Acta Biomaterialia xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

A nanovehicle developed for treating deep-seated bacteria via using low-dose X-ray

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ARTICLE INFO

Article history:
Received 23 May 2016
Received in revised form 6 September 2016
Accepted 2 October 2016
Available online xxxx

Keywords:
X-ray
Anti-bacterial
Graphene oxide quantum dots
Vancomycin
Protoporphyrin IX

ABSTRACT

Many non-antibiotic strategies, such as photocatalysis and photodynamic therapy, have been proposed to inhibit and/or kill bacteria. However, these approaches still have drawbacks such as insufficient bacterial specificity and the limited penetration depth of ultraviolet and near-infrared light. To overcome these limitations, we developed a bacteria-specific anti-bacterial technique via using low-dose X-ray. Graphene oxide quantum dots (GQDs, a multifunctional vehicle) conjugated with vancomycin (Van, a bacteria-targeting ligand) were assembled with Protoporphyrin IX (PpIX, a photo/radiation sensitizer) to yield a novel Van-GQDs/PpIX complex that specifically attached to *Escherichia coli* and efficiently generated intracellular reactive oxygen species following X-ray activation. Delivery using GQDs increased the PpIX/Van ratio in the target bacterial cell, damaged bacterial cell wall, and enhanced X-ray-induced PpIX activation. Hence, this approach allowed for the use of a low-dose X-ray to efficiently activate the Van-GQDs/PpIX complex to exert its bactericidal effects on *Escherichia coli* without damaging normal cells. Furthermore, the *E. coli* did not develop resistance to the proposed approach for at least 7 rounds of repeated administration during one week. Thus, this proposed vehicle exhibiting bacteria-specific X-ray-triggered toxicity is a promising alternative to antibiotics for treating serious bacterial infections occurring in deep-seated tissues/organs (e.g., osteomyelitis and peritonitis).

Statements of Significance

Administration of antibiotics is the most common treatment modality for bacterial infections. However, in some cases, patient attributes such as age, health, tolerance to antibiotics do not allow for the use of high-dose antibiotics. In addition, some bacteria develop resistance to antibiotics because of improper and long-term use of these agents. Therefore, non-antibiotic strategies to treat deeply situated bacterial infections, such as osteomyelitis, are urgently needed for avoiding amputation. To date, several non-antibiotic approaches, such as Ag nanoparticles, graphene-based materials, photocatalysis, and photodynamic therapy have been proposed to inhibit and/or kill bacteria. However, the major challenges of photochemical strategies, specificity and limited penetration depth of light source, still remain for treating the deep-seated bacteria. To overcome these problems, we developed a novel nanovehicle that exerted toxic effects specifically on bacteria following activation by a deeply penetrative low-dose X-ray, without damaging normal cells. As such, it realizes a deeply photochemical route for treating the deep-seated bacteria.

1. Introduction

Deeply bacterial infections, such as pyelonephritis, osteomyelitis and peritonitis, are common in hospitals. Without proper management, these infections might spread to the blood, leading to the life-threatening condition, septicemia [1–3]. Untreated septicemia

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http://dx.doi.org/10.1016/j.actbio.2016.10.003
1742-7061/

would result in the generation of lipopolysaccharides and quickly progress to sepsis, which can cause organ failure and even death in some cases. Therefore, inhibition of bacterial growth in deep-seated organs and/or blood is an important consideration in the field of medicine [4,5].

Currently, administration of antibiotics is the most common treatment modality for bacterial infections [6–8]. However, in some cases, patient attributes such as age, health, tolerance to antibiotics do not allow for the use of high-dose antibiotics [9]. In addition, some bacteria develop resistance to antibiotics because of improper and long-term use of these agents [10]. Therefore, non-antibiotic strategies to treat deeply situated bacterial infections, such as osteomyelitis and peritonitis, are urgently needed [11,12]. To date, several non-antibiotic approaches, such as Ag nanoparticles [13], graphene-based materials [14], photocatalysis [15], and photodynamic therapy [16] have been proposed to inhibit and/or kill bacteria. Akhavan et al. [17] utilized a novel graphene oxide/TiO_2 thin films as photocatalysts for degradation of *E. coli* bacteria under solar light irradiation. They also proposed that graphene nanosheets may serve as an effective photothermal agent for inactivation of the graphene-wrapped microorganisms [18]. However, the major challenges of photochemical strategies, specificity and limited penetration depth of light source, still remain for treating the deep-seated bacteria [19,20]. To overcome these problems, we developed a novel nanovehicle that exerted toxic effects specifically on bacteria following activation by a deeply penetrative low-dose X-ray, without damaging normal cells. Such a concept has not been reported previously.

The nanonvehicle was composed of graphene oxide quantum dots (GQDs), a multifunctional vehicle; vancomycin (Van), a bacteria-targeting ligand; and Protoporphyrin IX (PpIX), a photo/radiation sensitizer. PpIX has been used as a radiosensitizer that can generate reactive oxygen species (ROS) after X-ray activation to kill cancer cells [21]. However, this approach requires high doses of PpIX and X-ray irradiation, as evidenced from the results reported for cancer therapy [21,22], which might damage adjacent normal cells. Therefore, this approach has not been employed in anti-bacterial applications; thus, whether this approach is efficacious for bacteria is unknown. To lower the radiation dose, targeted delivery of PpIX to bacteria is a key successfully point. Hence, we considered using Van, a commonly used antibiotic, as a bacteria-targeting ligand [23]. However, Van is known to induce ototoxicity and nephrotoxicity [24]. Therefore, a vehicle that enabling bacteria-specific delivery of PpIX with a limited dose of Van (i.e., at a high PpIX/Van ratio) was needed. Thus, we developed the Van-GQDs/PpIX complex, a high PpIX/Van ratio nanovehicle in which Van-conjugated GQDs were assembled with PpIX. We hypothesized that the Van-GQDs/PpIX complex could specifically attach to and damage the cell wall of bacteria, and following X-ray activation, could efficiently induce ROS production to kill the bacteria. Currently, no nanovehicle that can exert bacteria-specific and X-ray-activated toxicity is available.

In the present study, we conducted a systemic investigation to confirm the dose of X-ray used whether activated the Van-GQDs/PpIX complex to exert synergistic anti-bacterial effects without damaging normal cells. Our finding, Van-GQDs/PpIX complex together with X-ray irradiation is a promising anti-bacterial approach, provides valuable information for treating deep-seated bacterial infections that might cause death (i.e., peritonitis) or amputation (i.e., osteomyelitis).

2. Materials and methods

1,3-Diphenylisobenzofuran (DPBF), 2′,7′-dichlorofluorescin diacetate (DCFDA, 97%), hydrochloric acid, MES hydrate, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), protoporphyrin IX, propidium iodide (PI), phosphate-buffered saline (PBS), sodium hydroxide, sodium nitrate, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 98%), trypsin, and vancomycin hydrochloride hydrate were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium (DMEM), LB Agar, Luria-Bertani (LB) broth, and fetal bovine serum (FBS) were purchased from Life Technologies. Acetone and absolute ethanol (99.5% up) were purchased from Acros Organics and JT Baker, respectively.

2.1. Preparation, modification, and characterization of GQDs

Graphite flakes were mixed with 30 mL of sulfuric acid and 10 mL of nitric acid under sonication for 2 h, and then heated to 100 °C and stirred for 24 h. Next, 400 mL of water was added, and the pH was adjusted to 8 using 0.5 M NaOH solution. The final solution was filled in a dialysis bag (2000 Da) and was dialyzed for 24 h to obtain the GQD suspension. Then, 5 mg of GQDs, 15 mg of EDC, and 12 mg of NHS were mixed in 18 mL of MES (2-morpholinoethanesulfonic acid) buffer and stirred for 6 h. Next, 1 mg of Van was added and the mixture was stirred for 24 h. Finally, 50 mL of acetone was added to the solution and the mixture was centrifuged for 10 min (6000 rpm). The resulting precipitate was Van-conjugated graphene oxide quantum dots (Van-GQDs).

Van-GQDs (10 mg) was suspended in 1 mL of deionized (DI) water. The resulting suspension was mixed with 1 mL of PpIX/DMSO solution (1 mg/mL) and stirred for 24 h. Finally, acetone was added to the suspension and the mixture was centrifuged for 1 min (6000 rpm). After removing the supernatant and drying in a vacuum desiccator, Van-GQDs/PpIX complex was obtained.

For subsequent investigations, Van-GQDs/PpIX complex was mixed with DI to form a suspension (sample name, Van-GQDs/PpIX or VGP) containing 0.8 μg/mL Van, 500 μg/mL GQDs, and 25 μg/mL PpIX. Sample solutions with various concentrations of Van, GQDs, and PpIX were prepared for comparison. GQDs sample was prepared by mixing GQDs and DI to form a suspension with 500 μg/mL GQDs. PpIX sample was prepared by mixing PpIX and DMSO/DI solution to form a suspension with 25 μg/mL PpIX. Van-GQDs sample was prepared by mixing Van-GQDs and DI to form a suspension with 0.8 μg/mL and 500 μg/mL Van and GQDs, respectively. Finally, the GQDs/PpIX sample was prepared by mixing GQDs with the PpIX sample to form a mixture with 500 μg/mL and 25 μg/mL GQDs and PpIX, respectively. In the present study, we used red light during experimental operation and employed aluminum foil as a shade during sample delivery and storage, by doing which most undesirable activation was avoided.

The samples prepared in the present study were characterized via transmission electron microscope (TEM; JEOL, JEM-2000EX II), high resolution TEM (HR-TEM; Tecnai G2) and dynamic scattering (DLS; Malvern Zerasizer Nano Series, ZS90) for morphology, crystal phase and particle size analysis, respectively.

2.2. Ex vitro ROS test

Ten micromolar solution of DPBF was added to PBS (control group) and the prepared samples of GQDs, Van-GQDs, PpIX, GQDs/PpIX, and Van-GQDs/PpIX, and the resulting mixtures were irradiated with 2 Gy X-ray (6 MV). Subsequent to irradiation, the fluorescence intensity of the supernatants was measured at 410 nm excitation and 455 nm emission using multimode microplate readers.

Please cite this article in press as: C.-L. Pan et al., A nanovehicle developed for treating deep-seated bacteria via using low-dose X-ray, Acta Biomater. (2016), http://dx.doi.org/10.1016/j.actbio.2016.10.003