Construction of Antibacterial N-Halamine Polymer Nanomaterials Capable of Bacterial Membrane Disruption for Efficient Anti-Infective Wound Therapy

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1. Introduction

Since micro-organisms can indulge in wanton persecution of skin and cause large-scale human microbial infections, millions or billions of people have suffered from skin tissue–related microbial infections.[1] Particularly, considerable patients feel a nagging sense of discomfort resulting not only from wound and skin tissue–related devastating form of trauma but also from the possible deterioration of micro-organism–originated microbial infections.[2] Depending on the wound type and associated requirements to the complete healing of wounds, the most suitable wound dressing needs to be selected, for which today, we are eager.[3]

For a rapid wound healing, an ideal wound dressing should be able to maintain a moist environment, act against microorganisms, allow for gaseous exchange, be nontoxic, nonallergenic and nonadherent, possess antimicrobial properties, and efficiently promote wound healing.[4]

To overcome the abuse of traditional antibiotics and solve the issue of antibiotic resistance, active antibacterial materials have unlocked a new chapter of wound dressing to prevent infection, control symptoms, and improve wound healing in clinical applications due to their inherent antimicrobial activity against a broad spectrum of bacteria.[5]

To prevent and control emerging infection-induced diseases, numerous researchers have been focusing on the development of highly effective antibacterial agents so that pathogens can be eliminated or neutralized before they become hazardous.[6,7] To date, many types of antibacterial agents, such as nanosilver,[8] zinc oxide,[9] chitosan,[10] cationic polymer,[11] and active halogen,[12] that could act as wound dressings to avoid infection-induced diseases have been recognized to be beneficial to the actual needs. Of the new antibacterial agents that effectively kill a wide spectrum of pathogens, halogen-based antibacterial agents are known to prevent lethal diseases from infectious pathogens and therefore have attracted significant research interest, as they can play an effective role in protecting human health and improving environmental hygiene.[13]

Being used predominantly as disinfectants, halogen-based antibacterial...
materials have been adopted throughout the world because of their cost-effectiveness and ability to rapidly kill the majority of micro-organisms. Most of the halogen-based antibacterial agents are composed of strong oxidants, such as salt of hypochlorite, povideone-iodine, and N-halamines. Among the available halogen-based biocides, N-halamines have become one of the most popular choices. Scientists and engineers have studied various N-halamines for different applications, including water disinfection, air purification, and textile products, and even some of these have been commercialized. Nevertheless, the effectiveness of N-halamines with respect to wound therapy has not been studied systematically until now.

After Berliner first discovered the success of N-halamines to water disinfection in 1931, N-halamines have been used globally as life-saving agents in the treatment of a broad spectrum of bacterial infectious diseases. In the early 1990s, Worley began to focus on the design and synthesis of N-halamine polymers capable of inactivating bacteria on contact. Then many research groups, especially Sun's group and Ren's group, developed various N-halamine polymers for wide and potent applications. Recently, N-halamine nanomaterials have gained increasing attention since they could offer higher antibacterial activity than their bulk counterparts. Definitely, N-halamines refer to inorganic or organic compounds in which halogens with “+1” oxidation state are bonded covalently onto nitrogen. Based on the unique antibacterial function of N-X covalent bonds, N-halamine polymers can provide antibacterial halogen atoms, resulting in the destruction of micro-organisms without the formation of multidrug-resistant superbugs, and have attracted intense research interests in their antibacterial function against pathogens owing to their advantages over other halogen-based biocides, such as long-term stability in an aqueous or dry surrounding, high durability, regenerability, lower corrosion than free halogens and sodium hypochlorite, good human and environmental safety, weakly toxic, and low cost. More recently, our comparison of antibacterial efficacy revealed that with powerful antibacterial function, N-halamine-based materials can exhibit their comparable antibacterial activities to other praised biocides, such as silver, zinc oxide, titania, quaternary ammonium/phosphonium salts, and chitosan, suggesting their great potential in biomedical applications, particularly in antibacterial wound dressing.

Unlike other halogen-based biocides, the N-halamines are more diverse in their molecular structures. Commonly, N-halamines are divided into three types: amine N-halamines, amide N-halamines, and imide N-halamines. Stability of three N-halamines toward the release of free halogen has been shown to decrease in the order: amine N-halamine > amide N-halamine > imide N-halamine, while antibacterial capability follows the reverse order. Among these three types, amide N-halamines are the most popular ones because they could afford both good antibacterial activity and high stability. On the basis of the structural characteristics, hydantoin-containing N-halamines belonging to a subcollection of amide N-halamines due to the presence of amide N-X bonds become attractive to many researchers. Owing to their N-X covalent bond, hydantoin-containing N-halamines could construct a good balance easily between antibacterial activity and stability, thus presenting a great deal of interest for use in various applications.

In this work, we designed and employed new antibacterial N-halamine polymer nanomaterials with capability of bacterial membrane disruption for anti-infective wound therapy. By manipulating the merits of artificial synthesis to arrange feasible functional groups and molecular chains in designed antibacterial polymer nanomaterials, we first demonstrated an innovative synthesis of amide N-halamine polymer nanomaterials for wound dressing via a radical copolymerization followed by chlorination, and enabled the unambiguous assessment for wound healing by antibacterial study and biological effect investigation.

2. Experimental Section

Materials: 5,5-Dimethylhydantoin (DMH), ethanol, and sodium hypochlorite (NaClO) were obtained from Tianjin Fengchuan Chemical Reagent Technologies Co., Ltd. MMA, potassium persulfate (KPS), 2,2′-azobisisobutyronitrile (AIBN), potassium hydroxide (KOH), dichloromethane, acetic acid, petroleum ether, potassium iodide, sulfuric acid, and sodium thiosulfate were purchased from Tianjin Beilian Fine chemicals development Co., Ltd. Starch and N,N-dimethyl formamide (DMF) were available from Tianjin Guangfu Fine Chemical Research Institute. Allyl bromide, methanol, acetone, glutaraldehyde, osmium tetroxide, and isomyl acetate were provided from Sinopharm Chemical Reagent Co., Ltd. Alkaline lead citrate was obtained from Shenyang Hengzhisheng Trading Co., Ltd.

Synthesis of ADMH: ADMH was synthesized using a modified Gabriel Reaction according to a previous report. Typically, 3.2 g of DMH was added into 12.5 mL of ultrapure water containing 1.4 g of KOH, followed by stirring the reaction mixture at 60 °C for 1 h. Then 2.2 mL of allyl bromide in 5 mL of methanol solution was added into the reaction system above, and the solution was continuously stirred at 60 °C for 2.5 h. After acidification with acetic acid to pH 7.0 and then dried with a rotary-evaporator at room temperature, the resulted solid was recrystallized from petroleum ether, yielding ADMH.

Synthesis of Poly(ADMH-co-MMA): Synthesis of poly(ADMH-co-MMA) was performed via a radical copolymerization method. Briefly, 2 g of ADMH and 0.314 g of potassium persulfate were dissolved in 60 mL of ultrapure water in a 250 mL three-neck flask with a condenser and an N2 gas inlet. After 0.595 mL of MMA was added drop-wise into the solution in the flask, the mixture was stirred at 75 °C for 12 h, leading to the formation of poly(ADMH-co-MMA). Corresponding to the optimal reaction condition in optimization (i.e., initiator type, feed ratio of two monomers, mass fractions of KPS, reaction temperatures, polymerization times, etc.), the poly(ADMH-co-MMA) material was prepared in the synthesis.

Synthesis of N-Halamine Polymer Nanomaterials: N-halamine polymer nanomaterials were obtained facilely through a chlorination reaction. Typically, 0.5 g of poly(ADMH-co-MMA) was immersed in a 70 mL of 10 wt% sodium hypochlorite solution and stirred for 12 h at room temperature. The as-prepared N-halamine polymer nanomaterials were centrifuged and...
washed thoroughly using ultrapure water, and then dried at 40 °C for 6 h to remove residual free chlorines from the sample surface. The active chlorine content (ACC) of the N-halamine polymer nanomaterials was controlled by tuning the aging time of chlorination.

**Structural Characterization:** FTIR spectra were obtained on a Thermo Nicolet (Woburn, MA) Avatar 370 FTIR spectrometer. 1H NMR spectra were recorded on a Bruker AVANCEIII-500 NMR spectrometer with DMSO as a solvent. X-ray photoelectron spectra (XPS) was carried out on a PHI-5000CESCA system with Mg Kα radiation. TEM images were taken on a Hitachi H-8100 transmission electron microscope at 200 kV after the sample was prepared by drying a drop of the sample suspension onto carbon-coated copper grid. Size distribution was measured using a ZetaPlus Zeta Potential Analyzer (ZZPA). Energy-dispersive X-ray (EDX) spectra and elemental mapping were measured on a Hitachi S-4800 field emission scanning electron microscope. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were performed using a Perkin-Elmer thermogravimetric analyzer. Number-average molecular weights (Mn) and polydispersity indices (PDI) of polymer sample were determined by gel permeation chromatography (GPC) using a Waters 1515 gel permeation chromatograph with DMF as an eluent.

**Determination of Available Chlorine Content:** Available chlorine content (ACC) of N-halamine polymer nanomaterials was determined by an iodometric/thiosulfate titration. The percentage of active chlorine (Cl⁺ %) for sample was calculated according to the following equation.

\[
\text{Cl}^+\% = \left( \frac{0.03545 \times C \times V}{2 \times W} \right) \times 100\%
\]  

where C and V are the concentration (mol L⁻¹) and volume (L) of Na₂S₂O₃ consumed in the titration, respectively, and W is the weight of sample (g).

**Stability Measurement of Available Chlorine:** In open and close environments, the stabilities of available chlorine in N-halamine polymer nanomaterials were evaluated separately by using the iodometric/thiosulfate titration method. In an open environment, N-halamine polymer nanomaterials were stored for 7 days at 37 °C, and ACC were measured every 1 h. In a close system, N-halamine polymer nanomaterials were stored at 37 °C for 15 days, and ACC was continuously measured every 1 day.
**Bacterial Culture:** *Staphylococcus aureus* (Gram-positive bacteria) and *Escherichia coli* (Gram-negative bacteria) were used as two model micro-organisms to check antibacterial activities of sample. Bacteria were grown overnight at 37 °C under agitation (220 rpm) in Luria-Bertani growth medium (LB). Cells were harvested by centrifugation, washed twice with normal saline, and then diluted to concentrations of 10^5 colony-forming units mL^{-1} (CFU·mL^{-1}).

**Plate Counting Method:** Plate counting method was carried out according to the literature.[41] Typically, bacteria were grown overnight at 37 °C in Luria-Bertani growth medium (LB) and the bacterial cells were harvested by centrifugation, washed with normal saline, and diluted to concentrations of 10^4 CFU·mL^{-1}. N-halamine polymer nanomaterials with Cl% of 0.966 wt% were dispersed in 1 mL of sterilized distilled water, and then sonicated for 5 min to obtain sample suspension. When an antibacterial test was run, 100 μL of bacteria suspension and 900 μL of sample suspension were well mixed, and then 1 mL of intermixture was moved out and dispersed onto LB agar plates. Colonies on the plates were counted after an incubation procedure at 37 °C for 24 h.

**Fabrication of Wound Dressing:** One square cotton fabric on the negative pressure suction filter was fixed according to the previous literature.[42] The suspension of N-halamine polymer nanomaterials (1.0 mg·mL^{-1} and Cl% of 0.966 wt%) was added into a metal cylinder until the suspension was completely absorbed by cotton fabric. Then, the square fabric disc was taken out and vacuum-dried at 60 °C. Finally, the N-halamine polymer nanomaterial-loaded cotton was fixed on a dressing purchased from drug store to form the N-halamine polymer nanomaterial-based wound dressing.

**Mouse Wound Model:** Mouse wound model was built based on the report.[43] To evaluate the anti-infective property of N-halamine polymer nanomaterial-based antibacterial system in vivo, a wound model was fabricated. The three groups of 12 male Kunming mice with about 4 mm^2 wound were divided into the control, poly(ADMH-co-MMA), and N-halamine polymer nanomaterial group. *S. aureus* (100 mL, 10^8 CFU·mL^{-1}) suspension was placed on the wound and then the different dressings were placed on the wound site, respectively. Photos were taken of the wounds of the mice in three different groups, while wound dressings were changed at 24 h interval. After 3 days, when all mice were sacrificed, the tissues of wounds were harvested and the number of bacteria was quantified. To determine the amount of bacteria in the tissues, the wound in three groups of mice were separated and homogenized in sterile PBS. Aliquots of diluted homogenized intestinal tissues were placed on agar, on which the grown colonies were counted for analysis. All animal procedures were in accord with the guidelines of the Institutional Animal Care and Use Committee.

**SEM Characterization in Bacterial Measurements:** For sample preparation, *E. coli* was cultured to an exponential phase, harvested by centrifugation, washed with PBS, and re-suspended. Cells (10^8 CFU·mL^{-1}) were incubated at 37 °C for 180 min and 60 mg of N-halamine polymer nanomaterials were dispersed in 10 mL of sterilized distilled water. Controls were run without N-halamine polymer nanomaterials. After incubation, the cells were mixed and centrifuged at 5050 × g for 7 min. Bacterial pellets were washed with PBS, and then centrifuged. Continuously, fixation of bacterial cells was performed with 2.5% w/v glutaraldehyde at 4 °C overnight, followed by washing with PBS. Cells were harvested and post-fixed with 1% osmium tetroxide for 1 h, and then they were dehydrated for 15 min in each of a graded ethanol series (50%, 70%, 80%, 90%, 95%, and 100%). After that, they were transferred to absolute acetone for 15 min, a mixture (ν/ν = 1:1) of alcohol and iso-amyl acetate for 30 min, and pure iso-amyl acetate for 1 h. Finally, specimens were dehydrated in a Hitachi Model HCP-2 critical point dryer with liquid CO2. The dehydrated specimens were coated with gold-palladium and observed in a Hitachi S-4800 field emission scanning electron microscope.

**TEM Observation in Bacterial Measurements:** In TEM (Model JEM-1230, JEOL, Japan) observation, pretreatment of bacteria samples was conducted in the same way as the treatment of SEM samples in bacterial measurements. After the treatment with a series of ethanol solutions (50%, 70%, 80%, 90%, 95%, and 100%) for 15 min each, bacterial cells were transferred to absolute acetone for 15 min. Their specimens were then put into 2:1 and 1:2 mixtures of absolute acetone and resin overnight. Finally, the specimens were placed in different capsules containing embedding medium, heated at 70 °C for about 9 h, and stained by uranyl acetate and alkaline lead citrate for 15 min.

**Confocal Laser Scanning Microscopy Study:** *E. coli* was conducted in the same way as that in the preparation of SEM samples in bacterial measurements. Bacteria were stained according to the requirements in Live/Dead Baclight Bacterial Viability Kits (L13152).[44] Briefly, SYTO 9 (2.5 mL) and propidium iodide (PI, 2.5 mL) were mixed and then added into bacterial solution with equal volume. After the mixture was incubated at room temperature for 15 min in dark, the stained bacterial suspension (5 μL) was trapped between a glass slide and cover slide. The excitation/emission wavelengths of SYTO 9 and PI are 480/500 nm and 490/635 nm, respectively. The confocal image was obtained by Zeiss 510 Meta laser scanning confocal microscope with 20× magnification. The green (SYTO 9) was obtained by 488 nm Ar excitation laser and BP 505–530, and the red (PI) was obtained by 543 nm HeNe excitation laser and BP 560–615.

**Release of Nucleic Acid:** Bacterial suspension (10 mL) with a concentration of 10^8 CFU·mL^{-1} was centrifuged, washed at three times with 0.85% saline, and then dispersed in 0.85% saline to obtain a bacterial concentration of 10^8 CFU·mL^{-1}. A solution of N-halamine polymer nanomaterials (6 mL) with a concentration of 6 mg mL^{-1} was added into the above bacterial suspension. After contacting for different times (5, 15, 30, 60, 120, and 180 min), 24 mL of 0.03 wt% sodium thiosulfate aqueous solution was added into the above mixture to neutralize the active chlorine and stop the antibacterial action of N-halamine polymer nanomaterials. After filtration of the above mixture with 0.22 μm microporous membrane, a UV-visible spectrophotometer was used to measure the optical density (OD_{560}) value of the filtrate at 260 nm.

**Detection of DNA Damage:** A DNA cleavage experiment was performed according to the literature related to an agarose gel electrophoresis.[45] Nutrient broth (peptone 10 g·L^{-1}, yeast extract 5 g·L^{-1}, NaCl 10 g·L^{-1}) was used as the media. Agarose (0.25 g) was dissolved in 25 mL of TAE buffer (4.84 g Tris base, pH 8.0, 0.5 M EDTA/1 L) by boiling. When the temperature was increased to 55 °C, the gel was poured into the gel.
cassette fitted with comb, and then was left for solidification. After the comb was carefully removed, the gel was placed in the electrophoresis chamber flooded with TAE buffer. DNA sample (20 µL) was loaded (mixed with bromophenol blue dye at the ratio of 1:1) carefully into the wells, along with standard DNA marker and a constant 60 V of electricity was passed for around 50 min. The gel was removed and carefully stained with ETBR solution (1 µg·L−1) for 10 min and the bands were observed under UV trans-illuminator. The results were then compared with standard DNA marker.

**Determination of Intracellular Ion Leakage:** Before the determination of intracellular ion leakage, a pre-treatment process was done following the pre-treatment procedure for the release of nucleic acid above. After filtration of *E. coli* suspension that treated with *N*-halamine polymer nanomaterials, atomic absorption spectrometry (AAS) was used to determine metal elements (i.e., K+, Mg2+, and Ca2+) of bacteria to estimate intracellular ion leakage.

**Statistical Analysis:** All data measurements and analyses are average ± standard deviation. For all comparisons, *p* of 0.05 was considered to be statistically significant.

### 3. Results and Discussion

#### 3.1. Preparation and Characterization of *N*-Halamine Polymer Nanomaterials

In our synthetic strategy with a step-by-step optimization, the self-prepared 3-allyl-5,5-dimethylhydantoin (ADMH) (Figures S1 and S2, Supporting Information) in a high yield (∼95%) offers a double bond easy for copolymerization, which can be chemically converted to form amide groups in the final fabrication of amide *N*-halamine polymer nanomaterials with both an effective antibacterial activity and a good structural stability under some circumstances (e.g., water, light, and heat). Based on experimental and theoretical studies of *N*-halamines,[46–52] the structural stability decreases in this order: amine *N*-halamines > amide *N*-halamines > imide *N*-halamines, while the antibacterial capability follows the reverse order: amine *N*-halamines > amide *N*-halamines > imide *N*-halamines. Upon using potassium persulfate and ultrapure water as initiator and solvent, respectively, methyl methacrylate (MMA) could react with ADMH at 75 °C for 12 h to give poly(3-allyl-5,5-dimethylhydantoin-co-methyl methacrylate) (poly(ADMH-co-MMA) (Figures S1 and S2, Supporting Information). Acting as the other monomer, MMA cannot only provide ester groups to stabilize the radicals and thereby promote the reactivity of allyl groups of ADMH toward chain propagation reaction in radical copolymerization,[29,53,54] but also tune the length of polymer chain, controlling the antibacterial efficiency of the resulting *N*–*X* bonds to some extent. As chlorination finally led to the formation of amide *N*-halamine polymer nanomaterials with antibacterial *N*–Cl covalent bonds, the structural change in the synthesis, coupled with a special transition of *N*–H to *N*–Cl, has been monitored and evidenced by Fourier transform infrared (FTIR) spectroscopy (Figure S1, Supporting Information), 1H nuclear magnetic resonance (1H NMR) spectroscopy (Figure S2, Supporting Information), X-ray photoelectron spectra (XPS, **Figure 2a**), scanning electron microscopy (SEM,

![Figure 2. a) XPS survey spectrum, b) SEM image and TEM image, and c) size distribution of *N*-halamine polymer nanomaterials. d) Number average molecular weights (*Mn*) and polydispersity index (PDI) in *N*-halamine polymer nanomaterials as function of the ADMH molar content (F<sub>ADMH %</sub>).](image-url)
Figure 2b), transmission electron microscopy (TEM, the insert in Figure 2b), energy-dispersive X-ray spectra (EDX, Figure S3, Supporting Information), and thermogravimetric analysis (TGA, Figure S4, Supporting Information). As can be seen from XPS spectrum (Figure 2a), N-halamine polymer nanomaterials exhibit a Cl 2p peak, indicating the incorporation of Cl and thereby the formation of N–Cl bond after chlorination in the synthesis. The N-halamine polymer nanomaterials show a quasi-sphere shape with an out-of-flatness surface (Figure 2b), displaying an average size of 438.2 nm (Figure 2c). Basically, it can be demonstrated that the as-prepared N-halamine polymer that exists in a nanoparticle form has a monodispersity in its nanoscale size.

Generally, it has been recognized that, for the synthesized N-halamine polymer nanomaterials, the higher the ADMH content is, the stronger the antibacterial activity should be. However, owing to the limitation of molecular weight related to molecular chain, the high-molecular-weight N-halamine polymer nanomaterials suffer from the steric-hindrance effect that decreases its permeation through the bacterial cell for antibacterial action even though there are many N–Cl groups. After the molecular weight exceeds a limited value, the relationship between the ADMH content and molecular weight will determine the antibacterial activity of the synthesized N–Cl bonds in the N-halamine polymer nanomaterials. In our optimized test (Figure 2d), the F_{ADMH} % was well tuned to control the number average molecular weights of N-halamine polymer nanomaterials in a range of 1 × 10^2 to 1 × 10^5 Da.

Strategies to obtain a high proportion of antibacterial N–Cl groups in final N-halamine polymer nanomaterials rely on the controlled molar percentage of N–H groups in poly(ADMH-co-MMA) while the N–H groups are only located in the monomer unit of ADMH. We became interested in the methods of retaining enough N–H groups in ADMH of poly(ADMH-co-MMA) to make the N–Cl groups efficient for antibacterial function. In a step-by-step optimization of synthesis conditions, i) the first comparison of two initiators (Table S1, Supporting Information) showed that potassium persulfate (KPS) as an initiator leads to a higher molar percentage of ADMH unit in poly(ADMH-co-MMA) and correspondingly a high content of N–H groups as compared with 2,2'-azobisobutyronitrile (AIBN). ii) When different reaction times (0, 2, 6, 12, 24, and 36 h) were chosen on a co-polymerization of ADMH and MMA, we found that, based on the combination of elemental analysis and NMR (Table S2, Supporting Information), the reaction time of 12 h can result in the maximum molar percentage of ADMH unit. iii) Although an increasing feeding ratio of ADMH to MMA (Table S3, Supporting Information) can favor the proportion of ADMH in the copolymer, we are aware of some trouble in the purification of copolymer product and thus demonstrate 2:1 as an optimal feeding ratio. iv) The evaluation (Table S4, Supporting Information) of the used KPS content reveals that 12 wt% was the optimal mass percentage of KPS in the total mass of two monomer units, corresponding to the maximum molar proportion of N–H group in the copolymer product. v) Finally, the analysis of reaction temperature (Table S5, Supporting Information) indicates that, compared to other temperatures (i.e., 55, 65, 85, and 95 °C), 75 °C is the best temperature associated with the highest molar percentage of ADMH.

Released from N–Cl covalent bonds, the available chlorine in the N-halamine polymer nanomaterials has been evaluated using an iodometric titration method. In a qualitative test (Figure S5a, Supporting Information), the N–Cl bonds in the initial N-halamine polymer nanomaterials can react with the added potassium iodide, resulting in the formation of iodine. After the addition of sodium thiosulfate, the N–Cl can be finally regenerated. In contrast, a quantitative analysis directly showed that based on an optimized condition, the synthesized N-halamine polymer nanomaterials can deliver the maximum content (≈0.966 wt%) of chlorine (Figure S5b, Supporting Information), corresponding to its strongest antibacterial function. As compared to commercial antibacterial NaClO, the available chlorine of the N-halamine polymer nanomaterials presents a better stability (Figure S6, Supporting Information), indicating that the antibacterial action of the N-halamine polymer nanomaterials is very stable under an open or close environment.

3.2. In Vitro Antibacterial Evaluation of N-Halamine Polymer Nanomaterials

Bactericidal ability of the N-halamine polymer nanomaterials was evaluated via a plate counting method with E. coli and S. aureus as two models with a bacterial concentration of 10^9 CFU·mL⁻¹, and it was found that similar to the control, the poly(ADMH-co-MMA) presents dense bacterial dots, suggesting that the poly(ADMH-co-MMA) polymer has no antibacterial function (Figure 3a). However, the 1.0 mg mL⁻¹ of N-halamine polymer nanomaterials almost offers no survival of bacterial dots on the control plate, which corresponds to the time needed to kill 10^9 CFU·mL⁻¹ of two bacteria owing to the formation of the antibacterial N–Cl bonds that were originated from the N–H bonds of the poly(ADMH-co-MMA). Even bacteria in a suspension with a concentration as high as 10^8 CFU·mL⁻¹ could also be completely killed after 3 h exposure to N-halamine polymer nanomaterials (Figure 3b). As a function of contact time, the antibacterial kinetic assay (Figure 3c and Figure S7, Supporting Information) reveals that in a period of 60 min, the survival percentages of both E. coli and S. aureus bacteria significantly decrease with increasing contact time, which can be associated with the gradual release of Cl from N–Cl bonds in the N-halamine polymer nanomaterials and thereby incremental antibacterial property. After 60 min, the survival percentages of the two bacteria are 0% and 12.38%, indicating a stronger and faster antibacterial activity of the N-halamine polymer nanomaterials on E. coli than that on S. aureus. Interestingly, when the contact time was fixed at 15 min, the increasing concentration of the N-halamine polymer nanomaterials led to an improved amount of N–Cl groups and thus enhanced antibacterial efficiency (Figure 3d and Figure S8, Supporting Information).

3.3. In Vivo Wound Therapy by N-Halamine Polymer Nanomaterials

In order to evaluate wound therapy ability of the N-halamine polymer nanomaterials in wounds of normal mice under in
vivo conditions, the rate of wound healing was monitored when the treatment was conducted by using poly(ADMH-co-MMA) and N-halamine polymer nanomaterials for comparison. As seen in Figure 4a,b, when the poly(ADMH-co-MMA) polymer has no significant effects on the reduction of wound size after day 1, day 2, and day 3, the N-halamine polymer nanomaterials can reduce the wound size by 39.74 ± 1.42%, 48.78 ± 6.95%, and 55.09 ± 1.56%, which is better than the control group (15.95 ± 9.46%, 25.3 ± 2.25%, and 34.57 ± 15.85%), respectively. This demonstrates an improved progress of wound therapy for the N-halamine polymer nanomaterials from day 3 onward, confirming that the bacterial activity is importantly ascribed to the N–Cl bonds rather than the N–H bonds. According to a histological investigation of wound specimens at day 3 (Figure 4c), obvious differences support the finding that with N–Cl bonds, the N-halamine polymer nanomaterials promote the wound healing while the poly(ADMH-co-MMA) polymer materials suppress the wound healing. For the control wounds (Figure 4c), an apparent edema can be clearly observed between the skin and the muscles, followed by diffuse hemorrhage and diffuse neutrophilic exudate and infiltration in which neutrophils perform cellular immune function to resist the attack of bacteria. This suggests the appearance of purulent bacteria in infection areas of the control wounds. For the poly(ADMH-co-MMA) (Figure 4c), a serious festering focal, including red stained necrotic granulation tissues in its center, exists between the skin and the muscles, resulting in the accumulation of neutrophils, probably because the disintegration of neutrophils released various lysosomes in which enzyme promoted the dissolution of surrounding tissue and formed the abscesses. This indicates the presence of considerable bacterial infections in the wounds, evidencing an inactive effect of poly(ADMH-co-MMA) on wound healing. Compared to the wounds treated with poly(ADMH-co-MMA), the wounds (Figure 4c) treated with the N-halamine polymer nanomaterials present the regeneration of epidermal cells with mature differentiation, along with a great amount of fibroblasts, granulation tissue hyperplasia, and compact collagen deposition. Moreover, few neutrophils are located in the intercellular space, demonstrating that due to unique antibacterial function of N–Cl, bacterial infections decline to small amount at day 3. These above mentioned results are well in accord with the analysis of residual bacteria from three different wound samples (Figure S9, Supporting Information). Therefore, the N-halamine polymer nanomaterials exhibit the best wound therapy performance, which can act as ideal candidate for wound dressing in clinical skin repair by reducing the pain and shortening their hospital stay.

3.4. Proposed Mechanism of N-Halamine Polymer Nanomaterials for Wound Therapy

To theoretically understand antibacterial action of the N-halamine polymer nanomaterials used as wound dressing, a...
proposed four-stage mechanism in the process of wound therapy can be illustrated in Figure 5a. Firstly, skin starts to lose its capacity of self-wound healing, when micro-organisms invade and form a lot of robust colonies, causing a serious wound infection.\[56\] Serving as an antimicrobial coating material, the N-halamine polymer nanomaterial-based wound dressing drives its antimicrobial function to prevent the damaged skin from a further infection. Unlike small molecule antibacterial agents that are used in traditional dressing to attack bacteria by releasing themselves into the wound system,\[57\] the coated N-halamine polymer nanomaterials produce their active chlorines bound at N\(\text{Cl}\) bonds, performing their antibacterial function. As bacteria are covered with a lipopolysaccharide layer, constituting its cytomembrane, and thereby carry negative charge, more and more positive charged chlorine (Cl\(^+\)) bound in the N\(\text{Cl}\) bond can move to arrest bacteria by an electrostatic interaction.\[58\] This behavior seems like a “trapping action” in which the bacteria tend to move automatically to the N-halamine polymer nanomaterials, evidencing a significant antibacterial ability of the N-halamine polymer nanomaterial-based dressing over other microbicides. Generally, only in a short period when the N-halamine polymer nanomaterial-based wound dressing is dressed on infected wounds, bacteria can stay at safety until the trapping action starts. Secondly, once N–Cl bonds are contacted with bacteria, chlorines can be released from the N–Cl bonds, enter the cell’s biological receptors, and perform their oxidative function in which an ion exchange reaction (i.e., N–Cl + Bacterium + H\(^+\) → N–H + Bacterium–Cl\(^-\)) occurs between the contacted N–Cl bond and bacterium, resulting in the exchanged Cl\(^-\) to stay on the surface of bacteria.\[59\] Interestingly, after the exchange reaction, these N–H bonds that were formed after treated to bacteria could be re-chlorinated to N–Cl bonds by the inner chlorines in the N-halamine polymer nanomaterials which can move out to be coordinated with the outer nitrogen sites in the N–Cl bonds releasing Cl\(^+\).\[60\] This suggests that in this stage of exchange reaction, the N-halamine polymer nanomaterials can provide active N–Cl bonds enough to react with bacteria by the exchange reaction. In this way, more and more bacteria start to take part in the exchange reaction and contact the antibacterial N–Cl bonds of the N-halamine polymer nanomaterials. Thirdly, as the ion exchange reaction spreads out over the bacteria suffering from the trapping action, the exchange reaction is immediately evolved into an incredible transfer reaction (Bacterium\(_1\)–Cl\(^-\) + Bacterium\(_2\) → Bacterium\(_1\) + Bacterium\(_2\)–Cl\(^-\)) between an exchanged bacterium and the other bacterium. Furthermore, this transfer reaction can also spread in the group of bacteria, leading to the transfer of Cl\(^-\) from one bacterium to
another one, until all bacteria were infected by Cl\(^{+}\). Finally, with their powerful oxidative activity, the Cl\(^{+}\) ions staying on the surface of bacteria look like sharp “mini-blades” to cut and destroy the cell membrane of each bacterium so that much intracellular content was spitted out endlessly in the death of bacteria.\(^{[61]}\)

As evidenced in several biological and chemical tests, the “mini-blades” derived from N–Cl bonds of the N-halamine polymer nanomaterials significantly cut and destroy the cell membranes of bacteria (Figure 5b–f and Figure 6), making a subsequent release of some stuffs (e.g., DNA, ions, Figure 7).

Figure 5. a) A proposed four-step mechanism illustration of N-halamine polymer nanomaterial-based dressing for wound healing. b–f) Morphology of E. coli exposed to N-halamine polymer nanomaterials. TEM images illustrating E. coli undergoing changes in morphology after incubation with 1.0 mg·mL\(^{-1}\) of N-halamine polymer nanomaterials a 37 °C for 1 h. The untreated E. coli (Figure 4a) displays a rod shape and membrane integrity, while different stages of destruction (red arrow) can be seen from Figure 4c–f after E. coli was exposed to N-halamine polymer nanomaterials.

Figure 6. Morphologies of E. coli before and after treated with the N-halamine polymer nanomaterials. a1) SEM images and b1) TEM images of normal E. coli before treated with the N-halamine polymer nanomaterials. a2) SEM images and b2) TEM images of E. coli after treated with the N-halamine polymer nanomaterials. c1–c7) Epifluorescence images and c8) percentages of live (green) and dead (red) E. coli after a treatment with 6 mg·mL\(^{-1}\) of the N-halamine polymer nanomaterials for different times. (c1) 0 min, (c2) 5 min, (c3) 15 min, (c4) 30 min, (c5) 60 min, (c6) 120 min, and (c7) 180 min.
Before the treatment with the N-halamine polymer nanomaterials, *E. coli* cells (Figure 6a1,b1) show a normal rod morphology with smooth cell surfaces. After the treatment with the N-halamine polymer nanomaterials (1 mg·mL⁻¹), *E. coli* cells (Figure 6a2,b2) lose their cellular integrity, in which a few distinct concaves can be observed on the cell membrane, along with the collapsed membrane and the released cytoplasm.

As a result, these membranes missed their cytoplasm, which are severely damaged and are becoming even. Fluorescence images (Figure 6c1–c7) of the *E. coli* treated with N-halamine polymer nanomaterials revealed that representing dead bacteria, red spots increase with increasing contact time from 5 to 180 min, as confirmed in the statistics (Figure 6c8). This suggests that as a biological study, the permeability of the treated...
bacteria increases with treating period, resulting in spitting out of intracellular contents from the treated bacteria (as schematically illustrated in Figure 7a). In an increasing treating period (Figure 7b), the leakages of nucleic acid from both E. coli and S. aureus significantly rise up, implying that both two treated bacterial strains should suffer the damage of membrane, becoming more and more serious almost with the increasing treating time. This result hints a biological effect in which the N-halamine polymer nanomaterials might destroy the cell membrane to kill bacteria, as a result causing the leakage of the intracellular contents, for example, DNA. So the possibility of DNA cleavage induced by direct or indirect contacting between the N-halamine polymer nanomaterials and DNA caught our attentions. Clearly, with the increase of treating period from 5 to 180 min, similar patterns of migration are observed in Figure 7c, suggesting that the N-halamine polymer nanomaterials have no effect on DNA cleavage. We think that the inactivation of N-halamine polymer nanomaterials meeting DNA might result from the exhaustion of all N-halamine polymer nanomaterials in bacterial outer structures (e.g., cell wall and membrane). To figure out whether the N-halamine polymer nanomaterials can really affect DNA at their direct contact with each other, we directly extracted DNA from E. coli, and then treated it with a 6 mg·mL\(^{-1}\) of N-halamine polymer nanomaterials in a period from 5 to 180 min. Astonishingly, no significant DNA damage is found in Figure 7d, demonstrating that in a fixed sample concentration of 6 mg·mL\(^{-1}\), the N-halamine polymer nanomaterials did not do any damage on E. coli. It was inferred that the effect of N-halamine polymer nanomaterials on DNA is not quite palpable in the present experimental conditions. Continuously, we designed another experiment (Figure 7e) in which the extracted DNA was treated by the N-halamine polymer nanomaterials with different concentrations (from 6 to 42 mg·mL\(^{-1}\)) in a fixed contact period of 180 min. In accord with the results in two experiments above, no change was detected for the patterns of migration with the increasing concentration of the N-halamine polymer nanomaterials in the extended period. So it was demonstrated that the N-halamine polymer nanomaterials cannot take any effect on the DNA cleavage. After the researches on both the damage of cellular structure and the release of DNA, we continue to study whether the damage of cell structure could result in the release of metal ions from cells, such as K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) in the field of biological effect of the N-halamine polymer nanomaterials, since the ions can leak out of bacterial cell and lead to the eventual death of bacteria. After testing the ion concentration in a period from 5 to 180 min, it can be found that in both bacterial suspension (E. coli and S. aureus) treated with N-halamine polymer nanomaterials, K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) were released more and more seriously with increasing contact time, as evidenced in the increasing ion content in Figure 7f,g. So we conclude that the N-halamine polymer nanomaterials definitely destroy the bacterial cyto-structure and results in the release of intracellular contents, including metal ions and DNA, so that the bacteria go to death finally.

Definitely, these tests revealed that bacterial cells spit out their intracellular content and lose their cellular integrity (even some of bacterial cells missed their cytoplasm entirely), after their membranes are severely damaged. Since the treatment by the N-halamine polymer nanomaterials, the death of bacteria cannot only benefit skin tissue regeneration but also accelerate the rate of healing and the aesthetic repair of wounds. It should be noted that the exact timing of each stage cannot be determined easily because of the resolution limit and the diversity within each individual cell. However, these rough but representative stages help us understand, to some degree, the dynamic process at the cellular level for this N-halamine polymer nanomaterial-induced antibacterial action in the process of wound therapy.

4. Conclusion

In summary, we have demonstrated that, for the N-halamine polymer nanomaterial-based wound dressing, its antibacterial function against bacteria, coupled with the behavior of wound therapy, is related closely to the diversity within each individual bacterial cell. Nevertheless, the proposed four-step mechanism significantly unlocked a detailed antibacterial action and healing process to the use of the N-halamine polymer nanomaterials as wound dressing in a biological and chemical surrounding. All the dynamic process at the cellular level for the N-halamine polymer nanomaterials importantly induces and activates the process of wound therapy after bacterial infection at wound sites of skin. Our findings suggest that depending on the unique antibacterial function of N–Cl bonds, the amide N-halamine polymer nanomaterial-based wound dressing has addressed a good wound therapy efficiency in skin tissue–related bacterial infections, which could be developed for the potential treatment of pathogen infections, demonstrating the successful synthesis of N-halamine polymer nanomaterials and their unambiguous ability in medical treatment and biological effect.

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.

Keywords

antibacterial, antibacterial dressing, bacterial membrane disruption, N-halamine polymer nanomaterials, wound therapy

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