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Facile Synthesis, Structure, Biocompatibility and Antimicrobial Property of Gold Nanoparticle Composites from Cellulose and Keratin

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Abstract

A novel, one-pot method was developed to synthesize gold nanoparticle composite from cellulose (CEL), wool keratin (KER) and chloroauric acid. Two ionic liquids, butylmethylimidazolium chloride and ethylmethylimidazolium bis(trifluoromethylsulfonyl)imide were used to dissolve CEL, KER and HAuCl₄. X-ray diffraction and X-ray photoelectron results show that Au³⁺ was completely reduced to Au⁰NPs with size of (5.5 ± 1) nm directly in the composite with NaBH₄. Spectroscopy and imaging results indicate that CEL and KER remained chemically intact and were homogeneously distributed in

the composites with Au⁰NPs. Encapsulating Au⁰NPs into [CEL+KER] composite made the composite fully biocompatible and their bactericidal capabilities were increased by the antibacterial activity of Au⁰NPs. Specifically, the [CEL+KER+Au⁰NPs] composite exhibited up to 97% and 98% reduction in growth of antibiotic resistant bacteria such as vancomycin resistant *Enterococcus faecalis* and methicillin resistant *Staphylococcus aureus*, and was not cytotoxic to human fibroblasts. While [CEL+KER] composite is known to possess some antibacterial activity, the enhanced antibacterial observed here was due solely to added Au⁰NPs. These results together with our previous finding that [CEL+KER] composites can be used for controlled delivery of drugs clearly indicate that the [CEL+KER+Au⁰NPs] composites possess all required properties for successful use as dressing to treat chronic ulcerous infected wounds.

Graphical abstract

Keywords

Ionic liquid, Green, Sustainable, Polysaccharide, Keratin, Wound dressing, Gold nanoparticles, Antibiotic-resistant bacteria

1. Introduction

Gold nanoparticles (AuNPs) have been the subject of intensive research in recent years, due to their intriguing optical, electrical, chemical and biochemical properties. For example, AuNPs are reported to exhibit high antimicrobial activity against both gram-positive and gram-negative bacteria. They have also shown to be an effective antiviral agent.^{1,2,3,4,5,6} The size, morphology and stability of AuNPs are known to strongly affect their antimicrobial and antiviral activity.^{1,2,3,4,5,6,7} It is known that colloidal AuNPs undergo coagulation and aggregation in solution, which, in turn, lead to changes in their size and morphology and hence their antibacterial and antiviral properties. As a consequence, intense

efforts have been made to control the morphologies of AuNPs. One possible remedy is to anchor the AuNPs into a supporting material in order to prevent their coagulation and aggregation so that they can maintain their activity. In fact, AuNPs have been encapsulated in various man-made polymers, and such systems have been reported to retain some of their antimicrobial activity.^{8,9,10,11} For example, anchoring AuNPs onto poly [2-(methacrylamido)-glycopyranose] and poly [2-(methacryloxy)ethyl trimethylammonium iodide] have proved to be effective against a few bacteria.^{8,9,10,11} Unfortunately, reported AuNPs-encapsulated polymers are based mainly on man-made polymers.^{1,2,3,4,5,6,7,8,9,10,11} As such they are not biocompatible, may exhibit some toxicity, and hence may not be used for biomedical applications. It is, therefore, of particular importance to develop a novel method to anchor AuNPs onto composites made from biopolymers such as polysaccharide (cellulose (CEL)) and protein (keratin (KER)) as these composites are not only biocompatible but also sustainable, as CEL and KER are the most abundant biorenewable biopolymers on the earth.

We have demonstrated recently that a simple ionic liquid (IL), butylmethylimidazolium chloride ([BMIm⁺Cl⁻]), can dissolve both CEL and KER and by use of this IL as the sole solvent, we developed a simple, GREEN and totally recyclable method to synthesize [CEL+KER] composites just by dissolution without using any chemical modifications or reactions. Spectroscopy (FTIR, NIR, ¹³C CP-MAS-NMR) results indicate that there was no chemical alteration in the structure of CEL and KER. The [CEL+KER] composites obtained were found to retain unique properties of their components, namely, superior mechanical strength from CEL and controlled release of drugs by KER.^{12,13,14,15,16,17} Because [BMIm⁺Cl⁻] can also dissolve metal salts such as silver chloride, it should be possible to use this IL as the solvent to synthesize [CEL+KER] composite which contains silver ions or silver nanoparticles. In fact, by use of [BMIm⁺Cl⁻] as the sole solvent, we have recently developed a novel method to synthesize composites containing CEL, KER and silver in the form of either ionic (Ag⁺) or Ag⁰ nanoparticles (Ag⁺NPs or Ag⁰NPs).¹⁸ The [CEL+KER+Ag⁰NPs] composite was found to inhibit growth of various bacteria. Unfortunately, both [CEL+KER+Ag⁺NPs] and [CEL+KER+Ag⁰NPs] composites were cytotoxic to human fibroblasts.¹⁸ However, [CEL+KER+Ag⁰NPs] composite was biocompatible when its Ag⁰NPs concentration was at or below 0.48 mmol.¹⁸ It is, therefore, tempting to use this synthetic method to synthesize [CEL+KER] composite which contains gold nanoparticles. This is because, as described above, gold nanoparticles are relatively less toxic and much more biocompatible and, more importantly, can inhibit growth of different types of bacteria and viruses than silver nanoparticles. Unfortunately, since gold metal salt such as chloroauric acid is not soluble in [BMIm⁺Cl⁻], it is not possible to use the synthetic method for silver nanoparticle composites to prepare [CEL+KER+AuNPs] composite.

The information presented clearly indicates that it is possible to use [CEL+KER] as a biocompatible composite to encapsulate AuNPs. Such considerations prompted us to initiate this study which aims to develop a novel, green, and one-pot synthesis to synthesize [CEL+KER+AuNPs] composite. It will be demonstrated in this paper that because another simple IL, ethylmethylimidazolium bis(trifluoromethylsulfonyl)imide ([EMIm⁺Tf₂N⁻]) can dissolve chloroauric acid and is mixable with [BMIm⁺Cl⁻], it was possible for us to develop a novel method in which both ILs, [BMIm⁺Cl⁻] and ([EMIm⁺Tf₂N⁻], were used as solvents to dissolve CEL, KER and HAuCl₄, respectively, to prepare [CEL+KER+Au³⁺] composite. The Au³⁺ was reduced to Au⁰NPs directly, in the composite by NaBH₄. Because the [CEL+KER+Au⁰NPs] composite obtained can prevent the Au⁰NPs from changing size and morphology as well as undergoing coagulation, it should, therefore, fully retain the unique property of the gold nanoparticles for repeated use without any complication of reduced activity and incomplete

recovery after each use. The synthesis, characterization, and property of the composite, including its antimicrobial activity and biocompatibility will be reported in this communication.

2. Materials and methods

2.1. Chemicals

Microcrystalline cellulose (DP $\approx$ 300) and HAuCl_4 were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Raw (untreated) sheep wool, obtained from a local farm, was cleaned by Soxhlet extraction using a 1:1 (v/v) acetone/ethanol mixture at 80 ± 3 °C for 48 h. The wool was then rinsed with distilled water and dried at 100 ± 1 °C for 12 h.^{12,13,14,15} 1-Methylimidazole, ethylimidazole and *n*-chlorobutane (both from Alfa Aesar, Ward Hill, MA) were distilled and subsequently used to synthesize $[\text{BMIm}^+\text{Cl}^-]$ and $[\text{EMIm}^+\text{Cl}^-]$. The latter was converted to $[\text{EMIm}^+\text{Tf}_2\text{N}^-]$ using method previously reported.¹⁹ Nutrient broth (NB) and nutrient agar (NA) were obtained from VWR (Radnor, PA). Minimal essential medium (MEM), Fetal Bovine Serum (FBS), and Penicillin-Streptomycin were obtained from Sigma-Aldrich (St. Louis, MO), whereas PBS, and trypsin solution (Gibco) were obtained from Thermo Fischer Scientific (Waltham, MA). CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay was obtained from Promega (Madison, WI).

2.2. Bacterial and cell cultures

The bacterial cultures of methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 33591, vancomycin resistant *Enterococcus faecalis* (VRE) ATCC 51299, and the cell culture of human fibroblasts ATCC CRL-2522 were purchased from the American Type Culture Collection (Rockville, MD).

2.3. Synthesis

$[\text{CEL}+\text{KER}+\text{AuNPs}]$ composites were synthesized with minor modifications to those used for $[\text{CEL}/\text{CS}+\text{KER}]$ composites.^{12,13,14,15,19} As shown in Scheme 1, washed wool was dissolved in $[\text{BMIm}^+\text{Cl}^-]$ at 100–110 °C. Once dissolved, CEL was then added to the KER solution. Using this procedure, $[\text{BMIm}^+\text{Cl}^-]$ solution of CEL and KER containing up to total concentration of 6 wt% (relative to IL) with various compositions and concentrations were prepared. Concurrently, in a separate flask, 240 mg of HAuCl_4 was dissolved in 2 mL of $[\text{EMIm}^+\text{Tf}_2\text{N}^-]$, and the mixture was then added dropwise to the $[\text{BMIm}^+\text{Cl}^-]$ solution of $[\text{CEL}+\text{KER}]$. The resulting solution was casted onto PTFE molds with desired thickness on Mylar films to produce thin composite film of $[\text{CEL}+\text{CS}+\text{Au}^{3+}]$. They were then kept at room temperature for 24 h to allow for gelation to yield Gel Films. The Gel Films were washed in 400 mL of 50:50 (v/v) THF:H₂O for 24 h to remove $[\text{EMIm}^+\text{Tf}_2\text{N}^-]$, and then with water for 4–6 days to completely remove $[\text{BMIm}^+\text{Cl}^-]$ to yield Wet Films. Washing water (2 L for a composite film of about 10cmX10cm) was repeatedly replaced with fresh water every 24 h until it was confirmed that IL was not detected in the washed water (by monitoring UV absorption of the IL at 290 nm). It was found that after washing for 72 h, no IL was detected in the washing water by UV measurements. Since the limit of detection of the spectrophotometer used in this work was estimated to be about 3×10^{-5} AU, and the molar absorptivity of $[\text{BMIm}^+\text{Cl}^-]$ at 290 nm is $2.6 \text{ M}^{-1}\text{cm}^{-1}$, it is estimated that if any $[\text{BMIm}^+\text{Cl}^-]$ remained, its concentration would be smaller than 2 $\mu\text{g}/\text{mL}$ of the washed water and 2 $\mu\text{g}/\text{g}$ of the composite film. Since this concentration is two orders of magnitude lower than the LD₅₀ value of the $[\text{BMIm}^+\text{Cl}^-]$,²⁰ if any IL remains in the composite films, it would not pose any harmful effect. Furthermore, as we have previously shown that results of UV–vis, FTIR and NIR techniques confirmed that when the composite films were washed with water, $[\text{BMIm}^+\text{Cl}^-]$ was removed from the films to a level not detectable by these techniques.^{12,13,14,15,16,17,18,19,20} Subsequently, the Au^{3+} doped Wet Films

were reduced with NaBH_4 to Au^0NPs . For example, the Wet Film, sandwiched between two PTFE meshes, was placed in 400 mL of 20 mM of NaBH_4 in methanol at room temperature for 24 h. The reduced film was then washed and dried slowly ($\sim 3\text{--}5$ days) at room temperature in a humidity-controlled chamber to yield $[\text{CEL}+\text{KER}+\text{Au}^0\text{NPs}]$ composite.

Scheme 1.

2.4. Analytical characterization

FTIR spectra (from 450 to 4000 cm^{-1}) were recorded on a Spectrum 100 Series FTIR spectrometer (Perkin Elmer, MA) at resolution of 2 cm^{-1} by the KBr method. Each spectrum was an average of 64 individual spectra. X-ray diffraction (XRD) measurements were taken on a Rigaku MiniFlex II diffractometer utilizing the Ni filtered $\text{Cu K}\alpha$ radiation ($1.54059\text{ \AA}$). The voltage and current of the X-ray tube were 30 kV and 15mA respectively. The samples were measured within the 2θ angle range from 2.0 to 40.00 . The scan rate was 5° per minute. Data processing procedures were performed with the Jade 8 program package.^{12,13,14,15,16,17,18,19,20} X-ray photoelectron (XPS) spectra were taken on a HP 5950 A ESCA spectrometer with Al monochromatic source and a flood gun used for charge suppression. The surface and cross-sectional morphologies of the composite films were examined under vacuum with a JEOL JSM-6510LV/LGS Scanning Electron Microscope with standard secondary

electron (SEI) and backscatter electron (BEI) detectors. Prior to SEM examination, the film specimens were made conductive by applying a 20 nm gold-palladium-coating onto their surfaces using an Emitech K575x Peltier Cooled Sputter Coater (Emitech Products, TX).

2.5. *In vitro* antibacterial assay

The composites [CEL+KER+Au⁰NPs] were tested for potential antibacterial activity against antibiotic resistant bacteria such as methicillin resistant *S. aureus* (ATCC 33591) (MRSA) and vancomycin resistant *Enterococcus faecalis* (ATCC 51299) (VRE), using previously published protocol.^{12,17,18,21} Prior to the assays, cultures were grown overnight at 37 °C and 150 rpm. Composites were cut into 3 mm × 20 mm strips and autoclaved at 121 °C, 15 psi for 20 min. The overnight cultures were diluted to 2 mL and put in contact with the material for 24 h. Test tubes with bacteria not exposed to any composite served as a control, whereas bacteria exposed to [CEL+KER] without Au⁰NPs served as a blank. The tubes were incubated for 24 h at 37 °C and 600 rpm. Before (at time 0) and after the exposure (24 h), the bacteria were diluted and plated onto nutrient agar plates, which were then incubated overnight at 37 °C. Colony forming units (CFUs) were counted the next day and compared to the corresponding CFU numbers at time 0. The results were expressed as Log of reduction in number of bacteria, calculated as $[\log (N_0/N_{24})]$, where N_0 is the number of CFUs at the beginning of the experiment, and N_{24} is the number of bacteria after 24 h. All experiments were carried out in triplicates; the variability between them was expressed as a standard error.

2.6. Biocompatibility assay

The biocompatibility of [CEL+KER+Au⁰NPs] composites was evaluated with the culture of human fibroblasts (ATCC CRL-2522) through 3 and 7 days as previously published.^{12,17,18,21} The composites in shape of circles with 7 mm in diameter were prior to the experiment thermally sterilized at 121 °C, 15 psi for 20 min. Human fibroblasts were grown in a sterile minimal essential medium (MEM) supplemented with 10% FBS and 1% Penicillin-Streptomycin according to ATCC guidelines, and incubated at 37 °C in a humidified atmosphere of 5% CO₂ until the 3rd passage. Cells were seeded in a 24-well plate at a concentration of 2×10^4 cells/mL as specified in guidelines for proliferation assay (Promega) and left for 1 day to allow for their attachment. The following day the sterilized composites were added to the wells and incubated with the cells for 3 and 7 days. Some wells did not contain composites and served as a control, whereas other wells contained [CEL+KER] composites without Au⁰NPs and served as a blank. After the incubation the viability and morphology of cells were evaluated with both, colorimetric CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay, and Olympus microscope camera with CellSens imaging software. The procedure for the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay was followed as specified in the manufacturer's manual. In brief, the MTS reagent was added in a 1:5 ratio to each well after the medium in wells was supplemented with a colorless MEM. The cells were incubated at standard culture conditions for 3 h, and the optical density was measured with a Perkin Elmer HTS 7000 Bio Assay Reader at 490 nm. The percent viability was calculated using the following equation:

(1)

$$\%cellviability = \frac{OD_{Testsample}}{OD_{Control}} \times 100$$

where $OD_{\text{Test sample}}$ is the measured OD of the test sample well, and OD_{Control} is the measured OD of the control well. Material was considered to be cytotoxic if viability of cells after the incubation was below 70% as specified in ISO 10993-5:2009(E).²² All experiments were carried out in triplicates; the variability between them was expressed as a standard error.

3. Results and discussion

3.1. FT-IR

FTIR spectrum of the [CEL+KER+Au⁰NPs] composite is shown as the black spectrum in Fig. 1. For reference, spectrum of the [CEL+KER] composite is also added (red spectrum). As expected, the red spectrum of the [CEL+KER] is similar to those previously observed for the [CEL+KER] composites, namely bands at $\sim 1650\text{ cm}^{-1}$ and $\sim 1530\text{ cm}^{-1}$ are due to amide C=O stretch (amide I) and C—N stretch (amide II) vibrations, and at $1300\text{--}1200\text{ cm}^{-1}$ are from the in-phase combination of the N—H bending and the C—N stretch vibrations (amide III).^{12,13,14,15} Major bands between 1200-- and 900 cm^{-1} are due to sugar ring deformations of the CEL.^{12,13,14,15} As shown, the black spectrum of the [CEL+KER+Au⁰NPs] composite is relatively similar to the red spectrum of the [CEL+KER] composite. It seems, therefore, that there may not be strong interaction between the Au⁰NPs and CEL and KER in the composite. However, careful inspection of the spectra, shown as four vertical dashed lines in the graph for clarification, reveals that there are, in fact, minor differences between the two spectra. It seems that interactions between Au⁰NP and C=O group lead to a shift in the intense amide band at 1646 cm^{-1} and the smaller band at 1529 cm^{-1} (of the [CEL+KER] composite) to 1649 cm^{-1} and 1532 cm^{-1} (of the [CEL+KER+Ag⁰NP] composite), respectively. Furthermore, bands due to CEL were also shifted when Au⁰NPs are present in the composite. Specifically, the sugar ring deformation band at 1062 cm^{-1} in [CEL+KER] composite shifted to 1065 cm^{-1} , and the O—H band at 2919 cm^{-1} shifted to 2023 cm^{-1} when Au⁰NPs were added to the composite. These results suggest that KER and CEL may interact with Au⁰NP through the amide groups of the former, and the O—H groups of the latter.^{12,13,14,15}

Fig. 1. FTIR spectra of [CEL+KER] composite (red) and [CEL+KER+705 μmol AuNPs] composite (black).

3.2. Powder X-ray diffraction (XRD)

X-ray diffractogram of [CEL+KER+Au⁰NPs] composite is shown in Fig. 2. Because both, CEL and KER, are present in the composite, as expected, the diffractogram exhibits a large peak at around $2\theta = 21.30^\circ$ which is due to CEL and KER. In addition to this peak, the diffractogram also has four peaks at (2θ) = 36.78° , 44.56° , 65.06° and 78.05° . The fact that these peaks correspond well with Miller indices of (1 1 1), (2 0 0) and (2 0 0) and (3 1 1) of metallic gold nanoparticles confirms that Au³⁺ were successfully reduced to Au⁰ and present as Au⁰NPs in the composite.^{23,24,25,26}

Fig. 2. Powder X-ray diffractogram of [CEL+KER+705 μ mol AuNPs] composite.

Scherrer equation was then used to determine the size (τ value) of the Au⁰NPs in the composites from the full width at half maximum (FWHM, β value in the equation) of its corresponding XRD peaks.^{27,28}

(2)

$$\tau = \frac{k\lambda}{\beta \cos \theta}$$

where τ is the size of the nanoparticle, λ is the X-ray wavelength, and k is a constant.^{27,28} The size of the metallic gold nanoparticle in the [CEL+KER+Au⁰] composite was found to be (5.5 ± 1) nm.

3.3. Scanning electron Microscope (SEM) images and Energy Disperse Spectroscopy (EDS) analysis

Fig. 3 shows the surface (left) and cross-sectional (right) SEM images of [CEL+KER+705 μ mol Au⁰NPs] composite. As expected, these images are similar to those reported previously for [CEL+KER] composite namely, the composite is homogenous, somewhat porous and has a rough surface.^{12,13,14,15} This may be due to the fact that while CEL exhibits smooth and homogeneous morphology without any pores, KER is known to have a rough and porous structure with a three dimensional interconnection throughout the film surface.^{12,13,14,15} This porous structure seems to reflect the physical properties of KER films such as its brittleness.^{12,13,14,15} As a consequence, incorporating CEL into KER matrix results in a composite which is rough and porous. More information on the chemical composition and

distribution of the Au⁰NPs can be found in Fig. 3B and C. Three images shown in Fig. 3B, are EDS image recorded for gold (left), carbon (center) and nitrogen (right). It is evident from this images that not only CEL and KER but also Au⁰NPs are homogenously distributed throughout the composite. The EDS spectrum (Fig. 3C) show that in addition to the two major bands at around 284 eV and 531 eV which are due to carbon and oxygen (of CEL and KER in composite).^{17,24} the third major band at ~2 eV eV can be assigned to Au as this band is similar to those reported previously for gold.²⁴

Fig. 3. (A) SEM images of [CEL+KER+705 μmol AuNPs] composite; (B) EDS images, recorded for gold (left), carbon (middle) and nitrogen (right) of [CEL+KER+705 μmol AuNPs] composite; and (C) EDS spectrum of the composite.

3.4. X-ray photoelectron Spectroscopy (XPS)

Fig. 4 shows the X-ray photoelectron of [CEL+KER+705 $\mu\text{mol Au}^0\text{NPs}$] composite. Two major bands at 284.4 eV and 532.0 eV, and their expanded view in Fig. 4C and 4D, can be assigned to C 1s and O 1s, respectively.^{25,29,30,31,32} Since the content of gold in the composite is rather low, it is not surprising that its signal is not clear in Fig. 4A. However, as shown in Fig. 4B, when the region around 85 eV was magnified and expanded, a prominent doublet at 83.8 eV and 87.5 eV was clearly seen. Based on the fact that this doublet is characteristic of Au^0 4f_{7/2} and 4f_{5/2}, respectively, and the absence of any band due to Au^{3+} at around 86 eV indicates that all Au^{3+} was reduced to Au^0 in the composite.

Fig. 4. X-ray photoelectron of [CEL+KER+705 $\mu\text{mol Au}^0\text{NPs}$] composite. (B), (C) and (D) are expanded plots of (A).

3.5. Antibacterial assay

As described in the introduction, various Au^0NPs encapsulated polymers have shown to be bactericide against both gram-positive and gram-negative bacteria such as *E. coli*, *S. aureus*, *Shigella flexneri*, *Proteus mirabilis*, *Bacillus cereus* and *Bacillus subtilis*.^{8,9,10,11} However, to date, antimicrobial activity of Au^0NPs -encapsulated composites/polymers against antibiotic resistant bacteria, such as methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) have not been investigated.^{8,9,10,11} Since growth inhibition of such antibiotic resistant bacteria is of particular importance, we decided to investigate antimicrobial activity of the [CEL+KER+705 $\mu\text{mol Au}^0\text{NPs}$] against these bacteria. To assess the antimicrobial properties of the composite, the bacteria were grown in the presence of the composite and then plated out onto nutrient agar and measured by the number of colonies formed compared to those for the blank ([CEL+KER] composite) and the control (no material). Each assay was carried out three times. The results were calculated as microbial log of reduction and are shown in Fig. 5. It is evident from the figure that the [CEL+KER+705 $\mu\text{mol Au}^0\text{NPs}$] composite effectively and substantially inhibited growth of both antibiotic resistant bacteria VRE and

MRSA. Specifically, up to (1.50 ± 0.03) and (1.66 ± 0.04) logs of reduction were found for VRE and MRSA, respectively, which correspond to 97% and 98% growth inhibition. It is important to point out that the antibacterial effect, which we report here, is due solely to the Au⁰NPs. As we have previously reported, [CEL+KER] composite also exhibits some antibacterial property.^{12,13,14,15} However, because the antibacterial activities of the [CEL+KER+705 μ mol Au⁰NPs] composite reported here were compared to those of the blank (i.e., [CEL+KER] composite without Au⁰NPs) and the control (no composite), the reported bactericidal effect is entirely due to the Au⁰NPs.

Fig. 5. Log of reduction for selected bacteria after 24 h of exposure to [CEL+KER+705 μ mol AuNPs]. Each bar represents an average $n = 3 \pm \text{SEM}$.

3.6. Biocompatibility assay

To assess a potential cytotoxicity of the [CEL+KER+705 μ mol Au⁰NPs] composite, the morphology and the proliferation capabilities of adherent human fibroblasts in presence and absence of biocomposites were analyzed. The proliferation capability was assessed using a colorimetric assay CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay, whereas the morphology of fibroblasts was examined microscopically. A material was considered to be cytotoxic if the viability of fibroblasts after the exposure was lower than 70% of control, as specified in ISO 10993-5: 2009(E).²² Fibroblasts were exposed to composites for 3 and 7 days. Viability of fibroblasts in the presence or absence of the [CEL+KER+705 μ mol Au⁰NPs] composite over time is shown in Fig. 6. Cells exposed to [CEL+KER+705 μ mol Au⁰NPs] showed no statistically significant difference (at 95% confidence interval) compared to the control. Neither at 3 or 7 days the viability of cells dropped under 70%, which indicates that [CEL+KER+705 μ mol Au⁰NPs] was not cytotoxic to human fibroblasts. Morphological data in Fig. 7 showed that the cells that were in contact with [CEL+KER+705 μ mol Au⁰NPs] composite looked relatively healthy. After 3 days they exhibited an unusual morphology to some extent with thickened central part of their long bodies (Fig. 7C), but were still adherent, whereas after 7 days their morphology looked normal (Fig. 7F) and was not different from that of the cells in control and blank wells (Fig. 7D and E).

Fig. 6. Fibroblast viability expressed as % of control after being exposed to either no composite, to blank ([CEL+KER]), or to [CEL+KER+705 μ mol AuNPs], for 3 (blue bars) and 7 days (red bars). Each bar represents an average of $n = 3 \pm \text{SEM}$. Materials causing $<70\%$ cell viability (dashed line) are considered cytotoxic.

Fig. 7. Images (100X) of human fibroblasts after 3 days (A, B, and C) and after 7 days (D, E and F): (A) and (D): in the absence of any composite; (B) and (E): with [CEL+KER] composite; and (C) and (F): with [CEL+KER+705 μ mol AuNPs] composite.

Reports on biocompatibility of biopolymer-bound AuNPs are rather limited, whereas studies on colloidal AuNPs report conflicting data regarding their biocompatibility.^{33,34,35,36,37} For example, studies using a range of larger colloidal AuNPs (30–90 nm) suggest their cytotoxicity is not size dependent,^{33,34,35,36,37} whereas others suggest that AuNPs of smaller sizes (<15 nm) penetrate the plasma membrane and cause adverse effects to mammal cells.^{33,34,35,36,37} In this study, we clearly and

unequivocally demonstrate, for the first time, that any possible cytotoxicity of Au⁰NPs can be removed by incorporating them into the [CEL+KER] composite. More importantly, the [CEL+KER+Au⁰NPs] composite is not only fully biocompatible but also fully retains its antimicrobial activity against antibiotic resistant bacteria such as VRE and MRSA.

It is of particular interest to compare the [CEL+KER+Au⁰NPs] composite to the [CEL+KER+Ag⁰NPs] composite which we reported recently.¹⁸ We have shown that the silver nanoparticle composite exhibits strong antimicrobial activity against various bacteria including *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, VRE and MRSA, and its bactericidal property was correlated with the concentration of Ag⁰NPs in the composite. While the composite exhibited excellent antimicrobial activity at high Ag⁰NPs content, it was rather cytotoxic to human fibroblasts. Fortunately, at Ag⁰NPs of 480 μmol or below, the composite became biocompatible and still exhibited antibacterial properties. However, at concentration of 480 μmol Ag⁰NPs, the composite exhibited reduced growth of VRE and MRSA by only (1.04 ± 0.08) and (0.28 ± 0.08) logs of reduction, respectively, which correspond to 90% and 47% growth inhibition. Conversely, as expected, the [CEL+KER+705 μmol Au⁰NPs] composite was not only bactericidal but was also much more biocompatible with human fibroblasts. In fact, at Au⁰NPs concentration of 705 μmol which is 1.5X higher than the 480 μmol of Ag⁰NPs, the [CEL+KER+Au⁰NPs] is not only fully biocompatible but also exhibits stronger antimicrobial activity (97% and 98% against VRE and MRSA, respectively) compared to the [CEL+KER+480 μmol Ag⁰NPs] composite for corresponding bacteria.

4. Conclusions

In summary, we have shown that gold nanoparticle composite was successfully and readily prepared from cellulose, wool keratin and chloroauric acid, in a simple one-pot synthesis in which two ionic liquids, [BMIm⁺Cl⁻] and [EMIm⁺Tf₂N⁻], were used as the solvents. XRD and XPS results show that Au³⁺ was completely reduced to Au⁰NPs with size of (5.5 ± 1) nm directly in the composite with NaBH₄. FTIR results indicate that CEL and KER remain chemically intact in the composites. SEM and EDS measurements confirm that CEL, KER and Au⁰NPs were homogeneously distributed in the composites. Results of antimicrobial assays and biocompatibility show that encapsulating Au⁰NPs in this [CEL+KER] composite enables the composite to be fully biocompatible while extending the bactericidal effect of the [CEL+KER] composite by adding Au⁰NPs. Specifically, the [CEL+KER+705 μmol Au⁰NPs] composite exhibited up to 97% and 98% reduction in growth of multidrug resistant bacteria, such as VRE and MRSA, and was not cytotoxic to human fibroblasts. While [CEL+KER] composite is known to possess some antibacterial activity,¹³ the enhanced antibacterial observed here was due solely to added Au⁰NPs. This is because reported antibacterial activities are those of the [CEL+KER+Au⁰NPs] composite compared to [CEL+KER]. It is of particular interest to compare the [CEL+KER+Au⁰NPs] composite to the [CEL+KER+Ag⁰NPs] composite which we reported recently [18]. While the [CEL+KER+Ag⁰NPs] composite exhibited high antimicrobial activity, it was rather cytotoxic to human fibroblasts at high Ag⁰NPs concentration. Because Au⁰NPs is relatively more biocompatible compared to Ag⁰NPs, the [CEL+KER+705 μmol Au⁰NPs] composite, which had a concentration of Ag⁰NPs 1.5X higher than Ag⁰NPs in [CEL+KER+480 μmol Ag⁰NPs] composite, was found not only fully biocompatible but also a stronger bactericide. These results, together with our previous finding that [CEL+KER] composites can be used for controlled delivery of drugs, such as ciprofloxacin¹³ clearly indicate that the [CEL+KER+Au⁰NPs] composite possesses all required properties to be successfully used as high-performance dressing to treat chronic ulcerous infected wounds. Furthermore, because of unique properties of Au⁰NPs, this biocompatible [CEL+KER+Au⁰NPs] composite can also be potentially used for many other applications,

including biosensors, therapeutic agents, and other drug delivery systems. These are subject of our current intense investigation.

Notes

The authors declare no competing financial interest.

Acknowledgment

The authors are grateful to Prof. Raymond Fournelle for his competent assistance to measure SEM, Gerald Benzi, Moven Mututuvvari and James Makuvaza for their assistance. One of us (FP) thanks the Fulbright Foundation for a fellowship support to spend time doing research at Marquette University.

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