water several times.

2.3. Preparation of SNPs/TC4 sheet (STS)

In this study, SNPs were immobilized on TC4 sheet surface in order to characterize the morphology, elemental composition, hydrophilicity, blood compatibility, and antimicrobial property.

First, the TC4 sheet was coated with dopamine (DA) (TC4/DA sheet), and the detailed procedures are described as follows: 20 mg DA was dissolved in 20 ml of Tris buffer (pH = 8.5); then TC4 sheet was immersed in this solution and continuously stirred at room temperature for 24 h. Then, the sheet was taken out from the solution, followed by continuously rinsing with DI water for 10 min to ensure that the adherent DA were eliminated absolutely. The whole process was repeated three times.

Secondly, the SNPs were immobilized on the TC4/DA sheet surface via carbodiimide reaction, and the detailed procedures are described as follows: 0.86 g (4.5 mM) EDC and 0.23 g (2 mM) NHS were added into 40 ml SNPs suspension (0.05 mg ml$^{-1}$), and the reaction was carried out for 12 h with vigorously stirring at room temperature. Then, the TC4/DA sheet was immersed into the suspension, and the reaction was carried out for 4 h. The resultant SNPs/TC4 sheet (STS) was rinsed with DI water and fully dried by freeze-drying.

2.4. Characterization of SNPs and STS

Attenuated total reflection-Fourier transform infrared spectra (ATR-FTIR) were obtained on a Nicolet-560 spectrophotometer (Nicol, US) between 4000 and 500 cm$^{-1}$ with the resolution of 2 cm$^{-1}$. The morphologies were observed by scanning electron microscope (SEM, quanta 250, USA). The elemental concentrations were determined by energy-dispersive X-ray spectroscopy (EDS, quanta 250, USA). The particle size was measured using a Zetasizer Nano ZS-90 instrument (Malvern, UK). The hydrophilicity was characterized by contact angle measurement using a contact angle goniometer (DSA20, KRUSS, Germany) equipped with video capture. To investigate the stability of STS, a peel-tape experiment was employed (see figure S3).

2.5. Blood compatibility

The blood compatibility of the SNPs was characterized by platelet adhesion and hemolysis test.

The collection of human blood, preparation of plasma and related experiments were all performed in the Institute of Blood Transfusion (IBT, a Chinese government authorized blood tests institution), Chinese
Academy of Medical Sciences. All experiments were performed in compliance with the relevant laws of Chinese government and institutional guidelines, and all the experiments are informed and approved by the institutional committee and performed by the staff of the institute.

Blood Collection: Human fresh blood was donated by a healthy man (age 24), the blood was collected by vacuum tubes with anticoagulant media (sodium citrate, anticoagulant media to blood ratio, 1:9). The collected blood was then centrifuged at 1000 rpm for 15 min to obtain platelet-rich plasma (PRP).

2.6. Cytotoxicity
Human umbilical vein endothelial cells (HUVECs), which were purchased from Fenghui Biotechnology, were derived from human umbilical vein. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was employed to estimate the cytotoxicity of SNP. The culture time of cell viability and proliferation was 3 d.

2.7. Antimicrobial property
The antimicrobial capability of SNP was investigated according to standard antimicrobial susceptibility test protocols (e.g. JIS Z 2801 and ISO 22196) [13]. Moreover, in order to characterize the long-term antibacterial property, an inhibition zone method was also employed.
Figure 3. ATR-FTIR spectrum for TC4 sheet, TC4/DA sheet, and STS.

Figure 4. Surface SEM micrographs for TC4 sheet (A), TC4/DA sheet (B), and STS (C-D); EDS mapping (Cl) of STS (E); Element compositions of TC4 sheet, TC4/DA sheet, and STS (F). See the supporting information for additional detail.
3. Results and discussion

3.1. Synthesis of SNPs

Figure 1 shows the procedure for the synthesis of SNPs. In this procedure, PAA was used as polyanion, and PDDA was used as polycation. The procedure was designed to incorporate blood compatibility and antimicrobial property into the SNP. After the process, both carboxyl groups and quaternary ammonium salt were introduced on the SNP surfaces. Hence, both blood compatibility and antimicrobial property were achieved.

3.2. Characterization of the SNP and STS

3.2.1. Particle size distribution

The size distribution of SNPs was characterized by zeta potential measurement system, as shown in figure 2. The average size of SNP was in the range of 200–300 nm, as revealed by particle size analysis, indicating that the SNP could be used in the field of biomedical material due to the nanoscale and narrow size distribution [14]. The morphologies of different size SNP were investigated using SEM, and the results are shown in figure S1 (available online at stacks.iop.org/MRX/8/075402/mmedia).

3.2.2. ATR-FTIR Spectra

Figure 3(A) shows the ATR-FTIR spectrum for TC4 sheet, TC4/DA sheet, and STS. As shown in the figure, for the TC4 sheet, no peak is found. For the TC4/DA sheet, three new peaks at 1400, 1569, and 3340 cm$^{-1}$, which were the characteristic peaks of DA, were found [15]. It confirmed that the DA was successfully coated on TC4 surface. After coating of the surface with SNPs, the C=O stretching vibration peak at 1730 cm$^{-1}$ was clearly visible, indicating the quaternary ammonium salt in PDDA [16].

3.2.3. Morphology and elemental composition.

Both morphology and elemental composition were investigated using a scanning electron microscope with energy-dispersive X-ray spectroscopy, and the results are shown in figure 4. As shown in the figure, TC4 sheet exhibited even surface (figure 4(A)). After coating of the TC4 sheet with DA, a lot of nanoparticles aggregated and accumulated on the TC4 sheet surface (figure 4(B)), which confirming that the TC4 sheet was successfully coated with DA. For STS, it was observed that lots of nano-cubes with side length of 400 nm, which might be SNPs, adhered to the surface (figures 4(C) and (D)). In order to prove the existence of the SNPs, EDS mapping (CI) of the STS was employed, and the results demonstrated that the SNPs (purple highlights) were successfully immobilized on the TC4/DA sheet surface (figure 4(E)). In addition, EDS mapping of other elements (C, O, Al, V, and Ti) of STS are shown in figure S2. Combining the results of the morphology and elemental composition, it was deduced that the nano-cubes adhered to the surface were SNPs.

Further investigation on EDS profile of the specimen exhibited the main element compositions of TC4 sheet, TC4/DA sheet, and STS. As shown in figure 4(F), the TC4 sheet showed the existence of C, O, Al, V, and Ti, indicating that the TC4 sheet had some amount of carbon contamination [17]. After coating of the TC4 sheet with DA, the atomic ratio of C raised from 3.94% to 17.29%, and the atomic ratio of O raised from 6.58% to 10.95%. The increase atomic ratios of the C and O were ascribed to the immobilization of DA. For STS, a new
element Cl is observed, which also demonstrated that the SNPs were successfully immobilized on the TC4/DA sheet surface.

3.2.4. Hydrophilicity
The water contact angles of TC4 sheet, TC4/DA sheet, STS are shown in figure 5. The contact angle of the TC4 sheet was about 78°. Coating with DA improved the surface hydrophilicity, and the water contact angle reduced to 40.3°, owing to the introducing of hydrophilic amino and hydroxyl groups. Further immobilizing SNPs on the surface suggested that the surface hydrophilicity was determined by the synergistic effect of DA and SNPs. Although PAA and PDDA were water-soluble, the assembled SNPs were insoluble, which suggested that the hydrophilicity was decreased. Hence, the STS hydrophilic nature was reduced, and the water contact angle raised to 61.6°. Another possible explanation for the result might be the surface structure of STS. As is apparently revealed in figure 4C above, a large number of SNPs adhered on the STS surface and constructed a lotus leaf-like structure, which might endow the surface with lotus effect [18]. The results also helped to confirm that SNPs were successfully immobilized on the surface of TC4/DA sheet.

3.2.5. Stability
To investigate the mechanical stability of the STS surface, adhesive tape test was performed. The results are shown in figure S3, and the detailed analyses are presented in the Supporting Information.
Figure 8. MTT tetrazolium assays of HUVECs treated with different concentrations of SNP for 3 d. (Values are expressed as mean ± SD, n = 3.).

Figure 9. Amounts of E. coli and S. aureus cultured for 24 h on TC4 sheet, TC4/DA sheet, and STS. Results were expressed as the relative viability, defined as the percentage of viable bacterial cells on TC4/DA sheet and STS relative to TC4 sheet. (Values are expressed as means ± SD, n = 3.).

Figure 10. Microbial colony picture for E. coli and S. aureus cultured for 24 h on TC4 sheet, TC4/DA sheet, and STS.
3.3. Blood compatibility

3.3.1. Platelet adhesion
For blood-contacting biomaterials, the extent of platelet adhesion and aggregation on the materials surface play key roles in thrombus formation. The aggregated platelets may further activate coagulation factors and accelerate thrombosis, which lead to further blood coagulation [19]. In the present study, in vitro platelet adhesion test was used to evaluate the blood compatibility of TC4 sheet, TC4/DA sheet, and STS.

As shown in figure 6, few platelets adhered on TC4 sheet surface, and the adherent platelets were dispersive and expressed rounded morphology with no pseudopodia and deformation, indicating that the bare TC4 sheet had relatively good hemocompatibility. After coating of the TC4 sheet with DA, the number of the adhered platelets slightly decreased. And STS displayed similar platelet adhesion level to TC4/DA sheet. The amounts of the platelets adhering on the samples are shown in figure S4. The suppressed platelet adhesion was attributed to the electronegative functional groups in DA and SNP [20].

3.3.2. Hemolysis ratios
To obtain further information of the hemocompatibility of SNP, hemolysis test was applied and the results are shown in figure 7. It was observed that TC4 sheet exhibited a low hemolysis ratio. After coating with DA, the sheet showed much better blood cell compatibility. It was remarkable that although the addition of SNP appeared to exacerbate hemolysis, the hemolysis ratio remained lower than the ISO standard 5% [21].

To sum up, compared with TC4 sheet, no significant difference was observed in the values of platelet adhesion and hemolysis ratio for the STS, which suggested that the blood compatibility of biological materials would be not affected by the addition of SNPs.

3.4. Cytotoxicity
The cytotoxicity of SNP was examined by MTT assay. As shown in figure 8, the viability of the cells was not significantly decreased till the concentration of SNP was up to 5 μg ml⁻¹. The results indicated that SNP showed low toxicity to human umbilical vein endothelial cells (HUVECs), implying that the SNPs have potential as antibiotics for tissue engineering.

3.5. Antimicrobial property
To confirm the antibacterial activity of the prepared samples, the bacterial amounts on the TC4 sheet, TC4/DA sheet, and STS were detected via the conventional test of bactericidal activity by using E.coli (Gram-negative) and S.aureus (Gram-positive) as the representative microorganisms, respectively. As shown in figure 9, the TC4 sheet showed the highest number of the adhered bacteria. The relative bacterial viability of E.coli and S.aureus decreased to 49.4% and 46.4% on TC4/DA surface, respectively, and further to 2.2% and 1.3% on STS surface, respectively. The antibacterial activity of TC4/DA sheet was attributed to the terminal amino groups in DA.
Amino group plays a critical role in ionic interactions with bacteria wall constituents, which leads to the occurrence of a hydrolysis of the peptidoglycans in the microorganism wall, provoking the leakage of intracellular electrolytes, leading the microorganism to death [22, 23]. The antibacterial activity of STS sheet was due to the synergistic effect of the terminal amino groups in DA and quaternary ammonium moiety in PDDA [24, 25].

The numbers of bacterial colonies were further tested to evaluate the antibacterial activity of the prepared samples. As shown in figure 10, abundant colonies were found on the culture plates treated by TC4 sheet, which indicated that the TC4 sheet was not bactericidal. For TC4/DA sheet, it was noticed that the TC4/DA sheet showed slightly increased antimicrobial activities against both E. coli and S. aureus compared with the TC4 sheet. In a sharp contrast, the colony numbers were significantly decreased after contacting with STS. To sum up, the results of antimicrobial property confirmed that the STS had good effect in the inhibition of the growth for both Gram-negative and Gram-positive bacterium. Furthermore, the long-term antimicrobial property of SNPs was tested as well, and the results are shown in figure S5, suggesting that the immobilized SNPs had low risk of antibacterial ingredient leaching occurrence.

In order to test the ‘substrate-independent’ property, SNPs were immobilized on polystyrene, glass and Ti surfaces by using poly(ethylene imine) as immobilizing agent. Then the antibacterial activity of all of the samples were evaluated, and the results are shown in figure 11. All of the coated samples exhibit significant effect of antibiotic sterilization, which indicated that the SNPs showed substrate-independent adhesive affinity to various solid surfaces because of the formation of irreversible covalent bonds, and the valid groups of antibacterial property was well retained.

4. Conclusion

In this study, we successfully synthesized a kind of biocompatible antimicrobial SNPs for biological application through a self-assembly method of PAA and PDDA. The blood evaluation results and cytotoxicity assays demonstrated that the SNPs had good blood compatibility and cytocompatibility. Furthermore, the SNPs exhibited excellent long-term stability and antimicrobial property. The multiple functions of the SNPs were ascribed to the bioactivities of PAA and PDDA. The present study offered a facile strategy to design antimicrobial nanoparticles which show great application potentials in various biological fields.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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