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Antimicrobial Activity and Biocompatibility of Polyurethane–Iodine Complexes

JIE LUO, YING DENG AND YUYU SUN*
Biomedical Engineering Program, University of South Dakota
South Dakota 57107, USA

ABSTRACT: Polyurethane (PU), one of the most versatile biomedical materials, strongly binds iodine, one of the most effective antiseptics, through the formation of a charge-transfer complex. The PU–Iodine complexes were characterized with UV/Vis study and X-ray photoelectron spectroscopy (XPS) analysis. The new materials evoked potent antimicrobial activity against Gram-negative and Gram-positive bacteria (including methicillin-resistant *Staphylococcus aureus,* vancomycin-resistant *Enterococcus faecium,* and bacterial spores), fungi, and viruses, as well as inhibited surface bacterial colonization and biofilm-formation. Based on the Kirby-Bauer test, the antimicrobial effects occurred through the slow release of iodine. The iodine release rate can be controlled by the preparation conditions of the PU–Iodine complex. Trypan blue exclusion analysis indicated that PU–Iodine has excellent mammalian cell viability. The PU–Iodine complexes have the potential for a wide range of medical, dental, and other related applications.

KEY WORDS: polyurethane, iodine, antimicrobial, biofilm-controlling, anti-infective, antibacterial, iodine release, controlled iodine release, biocompatible.

INTRODUCTION

Because of its wide availability, low cost, ease in fabrication, and excellent physical and biological properties, polyurethane (PU) is among the most widely used polymers in biomedicine [1–4]. Examples of its

*Author to whom correspondence should be addressed. E-mail: yuyu.sun@usd.edu

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medical applications include catheters, endotracheal tubes, hemodialysis, blood filters, and wound dressings. In dentistry, PU-based resins are employed to produce maxillofacial prosthetics and PU tubing is used to bring water to the air/water syringe, the ultrasonic scaler, and the high-speed hand piece. Unfortunately, because of microbial contamination and subsequent biofilm formation on the polymer surfaces, the use of these medical/dental devices is associated with increased risk of foreign body-related infections (FBRIs). Once biofilms are formed, the host defense systems are often unable to handle the FBRIs, in particular, to eliminate the microorganisms from the infected devices. In addition, microbes embedded in biofilms often have superior resistance to disinfection; it has been reported that microbes living in a biofilm are up to 10^3 times more resistant to biocides than free-floating microorganisms [5–8]. As a result, FBRIs are difficult to treat, often leading to serious morbidity and mortality, long hospital stays, and extra medical costs [5–17]. A total of 250,000 cases of central vascular catheter-related bloodstream infections are estimated to occur each year; mortality from these infections is 12–25% and the marginal cost to the healthcare system is $25,000 per infection [12,13]. Contamination of endotracheal tubes leads to ventilator-associated pneumonia, a serious complication with a mortality rate up to 48% [14,15]. Biofilms on dental tubing lead to heavy microbial contamination of dental treatment water and aerosols [16] and biofilms on dentures cause Candida-associated denture stomatitis, a common recurring disease that infects 11–67% of denture wearers [17].

Introducing antimicrobial functions into such devices is a potential solution to these problems [5–8,18]. Consequently, a number of antimicrobial PU and other polymeric device materials have been developed by either physically mixing antimicrobial agents (e.g., antibiotics, metal ions, quaternary ammonium salts, and N-halamine compounds) into or covalently binding the antimicrobial agents onto the polymers. While some of these studies have achieved encouraging results [18–22], extensive physical/chemical treatment procedures of medical/dental devices are often not accepted well in real biomedical applications due to feasibility, biocompatibility, safety/toxicity, cost, and regulation restrictions.

These hurdles stimulated our interest in PU–Iodine charge-transfer complexes [23], which can be formed by directly treating PU polymers with iodine in aqueous solutions for a short period of time under very mild conditions, regardless of the type and shape of the PU-based device. Iodine is one of the most widely used antiseptics in biomedical settings with low cost and high efficacy, which has been safely used for more than 150 years. Since free iodine has a high sublimation potential that causes

stability and irritation/toxicity concerns, a wide range of iodine complexes, such as polyvinylpyrrolidone–iodine, nylon–iodine, cellulose derivative–iodine, natural rubber–iodine and PU–Iodine, have been reported to increase stability [24–34]. Among these, PU–Iodine complexes were first disclosed in patents [28,29] and the antimicrobial activities were reported in medical literature in the middle 1990s [30,31].

To date, systemic investigation of the antibacterial (such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium,* and bacterial spores), antifungal, and antiviral functions of PU–Iodine complexes have not been reported and biofilm-controlling effects of these complexes have never been studied. Moreover, very little information is available about the durability of the antiinfective activities and the effects of these complexes on mammalian cell viability. The purpose of the current study is to address these issues and to develop this class of antimicrobial polymeric materials for biomedical applications.

MATERIALS AND METHODS

Materials

Polyether-based medical grade PU was a gift from The Lubrizol Corporation (Estane 5714, Wickliffe, OH). Iodine (ACS reagent, $\geq$99.8%) was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were analytical grade and used as received.

Preparation of PU–Iodine Complex Membranes

PU membranes were prepared by solvent casting from a 10% solution of PU in tetrahydrofuran (THF). After air-drying in a fume hood for 24 h, the membranes were dried under vacuum at 40°C for 24 h, conditioned in a desiccator for 24 h to reach room temperature equilibrium and then weighed before iodine treatment. Different iodine content solutions were prepared by dissolving a predetermined amount of iodine in 4% potassium iodide (KI) aqueous solution. At each iodine concentration, a series of PU membrane samples were immersed in the iodine aqueous solution at room temperature for a specific period of time. The membranes were taken out and rinsed thoroughly with Millipore water to remove un-bound free iodine (the rinsing solution was tested with 1% of starch solution to ensure that no further free iodine could be washed out from the membranes). The PU–Iodine complex membranes were air dried, conditioned in a desiccator for 24 h at room

temperature and weighed. The percent iodine content was calculated according to the following equation:

$$I_2\% = (W_2 - W_1)/W_1 \times 100 \quad (1)$$

where W_1 and W_2 were the weights of the PU membrane before and after iodine binding, respectively.

Characterization of the PU–Iodine Complex Membranes

UV/Vis spectra of the samples were recorded on a Beckman Coulter DU 520 UV/Vis spectrophotometer. X-ray photoelectron spectra (XPS) of the samples were obtained by using a PHI 5700 X-ray photoelectron spectroscopy system equipped with dual Mg X-ray source and monochromated Al X-ray source.

Antimicrobial Tests

The bacterial, fungal, and viral species were purchased from American Type Culture Collection (ATCC, Manassas, VA) and the spores were provided by North American Science Associates (Northwood, Ohio). All microbial tests were performed in a Biosafety Level 2 hood to ensure laboratory safety. The guidelines provided by the U. S. Department of Health and Human Services were followed [35] with appropriate personal protective equipment, including gowns and gloves being used in all the microbial studies. The microbial tests were conducted following AATCC (American Association of Textile Chemists and Colorists) Test Method 100-1999 with modifications as specified below.

Antibacterial Functions

Pseudomonas aeruginosa (ATCC 10145, Gram-negative), *Legionella pneumophila* (ATCC 33155, Gram-negative), and *S. aureus* (ATCC 6538, Gram-positive) were used as typical examples of nonresistant Gram-negative and Gram-positive bacteria, respectively. Methicillin-resistant *S. aureus* (MRSA, ATCC BAA-811) and vancomycin-resistant *Enterococcus faecium* (VRE, ATCC 700221) were selected to represent drug-resistant strains because these species have caused serious problems in both healthcare and various community settings. To prepare the bacteria suspensions, *P. aeruginosa* 10145 was grown in nutrient broth at 37°C for 24 h, *S. aureus* 6538, MRSA BAA-81 and VRE 700221 were grown in tryptic soy broth at 37°C for 24 h and

L. pneumophila 33155 was grown in 1099 charcoal yeast extract (CYE) buffered broth at 37°C for 48 h, respectively, according to the ATCC's recommendation. The bacteria were harvested by centrifuge, washed with phosphate buffered saline (PBS) and then resuspended in PBS with 10^7–10^8 colony forming units/mL (CFU/mL). 10 μL of the freshly prepared bacterial suspensions were placed between two identical PU–Iodine complex membranes ($2.0 \pm 0.1\,cm^2$) for specific contact times. The membranes then were transferred into 10 mL of sterilized sodium thiosulfate ($Na_2S_2O_3$) aqueous solution (0.03 wt%) to quench the active iodine, vortexed for 1 min, sonicated for 5 min to separate the films and to detach adherent cells from the membrane surfaces into the solution (this treatment does not affect the viability of the microorganism [22]). The solution was then serially diluted and 100 μL of each diluent were placed onto agar plates (nutrient agar for *P. aeruginosa* 10145, tryptic soy agar for *S. aureus* 6538, MRSA BAA-81 and VRE 700221 and 1099 CYE buffered agar for *L. pneumophila* 33155). The colony forming units on the agar plates were counted after incubation at 37°C for 24 h for *P. aeruginosa* 10145, *S. aureus* 6538, MRSA BAA-81 and VRE 700221 and 48 h for *L. pneumophila* 33155. Pure PU membranes were tested under the same conditions served as controls. Each test was repeated three times.

Antifungal Functions

Candida albicans (ATCC 10231) and *Aspergillus niger* (ATCC 1004) were used as representative examples of fungi. In the antifungal tests, cells were grown in broth solutions (YM broth for *C. albicans* 10231 and potato dextrose broth for *A. niger* 1004) at 26°C for 48 h, harvested, washed and resuspended in PBS to densities of 10^7–10^8 CFU/mL, as described above. 10 μL of the freshly prepared fungal suspensions were placed between two identical PU–Iodine complex membranes ($2.0 \pm 0.1\,cm^2$). After a specific contact time, the membranes were transferred to a 10 mL sterilized $Na_2S_2O_3$ aqueous solution (0.03 wt%), vortexed and sonicated. The solution was serially diluted and 100 μL of each diluent were placed onto agar plates (YM agar for *C. albicans* 10231 and potato dextrose agar for *A. niger* 1004). The colony forming units on the agar plates were counted after incubation at 26°C for 48 h. Pure PU membranes were tested as controls. Each antifungal test was repeated three times and the longest minimum contact time of the three tests for a total kill of the fungi (the weakest antifungal efficacy observed) was reported.

Antiviral Functions

Stock solutions of MS2 virus (ATCC 15597-B1) were prepared using the agar overlay method suggested by ATCC; *E. coli* (ATCC 15597) was employed as the host for the MS2 virus. The stock solutions were diluted with PBS to 10^6–10^7 plaque forming units per milliliter (PFU/mL) of the virus. 10 μL of the freshly prepared viral suspensions were placed between two identical PU–Iodine complex membranes ($2.0 \pm 0.1\,cm^2$). After a specific contact time, the membranes were transferred into 10 mL of sterilized $Na_2S_2O_3$ aqueous solution (0.03 wt%), vortexed and sonicated. The solution was then serially diluted and 100 μL of each diluent were placed onto LB agar plates containing a 'lawn' of 4-hour-old *E. coli* 15597 as the host. Plaque forming units on the agar plates were counted after incubation at 37°C for 24 h. Pure PU membranes were tested under the same conditions as controls. Each antiviral test was repeated three times and the longest minimum contact time of the three tests for a total kill of the virus (the weakest antiviral efficacy observed) was reported.

Antispore Functions

Bacillus subtilis spores (lot no. N24609) were used to challenge the antispore functions of the PU–Iodine complex membranes. 10 μL of the spore solutions (10^4–10^5 spores/mL) were placed between two identical PU–Iodine complex membranes ($2.0 \pm 0.1\,cm^2$). After specific times, the membranes were transferred into 10 mL of sterilized $Na_2S_2O_3$ aqueous solution (0.03 wt%), vortexed and sonicated. The solution was then serially diluted and 100 μL of each diluent were placed onto tryptic soy agar plates. Colony forming units on the agar plates were counted after incubation at 37°C for 24 h. Pure PU membranes were tested under the same conditions as the controls. Each antispore test was repeated three times and the longest minimum contact time of the three tests for a total inactivation of the spores (the weakest antispore efficacy observed) was reported.

Kirby–Bauer Test

The antimicrobial function of the samples was also assessed by a Kirby–Bauer (KB) technique. In this study, the surface of a tryptic soy agar plate was overlaid with 1 mL of 10^7–10^8 CFU/mL *S. aureus* 6538. The plates were then allowed to stand at 37°C for 2 h. A series of PU–Iodine discs (*c.* 8 mm) with different iodine contents were placed onto the surface of the bacteria-containing agar plate. The membranes were

gently pressed with a sterile forceps to ensure full contact between the membranes and the agar. The same procedure was also applied to the pure PU membranes to serve as controls. After incubation at 37°C for 24 h, the inhibition zone around the membranes (if any) was measured.

Biofilm-controlling Functions

The ability of the PU–Iodine complex membranes to prevent microbial biofilm formation was evaluated against *S. aureus* 6538 (Gram-positive) and *P. aeruginosa* 10145 (Gram-negative); both species are known to readily form biofilms [5,7–9]. In this test, *S. aureus* 6538 was grown in tryptic soy broth and *P. aeruginosa* 10145 was grown in nutrient broth at 37°C for 24 h, respectively. The bacteria were harvested and resuspended into sterile PBS, as described above. Each PU–Iodine complex membrane to be tested ($1.0 \pm 0.1\,cm^2$) was immersed individually in a vial containing 10 mL of 10^7–10^8 CFU/mL of *S. aureus* 6538 or *P. aeruginosa* 10145 suspension in PBS. The vials were shaken gently at 37°C for 1 h to allow initial bacterial adhesion. Each membrane was taken out of the bacterial suspension with sterile forceps and gently washed three times with PBS to remove any nonadherent bacteria. The membranes were then immersed into 10 mL of tryptic soy broth (for *S. aureus* 6538) or nutrient broth (for *P. aeruginosa* 10145) at 37°C for 24 h. Afterwards, some of the immersed membranes were removed sterilely from the broth solutions, washed gently with nonflowing PBS (3×10 mL) to remove loosely attached cells and sonicated in 10 mL of PBS for 5 min to transfer the adherent cells into the solution. The solution was serially diluted and 100 μL of each diluent were plated onto the correspondent agar plates. Recoverable microbial colonies were counted after incubation at 37°C for 24 h.

The remaining membranes were washed gently with PBS, fixed with 2.5% of glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB) and stored at 4°C overnight. At the end of fixation, the membranes were taken out, gently rinsed three times with PBS and dehydrated through an alcohol gradient [36]. The samples were then mounted onto sample holders, sputter coated with gold and observed under a Hitachi S-3200N scanning electron microscope. The same procedure was also applied to pure PU membranes as controls.

Mammalian Cell Viability Analysis

The effect of the PU–Iodine complexes on mammalian cell viability was assayed by the trypan blue dye exclusion method [37]. Briefly, test membranes were cut under sterile conditions and placed into a

96-well plate. The rat skin cell line CRL-1213^{TM} (from ATCC) was cultured in DMEM/high glucose medium supplemented with 10% fetal bovine serum at 37°C in humidified air atmosphere of 5% CO_2, trypsinized, counted and then plated into the study wells of the 96-well plate. Cells seeded in wells without the membranes were designated as untreated controls. Each well was plated with 1.25×10^4 cells in 200 μL of the medium. After the treatment, the cells were exposed for 5 min to trypan blue 0.2% (diluted from 0.4% solution; Sigma-Aldrich). The numbers of stain-positive (dead and dying cells) and stain-negative cells in each culture were counted in a hemocytometer chamber. Each membrane was exposed to the four cultures for 6 or 24 h, respectively. The data were analyzed with Student's *t*-test for statistical significance.

Stability and Rechargeability of the PU–Iodine Complexes

The stability of the PU–Iodine complexes in aqueous medium was monitored under static conditions. Membranes with different iodine contents weighing 0.20 g each were suspended individually in 10 mL of PBS at 37°C for specific times. The iodine contents at different time intervals in the immersing solution were determined by UV/Vis. The calibration curves of standard iodine solutions were prepared by measuring the absorbance of iodine solutions with concentrations varying between 0.05 and 100 ppm.

The PU–Iodine complexes were tested for retention of iodine and antimicrobial functions under storage. Membranes with known iodine contents were stored under normal lab conditions (25°C, 30–90% RH). The iodine contents and the antiinfective functions were tested periodically.

To recharge the PU–Iodine membranes, they were treated with 10% sodium thiosulfate aqueous solution at room temperature for 24 h to partially quench the bound iodine. The membranes were washed with Millipore water, air dried, and stored in a desiccator for 24 h to reach constant weight. The membranes were then treated with iodine aqueous solution to regenerate the PU–Iodine complex structure using the same conditions as above. After several cycles of this 'quenching–recharging' treatment, the iodine contents and antiinfective functions of the resultant membranes were re-evaluated.

RESULTS AND DISCUSSION

Pure PU membranes are colorless, but upon immersing the membranes into iodine aqueous solution at room temperature, the

membranes turned yellow and gradually dark purple with the increase of immersion time. The color was not removed by repeated washing, suggesting strong I_2 bonding, as reported [28–31]. A charge-transfer complex between iodine and the benzene ring of PU apparently forms, a well-known behavior of iodine with electron-rich compounds [38–40]. It was found that iodine also binds to other benzene-ring containing polymers including polystyrene and poly(ethylene terephthalate).

Characterization of PU–Iodine Complexes

The effect of iodine concentration in the binding solution is shown in Figure 1. In the test range (0–12.7 g/L of iodine in the solution), the complex formation was clearly iodine-concentration dependent. After the initial rapidly increasing stage, a near linear relationship was observed between iodine concentration in the solution and iodine content in PU.

With time, the iodine content in the membranes increased rapidly until a relatively constant value was reached within about 1 h (Figure 2). Initial iodine concentration in the solution did not seem to significantly affect the time needed to reach this equilibrium. In this system, iodine binding was affected by three stages: (1) diffusion of iodine through the bulk solution to the surface of the membranes, (2) absorption of iodine molecules on the membrane surface, and (3) diffusion of iodine from the

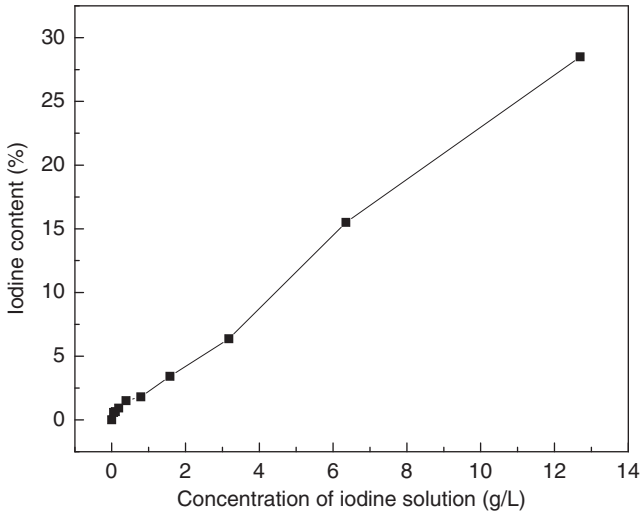

Figure 1. The effects of initial iodine concentration in the solution on iodine content in the resultant PU–Iodine complexes ($T = 21^{\circ}C$; $t = 2\,h$).

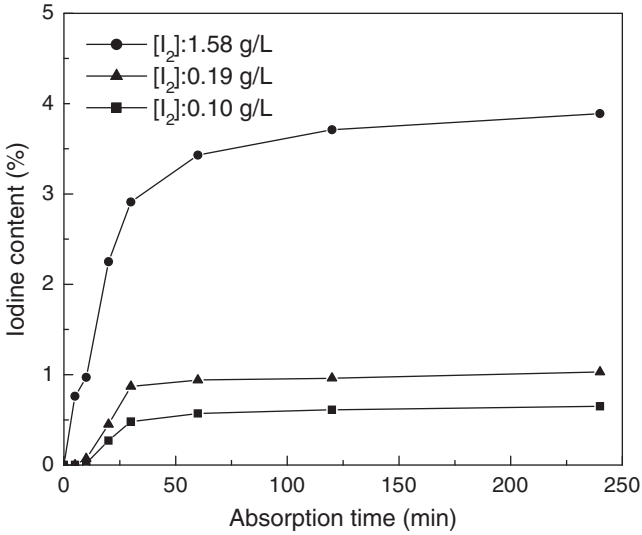

Figure 2. The effects of binding time on iodine content in the resultant PU–Iodine complexes at different iodine concentration in the solution (T = 21°C).

surface to the interior of the PU sample. The later would be the rate controlling stage due to the high hydrophobicity and low swellability of PU polymers in aqueous solution and the interior free volumes accessible to the iodine solutions may be limited. However, due to the relatively short period of time needed to reach absorption equilibrium and the relatively low iodine content in PU, it seems that the iodine may be primarily bound to the surface of the membranes.

Factors to promote the third stage, such as using porous PU foams instead of dense PU membranes, organic solvents instead of water and iodine sublimation instead of solution absorption, all significantly increased in the iodine content in PU–Iodine complexes (even >100% iodine content) [23]. However, our purpose was to prepare PU–Iodine complexes using simple and practical methods under mild conditions either before or after the medical devices were fabricated in order to achieve durable and rechargeable antimicrobial and biofilm-controlling functions with little negative cytotoxicity effects. We found that increasing the iodine content beyond a certain amount in the PU–Iodine composite minimally increased the antimicrobial potency, but adversely affected cell viability.

The XPS survey scans of the original PU membrane (Figure 3(a)) showed three peaks corresponding to C-1s (285 eV), N-1s (403 eV), and O-1s (533 eV), respectively, in good agreement with the literature [41].

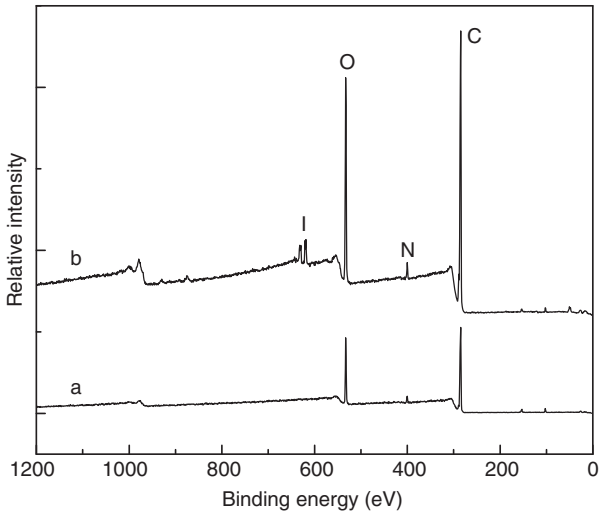

Figure 3. XPS spectra of (a) pure PU membrane and (b) PU–Iodine complex membrane containing 0.96% of iodine.

After iodine binding, two new peaks at 618.8 eV (I3d3) and 630.0 eV (I3d5) were observed in the PU–Iodine membrane (Figure 3(b)), confirming the formation of PU–Iodine charge-transfer complexes [42].

The UV/Vis spectrum of the original PU membrane (Figure 4(a)) had very little absorption >300 nm. After iodination, a broad band centered at 350 nm was observed in the UV spectrum (Figure 4(b)). The new band was assigned to the absorption of I_3^- [43], a known polyiodide formation in iodine-based charge-transfer complexes [23,38–41].

Antimicrobial Activities of the PU–Iodine Complexes

The antimicrobial efficacies of the PU–Iodine complexes were evaluated against Gram-positive and Gram-negative bacteria (including drug-resistant species and bacterial spores), fungi and viruses. The pure PU membranes, used as controls throughout this study, did not show any antimicrobial effects; however, the PU–Iodine complexes were antimicrobial. The minimum contact times of the PU–Iodine complexes with different iodine contents for a total kill of each microbial species are listed in Table 1. Higher iodine content generally led to more rapid antimicrobial effects. With 0.59% iodine, it took the PU–Iodine complexes 10 min to provide a total kill of 10^7–10^8 CFU/mL of *P. aeruginosa* 10145 (Gram-negative bacteria) and *S. aureus* 6538 (Gram-positive bacteria) while 3.46% iodine, was 2 min for a total kill for the same species.

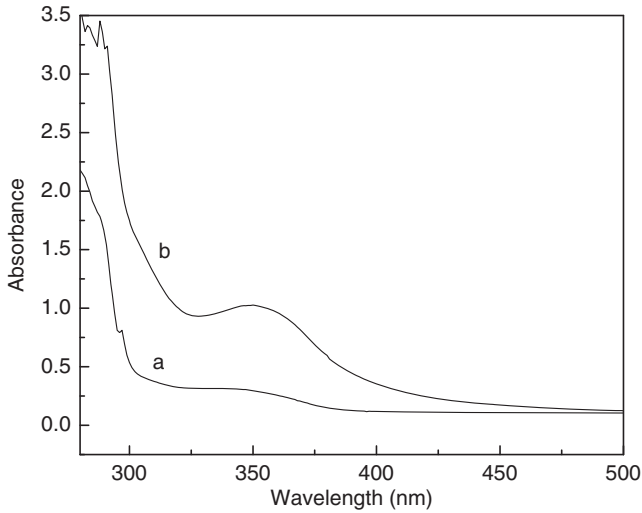

Figure 4. UV/VIS spectra of (a) pure PU membrane and (b) PU–Iodine complex membrane containing 0.96% of iodine.

Table 1. Antimicrobial efficacies of the PU–Iodine complex membranes with different iodine contents.

	Minimum contact time for a total kill of the microorganisms at different iodine contents		
	Percentage iodine in the membrane		
Microorganisms	3.46%	0.96%	0.59%
P. aeruginosa 10145[a]	2 min	5 min	10 min
S. aureus 6538[a]	2 min	5 min	10 min
L. pneumophila 33155[a]	20 min	40 min	40 min
MRSA BAA-81[a]	2 min	5 min	10 min
VRE 700221[a]	2 min	5 min	10 min
C. albicans 10231[a]	2 min	5 min	10 min
A. niger 1004[a]	20 min	40 min	40 min
MS2 virus 15597-B1[b]	15 min	20 min	40 min
B. subtilis[c]	16 h	24 h	24 h[a]

[a]Bacterial or fungal concentration: 10^7–10^8 CFU/mL.
[b]Viral concentration: 10^6–10^7 PFU/mL.
[c]Spore concentration: 10^5–10^6 spores/mL.
[d]After 24 h, this sample inactivated 99% of the spores.

L. pneumophila (Gram-negative bacteria), the leading cause of Legionnaires' disease [44], showed relatively higher resistance toward the complexes; it took the 0.59% iodine complexes 40 min and the 3.46% iodine 20 min to totally kill 10^7–10^8 CFU/mL of the organisms.

The PU–Iodine complexes provided potent antibacterial activity against drug-resistant species, including MRSA BAA-811 and VRE 700221 (Table 1), which are major concerns in healthcare settings and community facilities [45,46]. These results pointed to the potential of these PU–Iodine complexes for a wide range of high-touch, high-risk applications to achieve antimicrobial effects.

The antifungal function of the PU–Iodine complexes was evaluated with *C. albicans*, a mucosal organism and opportunistic pathogen of the immunocompromised and *A. niger*, a common microbial contaminant of food and an important cause of otomycosis and aspergillosis [46,47]. The 0.59% iodine provided a total kill of 10^7–10^8 CFU/mL of *C. albicans* 10231 and *A. niger* 1004 in 10 and 40 min, respectively. The higher iodine contents led to faster antifungal action. The 0.59% iodine achieved a total kill of 10^6–10^7 PFU/mL of the virus, *E. coli* bacteriophage MS2, in 40 min, while for the 3.46% iodine, only 15 min for a total kill of the same species was needed.

Bacteria spores are highly resistant to disinfectant, chemical disinfectants (phenolics, quaternary ammonium compounds, and alcohols) are effective antibacterial agents, but have little or no sporicidal activity [48,49]. Glutaraldehyde, chlorine, and iodine inactivate both bacteria and spores, but the sporicidal effects require higher concentrations and longer contact times. Sporicidal efficacies and inactivation mechanisms of *monomeric* disinfectants, including iodine, have been reported [48,50,51], however, sporicidal activities of polymeric biocides studies are still lacking.

Since sporicidal efficacies are regarded as a direct indication of the antimicrobial power of antiinfective polymers, the PU–Iodine complexes were challenged with *B. subtilis* spores. At 0.59% iodine, the samples inactivated 99% of the spores (10^5–10^6 spores/mL) after 24 h; at 0.96% and 3.46% iodine, total inactivation of the same spores was achieved within 24 and 16 h, respectively.

Biofilm-controlling Functions of PU–Iodine Complex Membranes

Based on the PU–Iodine complexes' ability to effectively kill microbes, their prevention of the formation and development of biofilms was tested. The pure PU membrane and the PU–Iodine membranes containing different amounts of iodine were contacted with *S. aureus* 6538 or *P. aeruginosa* 10145 (both form biofilms [46]) for 1 h to allow initial adhesion and then immersed in the corresponding broth solutions for 24 h. The pure PU membrane surfaces had the highest level of

Table 2. Recoverable S. aureus 6538 and P. aeruginosa 10145 from the pure PU membrane and PU–Iodine complex membranes with different iodine contents after incubation in broth solutions for 24 h.

	Recoverable adherent microorganisms (CFU/cm^2)[a]			
Microorganisms	Pure PU	PU–I_2 complex (0.59%)	PU–I_2 complex (0.96%)	PU–I_2 complex (3.46%)
S. aureus[b]	$(1.7 \pm 0.37) \times 10^8$	$(9.2 \pm 0.18) \times 10^4$	0	0
P. aeruginosa	$(4.2 \pm 0.87) \times 10^6$	$(7.1 \pm 0.15) \times 10^4$	0	0

[a]Every recoverable test was repeated three times.
[b]The *S. aureus* and *P. aeruginosa* concentrations were 10^7–10^8 CFU/mL.

recoverable bacteria: after sonication, 10^8 CFU/cm^2 of adherent *S. aureus* 6538 and 10^6 CFU/cm^2 of adherent *P. aeruginosa* 10145 were recovered after 24 h of contact (Table 2); both bacteria had strongly adhered onto the pure PU surfaces. However, the recoverable levels from the PU–Iodine membranes with 0.59% iodine decreased to 10^4 CFU/cm^2, while no bacteria was recovered from the membrane surfaces with 0.96% and 3.46% iodine, after 24 h of incubation with the bacteria.

The recovery results agreed well with SEM observations (Figure 5) after 24 h of incubation, both species demonstrated a strong tendency to adhere onto the surface of the pure PU, forming layered (Figure 5(a)) or aggregated (Figure 5(b)) micro-colonies that develop into continuous bacterial biofilms. In contrast, the PU–Iodine membrane surfaces with 0.96% iodine were much clearer (Figure 5(c) and (d)); no adherent bacteria or biofilms were observed.

Zone of Inhibition and *In Vitro* Iodine Release

Kirby–Bauer tests with different iodine contents were performed against *S. aureus* 6538 after incubation at 37°C for 24 h. No inhibition zone were observed around pure PU control membrane (Figure 6(a)) and barely detectable with 0.59% iodine (Figure 6(b)). However, the PU–Iodine membrane containing 0.96% iodine generated a 0.8 ± 0.1 mm zone (Figure 6(c)) and for 3.46% iodine, the zone was 7.0 ± 0.6 mm (Figure 6(d)).

The release rate of iodine from the polymer could have opposing effects: for antimicrobial action, a fast release was preferred for powerful and instant efficacy. On the other hand, however, a fast iodine release had lead to a short antimicrobial duration, which was a very important factor that should be taken into account in the design of antimicrobial polymeric systems.

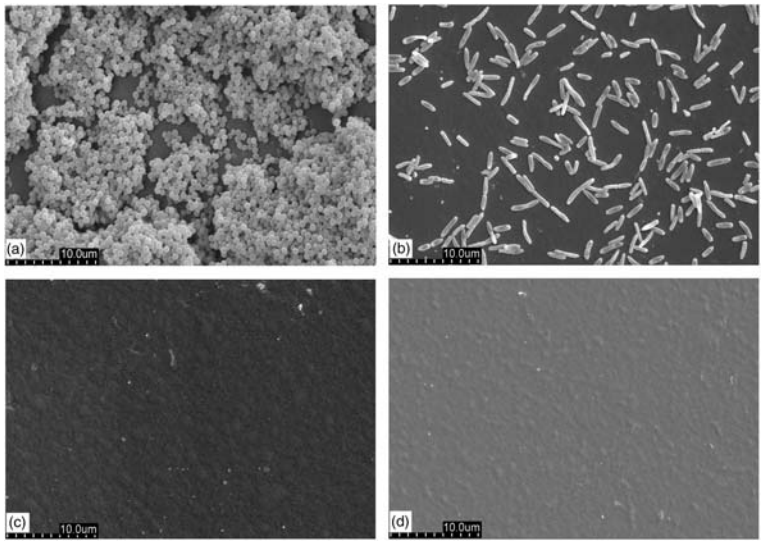

Figure 5. SEM results of biofilm-controlling activities of: (a) pure PU membrane challenged with *S. aureus* 6538, (b) pure PU membrane challenged with *P. aeruginosa* 10145, (c) PU–Iodine complex membrane containing 0.96% of iodine challenged with *S. aureus* 6538, and (d) PU–Iodine complex membrane containing 0.96% of iodine challenged with *P. aeruginosa* 10145; the contact time was 24 h.

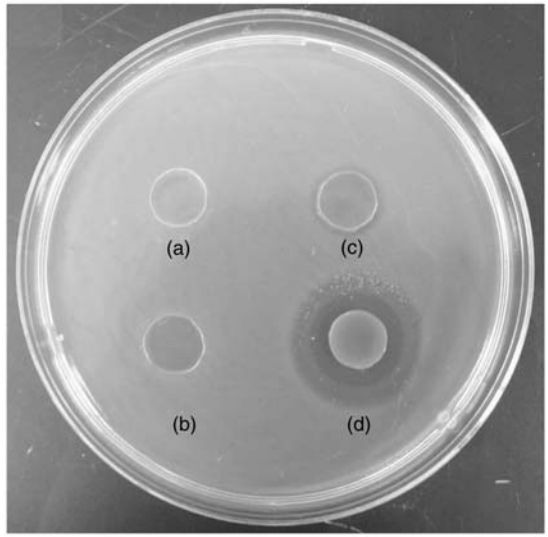

Figure 6. Zone of inhibition test results of: (a) pure PU membrane, (b) PU–Iodine complex membrane with 0.59% of iodine content, (c) PU–Iodine complex membrane with 0.96% of iodine content, and (d) PU–Iodine complex membrane with 3.46% of iodine content.

A quantitative evaluation of the iodine released from the different samples was conducted *in vitro* at 37°C in PBS (Figure 7). For the 0.59% samples (wt%), iodine was continuously increased for 3 days in the PBS solution; after that, the iodine content in the solution was constant at ~4.5 μg/mL (4.5 ppm). These results indicate that the disassociation rate constant of the charge-transfer complex may be very small and this could also be the cause that the PU–Iodine complex did not provide any significant inhibition zone in the Kirby–Bauer test (Figure 6(b)).

In the iodine release tests of PU–Iodine complexes with higher initial iodine contents, however, an interesting 'increasing → decreasing → constant' trend was observed (Figure 7). For example, with 3.46% iodine content in the membrane, 8.0 μg/mL of iodine was released after 2 h and 12.0 μg/mL after 24 h. After that, the iodine content in the solution began to decrease to a relatively constant value ~6.5 μg/mL after 3 days. Similarly, with 0.96% of initial iodine content in the membrane, the solution iodine content of 6.0 μg/mL was achieved on day 3. Afterwards, a lower value of 5.5 μg/mL was observed and remained constant for 7 days. The decreasing trend could be caused by the sublimation of the released free iodine form the solution, particularly at high iodine contents.

Apparently, the release of iodine gradually reduces antimicrobial activity, but many real applications need long-term antimicrobial effects [16]. One potential solution to this problem is to repeatedly recharge the apparatous. To confirm rechargeability, freshly prepared

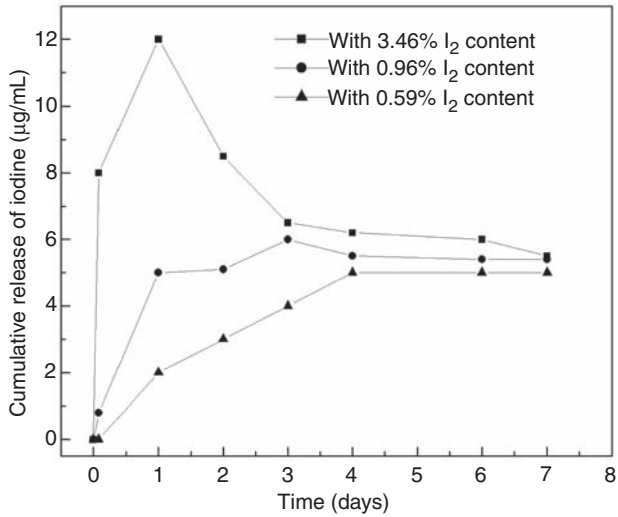

Figure 7. Iodine release into PBS at 37°C from PU–Iodine complex membranes with different iodine contents.

PU–Iodine with different iodine contents (0.59%, 0.96%, and 3.46%) were first treated with 10% sodium thiosulfate aqueous solution at room temperature for 24 h to quench the bound iodine and were then treated with iodine solutions to regenerate the complex, using the same conditions in the preparation of the first generation of the PU–Iodine complex membranes. After 5 cycles of this 'quenching–recharging' treatment, the iodine content in the complexes was essentially unchanged. While many applications may not need this 'rechargeable' feature while some applications may find this function attractive for long-term antimicrobial effects.

Mammalian Cell Viability

Only a few of the CRL-1213^{TM} cells exposed to PU–Iodine complex membranes had trypan blue-stained nuclei (indicating cell death); when viewed by phase-contrast microscopy, the stained cells were similar in size and shape as the unstained cells. Cultures of the cells exposed to pure PU membrane and PU–Iodine complex membranes with different iodine content (0.59%, 0.96%, or 3.46%) showed similar percent of stained cells and intact cellular morphology as seen in Table 3. There were no significant differences in the proportions of trypan blue-stainable cells in cultures exposed for 6 h to the control or any test groups. On the other hand, after 24 h of exposure, the proportion of trypan blue–stained cells in most solutions decreased and there were no significant differences among control and PU–Iodine complex membranes with 0.59% and 0.96% of initial iodine contents. With 3.46% iodine, however, the proportion of trypan blue–stained cells were greater with a statistically significant ($p < 0.05$) cell cytotoxicity effect on CRL-1213^{TM} cells after 24 h. This was attributed the higher concentration of iodine from PU–Iodine complex membranes (Figure 7) which could affect cell viability [51].

Table 3. The effects of PU- iodine complex membranes on CRL-1213^{TM} cell viability evaluated with the trypan blue assay.

Samples	% of undamaged cells after 6 h	% of undamaged cells after 24 h
Cell-only Control	93.7 ± 4.7	97.8 ± 2.7
Pure PU membrane	90.9 ± 7.5	95.4 ± 1.4
PU–Iodine with 0.59% I_2	85.2 ± 8.2	95.3 ± 4.2
PU–Iodine with 0.96% I_2	86.4 ± 4.6	94.7 ± 1.6
PU–Iodine with 3.46% I_2	89.3 ± 10.3	67.9 ± 9.9[a]

[a]Significant difference ($p < 0.05$).

Storage Durability of the PU–Iodine Complexes

Free iodine sublimes readily and so has low storage stability. However, the PU–Iodine complex in the membranes exhibited good stability. Under normal laboratory conditions (25°C, 30–90% RH), PU–Iodine samples, with iodine contents of 0.59%, 0.96%, and 3.46%, were stored for more than 6 months in a closed container. More than 95.5% of the initial iodine was retained and no significant changes in the antimicrobial efficacies were observed.

CONCLUSIONS

PU readily forms charge-transfer complexes with iodine under mild conditions, providing a simple and practical strategy to introduce antimicrobial functionality to biomaterials/devices. The chemical structures of the PU–Iodine complexes were confirmed by UV/Vis study and XPS analysis. The PU–Iodine complexes demonstrated potent antimicrobial activity against Gram-negative and Gram-positive bacteria, including multi-drug resistant species, fungi, and viruses; they also inactivated bacterial spores and inhibited the formation of bacterial biofilms. The antimicrobial effects were due to the slow release of iodine. Both antimicrobial potency and iodine release rates were dependent on the initial iodine content of the complexes. The PU–Iodine complexes did not affect the viability of rat skin cell line CRL-1213^{TM} at <0.96% iodine content. The depleted iodine was repeatedly recharged. The PU–Iodine complex structures were stable under normal storage conditions for at least 6 months. The easy PU–Iodine complex preparation and the unique antiseptic properties of the PU–Iodine complexes denote their potential for a broad range of antimicrobial biomedical applications.

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