ENGINEERING AND NANO-ENGINEERING APPROACHES FOR MEDICAL DEVICES

Preventing bacterial growth on implanted device with an interfacial metallic film and penetrating X-rays

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Abstract Device-related infections have been a big problem for a long time. This paper describes a new method to inhibit bacterial growth on implanted device with tissue-penetrating X-ray radiation, where a thin metallic film deposited on the device is used as a radio-sensitizing film for bacterial inhibition. At a given dose of X-ray, the bacterial viability decreases as the thickness of metal film (bismuth) increases. The bacterial viability decreases with X-ray dose increases. At X-ray dose of 2.5 Gy, 98 % of bacteria on 10 nm thick bismuth film are killed; while it is only 25 % of bacteria are killed on the bare petri dish. The same dose of X-ray kills 8 % fibroblast cells that are within a short distance from bismuth film (4 mm). These results suggest that penetrating X-rays can kill bacteria on bismuth thin film deposited on surface of implant device efficiently.

1 Introduction

Surgically implanted devices are often used to improve quality of life, and enhance patient survival rate. A big issue of medical implants is that bacteria can grow on these devices to form biofilm, and cause infections [1–4]. Bacteria reside and proliferate at surfaces of implants, causing local damages such as device loosening, wound dehiscence, disruption of prosthetic valves, as well as inflammation and fever [5, 6]. Device-related infection is hard to eradicate because bacteria live in well-developed films, and are more resistant to treatment with antimicrobial agents than their planktonic counterparts. Routine antibiotics are often incapable of reducing implant-associated infection [7, 8]. As the result, patients have to be managed with hospitalization, prolonged courses of antibiotics, and surgical intervention [9]. In many cases, implant removal is often the only effective way to eradicate the problem. An important strategy to reduce device-related infection is to prevent initial bacterial adhesion onto the implant surface. Ionizing radiation such as X-rays and gamma rays can easily penetrate most tissues, and kill bacteria by causing irreparable DNA damage. Many Gram-negative bacteria such as Escherichia coli (E. coli), Salmonella, and Pseudomonas aeruginosa (P. aeruginosa) can be effectively killed by X-rays [10–12]. High energy X-ray radiation has also been used to eliminate bacteria in living oysters without killing oysters [13, 14]. However, X-rays cannot be used directly to prevent bacteria group in human due to concerns of high dose radiation.

Bismuth has large cross-sections for X-ray absorption [15], and electron generation, and thus it already had been used as radio sensitizers to enhance radiation damage of bacteria and cancer cells [12, 16]. This paper describes a new method to inhibit bacterial growth on implanted device using X-ray radiation that can penetrate into tissue. A thin metallic film (bismuth) is deposited onto the device, and used as radio-sensitizing film that can absorb X-ray, and generate electrons and free radicals for bacterial inhibition. The results indicate that a given dosage of X-ray can effectively kill bacteria deposited on bismuth film without
causing much damage to normal cells (i.e., fibroblast cells). Thus this method has the potential for inhibiting bacterial growth on implanted medical device.

2 Materials and methods

LIVE/DEAD BacLight bacterial viability kit and LIVE/DEAD viability/Cytotoxicity kit are obtained from Invitrogen (Carlsbad, CA). Bismuth powder (200 meshes) is obtained from Sigma-Aldrich (St. Louis, MO). RPMI 1640 culture media, penicillin, streptomycin, fetal bovine serum (FBS), and Dulbecco’s phosphate buffered saline (D-PBS) are from Thermo Scientific (Logan, UT). Sodium chloride (NaCl), yeast extract, bacteriological agar, and BD falcon multiwell flat-bottom plate are obtained from VWR (West Chester, PA). Tryptone powder is from MO BIO (Carlsbad, CA). P. aeruginosa strain (ATCC 15442) and fibroblast cells are obtained from American Type Culture Collection (ATCC, Manassas, VA). Ultrapure water with resistivity of 18.2 MΩ cm-1 is obtained from a Millipore system (Marlborough, MA) and used for making solutions throughout all of the experiments. All chemicals used in this study are of analytical grade and are used without further purification. 1420 multi-label counter from PerkinElmer (Santa Clara, CA) is used for optical measurements. Inverted optical microscope is used to observe cultured cells, and a fluorescence microscope from Olympus is used to take fluorescent images.

_Pseudomonas aeruginosa_ bacteria are grown aerobically at 37 °C in Luria–Bertani broth (LB) medium under 200 rpm agitation. Prior to treatment, the bacteria are cultured overnight in 5 ml of LB in a shaker at 37 °C until the optical density (OD) of the culture reaches 1.0 at 600 nm, which indicates 10⁹ CFU ml⁻¹. The overnight cultured cell suspensions are diluted to 10⁷ CFU ml⁻¹ with sterile broth, and inoculated onto LB agar coated petri dish. Fibroblast cells are cultured under standard conditions (5 % CO₂ in air at 37 °C) in RPMI-1640 medium supplemented with 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin. After reaching 70–80 % confluence, cells are trypsinized with 0.25 % trypsin/0.53 mM EDTA solution at 37 °C for 3 min, followed by adding fresh medium at room temperature to neutralize trypsin. After centrifugation and removing supernatant, the cells are resuspended in fresh medium. The cell viability is determined by staining with Trypan blue, and cell number is counted with hemocytometer (Hosham, PA).

A Mini-X X-ray tube from Amptek (Bedford, MA) with a silver anode operating at 40 kV and 100 μA is used to generate X-rays. The tube is fitted with a brass collimator (with 2 mm diameter pinhole) to focus X-rays onto the target. An X-ray spectrometer (Amptek X-123) with a Si-PIN photodiode is used to analyze XRF emissions in transmission mode. The spectrometer contains a solid-state detector, a digital pulse processor and a multichannel analyzer, which are interfaced with a computer for data acquisition and analysis. The combination of a 25 mm thick silver filter and a 250 mm thick aluminum filter is used to reduce the background and improve the signal-to-noise ratio in a low energy (0.15 keV) region of the spectrum. The surface dose rate is measured with a handheld radiation dosimeter Mirion RAD-60 from Freshwater Systems (Greenville, SC).

A thermal evaporator (Denton) is used to deposit bismuth film on petri dish under vacuum (2.0 × 10⁻⁶ Pa). The thicknesses of deposited bismuth film are monitored by a quartz crystal microbalance. Briefly, a certain thickness (10, 30 and 100 nm) of bismuth film is deposited on the petri dish. The morphologies of film are examined with an atomic force microscope (AFM). The AFM images are collected at ambient conditions using a multimode Nanoscope (Veeco, CA). A micro-fabricated silicon probe integrated on a rectangular beam is used for imaging in tapping mode. The spring constant and the resonance frequency of the beam are 1 nm⁻¹ and 276 kHz, respectively.

The bactericidal activities of bismuth films against _P. aeruginosa_ are studied to proof the concept. Four different samples (bacteria on petri dish; bacteria on petri dish coated bismuth film; bacteria exposed to X-ray on petri dish; bacteria on petri dish coated bismuth film are exposed to X-ray) are used. Briefly, 200 μl _P. aeruginosa_ bacterial suspensions are divided to two groups equally. One group of bacterial suspension (100 μl) is inoculated onto different samples and then exposed to X-ray radiation immediately, the colony numbers are counted after incubated for overnight under 37 °C. Another group of bacterial suspension (100 μl) is inoculated onto different samples, and allowed to grow overnight at 37 °C before X-ray exposure. The diameters of colony are measured after 1–5 days. Bacteria without X-ray exposure are inoculated onto LB agar coated dish in absence of and in presence of bismuth film.

Bacteria viability assays are performed using the protocol provided in the bacteria viability kit. For dual staining of the bacteria, the bacteria in the broth are diluted to the density is 10⁴ CFU ml⁻¹, verified by the absorbance at 600 nm. 3 μl of a dye mixture (containing an equal volume of green-fluorescent SYTO® 9 stain and red-fluorescent propidium iodide stain) is mixed with 1 ml bacterial suspension and incubated at 37 °C in the dark for 15 min, followed by observation under fluorescence microscope. For live/dead cell calibration, 0.6 μl of the dye mixture and 200 μl of the bacterial suspension are added into each well of the 96-well culture plate. After incubation for 15 min, the fluorescent signals are measured at 530 nm/630 nm (dual fluorescence). The fluorescent backgrounds are
subtracted before calculation by measuring a cell-free control. The percentages of live and dead cells are calculated by dividing the fluorescence intensities of live or dead cells by the values obtained for controls.

Six independent duplicates are performed for each test and the data are shown as mean ± standard error. All the figures are plotted using the Origin 8.5 software (OriginLab, Northampton, MA) and statistical analyses are performed using SPSS 16.0 (SPSS Inc, Chicago, Illinois). One-way ANOVA and LSD tests are applied to compare the difference between the numbers of bacterial colony after different treatments. \( P < 0.05 \) is considered statistically significant.

3 Results and discussions

A thermal evaporator (Denton) is used to deposit a thin layer of bismuth film on clean petri dish in vacuum. The bismuth film is visible to naked eye, smooth and reflective, which is characteristic of bismuth. Figure 1a demonstrates the scheme of bismuth film enhanced X-ray radiation killing of \( P. aeruginosa \) on the surface of substrate. After incubation, \( P. aeruginosa \) seeded on agar film are exposed to X-rays. The in vitro bactericidal activity of this method is confirmed using LIVE/DEAD assay. The cytotoxicity of bismuth film and X-rays on human cell lines has been tested. Figure 1b is an atomic force microscopy (AFM) image showing morphology of the bismuth film, consisting small granular particles with diameter of 30 nm. Figure 1c is X-ray fluorescence (XRF) spectrum, where the characteristic \( L_{α1} \) and \( L_{β1} \) peaks of bismuth can be found at 10.82 and 13.02 keV, respectively. The intensity of XRF signals on bismuth film covered with LB agar is slightly lower than that without agar, suggesting that the gel itself can absorb photoelectrons.

The cytotoxicity of bismuth film has been tested on both bacteria and fibroblast cells. X-ray exposed \( P. aeruginosa \) LB agar plates are incubated for 24 h, and the growth of bacteria is monitored. At the same time, 80 % confluent fibroblast cells are used as control. To evaluate instant bactericidal activity, LIVE/DEAD viability assay is carried out to discriminate dead (stained red) and live (stained green) bacteria and cells. Four different \( P. aeruginosa \) samples in LB media are made as follows: (1) those seeded on dish; (2) those seeded on bismuth film; (3) those seeded on dish and exposed to 2.5 Gy X-ray; and (4) those seeded on bismuth film and exposed to 2.5 Gy X-ray. Figure 2a shows the fluorescent image of bacteria on bismuth film taken after exposing to X-ray, followed by overnight culture, where almost all bacterial cells are dead. Figure 2b shows bacteria grown on petri dish exposed to X-ray radiations, followed by overnight culture, where only a fraction of bacterial cells are dead, and many are still alive.

![Fig. 1 Metal film assisted bacterial inhibition on implanted device with penetrating X-ray (a); AFM image of 10 nm bismuth film on petri dish (b); XRF spectra of bismuth film deposited on petri dish with (red line) and without LB gel (c) (Color figure online)](image-url)
Figure 2c shows the viabilities of bacteria of four samples. Compared to the control, the combined use of radiation and bismuth film can kill over 98% \( P. \) \textit{aeruginosa} cells; the sample with X-ray radiation alone kills 25% bacterial cells, and the sample with bismuth film alone kills 2% bacterial cells. These results indicate that neither X-ray radiation nor bismuth film alone has high bactericidal activity, but the combination of X-ray and bismuth film can significantly enhance instant bactericidal activity. Figure 2d shows a fluorescent image of fibroblast cells exposed to 2.5 Gy radiations, where many fibroblast cells are alive. Figure 2e shows a fluorescent image of fibroblast cells on petri dish, where many fibroblast cells are alive. Figure 2f shows the viabilities of fibroblast cells after different treatment (e); fluorescence images of fibroblast cells with (d) and without X-ray radiation (e); viabilities of fibroblast cells without and with 2.5 Gy X-ray radiation (f).

The growth of X-ray irradiated bacteria is monitored by counting \( P. \) \textit{aeruginosa} colonies on the LB agar plate. Figure 3a shows the image of bacteria grown on bismuth film after exposure to X-ray radiation, where only a small amount of colonies is found after treating. Figure 3b shows the image of bacteria grown on petri dish after exposure to X-ray radiation, where the colony number has reduced. Figure 3c and d are the optical images of bacteria grown on petri dish and bismuth film alone, respectively. The bacterial density and coverage are similar in both images, suggesting bismuth film does not affect bacterial growth. Figure 3e shows that the relative colony number shows a decrease of 42% for X-ray radiation group, while the reduction of colony number is 87% in the group on bismuth film and exposed to X-ray radiation. Thus, the combined use of X-ray and bismuth film can significantly reduce the proliferation of \( P. \) \textit{aeruginosa}. The effect of X-ray dose on colony number has also been studied. Figure 3f shows that the relative colony number of \( P. \) \textit{aeruginosa} depends on the X-ray radiation dose: only 40% bacteria have been killed after exposed with 1.25 Gy X-ray radiations on petri dish; while, 60, 85, 95 and 99.5% bacteria have been killed on bismuth film at X-ray dosage of 1.25, 2.5, 5 and 10 Gy, respectively, suggesting bismuth film can significantly enhance bactericidal activity of X-ray radiation. Since photoelectrons produced by interaction of X-ray and bismuth can be absorbed by LB agar, the effect of LB gel thickness on colony number has been studied. Figure 3g shows the colony number of \( P. \) \textit{aeruginosa} after exposure to different dose of X-ray radiation as function of gel thickness, where black and red lines are viability in the presence and absence of bismuth film. As the thickness of LB agar increases, the colony number increases in the absence of bismuth film. At gel thickness of ~4 mm, the colony number is comparable to that without bismuth, indicating that almost photoelectrons and Auger electrons may have been absorbed by 4 mm LB agar. These results also suggest that X-ray irradiation decreases bacterial...
reproduction rather than killing bacteria instantly. This is because photoelectrons and Auger electrons damage double-stranded DNA [17], which has more effect on decreasing the reproduction capability of bacteria during the double session process than causing immediate bacterial death.

**Fig. 3** Optical images of *P. aeruginosa* grown on 10 nm bismuth film with 2.5 Gy X-ray (a), petri dish with 2.5 Gy X-ray (b), bismuth film (c) and petri dish (d), respectively; colony numbers of bacterial after different treatment (e); colony numbers of bacterial after exposure to different dosage of X-ray radiation at petri dish and on bismuth film (f); colony numbers of bacterial exposed to X-ray radiation as function of LB agar thickness on bismuth film (g), where black and red lines are viability in the presence and absence of bismuth film (Color figure online)

**Fig. 4** Optical images of *P. aeruginosa* colony of different samples: bismuth film exposed to 2.5 Gy X-ray (a), exposed to 2.5 Gy X-ray on petri dish (b), bismuth film (c), and petri dish (d), respectively; colony diameter of *P. aeruginosa* at 1–5 days after exposed to different dosage of X-ray under 10 nm bismuth film (e); colony diameter of *P. aeruginosa* at 1–5 days after exposed to 2.5 Gy X-ray on bismuth film of different thickness (f)
In order to test whether bacterial cells can be killed after grow on the surface, and then \textit{P. aeruginosa} is seeded on petri dish and bismuth film for overnight culture. After growing into bacterial colony, the samples are exposed to X-ray radiation. Figure 4a shows an optical image of colony taken during a 5 day period on bismuth film, where bacteria have been exposed to 2.5 Gy X-ray radiations. The dead bacteria stay in their original location and are finally dried. Bacteria grown on petri dish has been exposed to X-ray (Fig. 4b), where the edge of colony becomes transparent and the diameter decreases, because some bacterial cells are dead. In comparison, Fig. 4c shows the image of bacterial colony on bismuth film without X-ray exposure, where bacteria grow normally and colonies are still non-transparent after 5 days. In addition, Fig. 4d shows that those grown on petri dish and without X-ray exposure show similar appearance as those on bismuth film. Figure 4e shows colony diameter of \textit{P. aeruginosa} as a function of culture time, where bacteria have been formed on 10 nm bismuth film and exposed to different dosage of X-ray. The results show that the diameter of colony decreases as X-ray dosage increases on bismuth film. In the absence of X-ray, bacteria colony can grow slightly. Figure 4f shows colony diameter of \textit{P. aeruginosa} as a function of thickness (10, 30 and 100 nm) and exposed to 2.5 Gy of X-ray radiations. As bismuth thickness increases from 10, 30 to 100 nm, the diameter of colony decreases from 0.63, 0.41 to 0.22 mm. Because thicker film can generate more photoelectrons and Auger electron than the thinner one, the thicker bismuth film can kill more bacteria.

4 Conclusions

Bismuth film can enhance X-ray radiation based killing of bacteria. The use of pre-deposited metal film can significantly enhance deposition of X-ray energy close to bacteria without damaging normal cells that are relatively farther away from bismuth film. Bismuth film can enhance radiation and cause more bacteria dead. The percentage of dead bacteria and colony number decrease as the thickness of bismuth film increases. 98% of bacteria on bismuth film are killed, while it is only 25% of bacteria are killed on the bare petri dish. The X-ray dose can be enhanced several times in bacterial killing through using bismuth coated substrate. And there only have 8% fibroblast cells are killed show that mammalian cells are not significantly affected under the same conditions. Although only \textit{P. aeruginosa} is used in this study, the method can be developed to kill other types of microorganisms for patient with implant device.

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References