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Antibacterial and Cytotoxicity Effects of TiO₂-Grafted Cellulose Nanocomposite

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Abstract

TiO₂-grafted cellulose bio-nanocomposite (Cell.Com) was successfully prepared by combining modified cellulose and TiO₂ nanoparticles via copper-catalyzed alkyne-azide click reaction. The antibacterial and cytotoxicity effects of Cell.Com containing cellulose, triazole ring and TiO₂ moieties are reported in this work. The antibacterial activity of Cell.Com was investigated against gram positive (*S. aureus* and *B. subtilis*) and gram negative (*E. coli* and *P. aeruginosa*) bacteria. The antibacterial results indicate that the Cell.Com exhibits a wide range of antibacterial activity against both gram positive and gram negative bacteria which is due to the presence of TiO₂ and triazole ring moieties in its structure. In addition, the cytotoxicity effect of Cell.Com was studied in vitro on cervical cancer (HeLa), breast carcinoma (MCF-7), sarcoma osteogenic (Saos) and fibroblast cells by MTT assay. The cell viability results show that the Cell. Com diminishes relatively cancer cells growth in a dosage and time dependent way probably through triazole moiety, while no apparent cytotoxicity effect is observed on the normal fibroblast cells.

Keywords: *Nanocomposite, Cellulose, TiO₂, Antibacterial, Cytotoxicity.*

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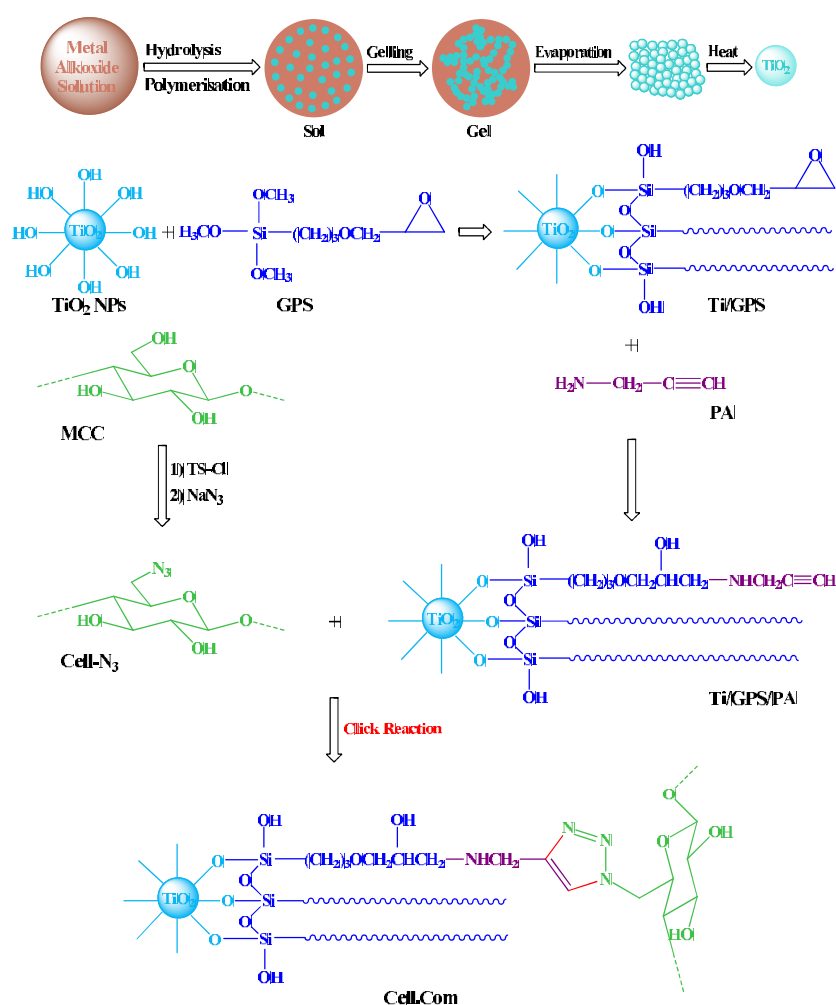
Introduction

With growing environmental awareness, green composites based on biodegradable and renewable natural resources have received increasing attention due to being harmless for ecological system than synthetic petroleum based polymers. Bio-nanocomposites are an important type of environmentally benign hybrid materials which are prepared from natural polymers and nano organic/inorganic fillers [1]. Nanocomposites often present the best properties of each of their components in a synergic way and offer outstanding advantages, efficiency, versatility and applicability in a broad range of chemistry and biology [2]. Recently, cellulose nanocomposites have attracted immense consideration owing to the renewability, biodegradability and biocompatibility [3]. Cellulose with many hydroxyl groups has been applied as a suitable substrate to embed metal oxide fillers to produce composites [4, 5]. Among the nanofillers, TiO₂ nanoparticle (TiO₂ NP) is a promising biocompatible metal oxide that is in the center of attentions because of its unique physicochemical properties [6] and multipurpose bioactivities such as antibacterial effect [7-10]. The antibacterial effect of TiO₂ NPs can be attributed to their small size that easily penetrates the cell wall structure [11]. TiO₂ nanoscale size leads to agglomeration and reduces surface area [12], whereas it is known that the efficiency of nanofillers strongly depends on their dispersion as well as on the interfacial strength with polymer matrix [13]. Modification of TiO₂ NPs with silane coupling agents is the useful method for homogeneous immobilization of nanoparticles on the cellulose matrix [14, 15]. In addition, it provides the possibility of further chemical modifications to achieve more efficiencies [16]. It could be accomplished by anchoring the TiO₂ NPs with triazole rings [17], exhibiting important biological activities including antimicrobial and anticancer [18-20]. Cu-catalyzed click chemistry is an attractive efficient chemical reaction for the nanocomposite preparation [21] and has great relevance to enhance the properties of nanocomposites via chemical grafting of modified nanoparticles with functionalized polymers by triazole rings [22]. There is a need to screen new synthesized compounds with different modes of action that might show antibacterial and anticancer activity. For this reason, we have prepared a bio-nanocomposite of microcrystalline cellulose/TiO₂ via click reaction and its antibacterial and cytotoxicity activities were investigated against gram positive (*S. aureus* and *B. subtilis*) and gram negative (*E. coli* and *P. aeruginosa*) bacteria, and against three cancer cell lines (HeLa, MCF-7 and Saos) and normal cell (human foreskin fibroblast).

Experimental

Materials

Dimethyl sulfoxide (DMSO) and nutrient Mueller-Hinton agar were purchased from Merck (Germany). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was provided from Alfa Aesar (Lancashire, UK). Dulbecco's modified eagle's medium (DMEM), penicillin and streptomycin were purchased Biowest (France). Fetal bovine serum (FBS) was from Gibco (Germany). Cervical cancer (HeLa), breast carcinoma (MCF-7) and sarcoma osteogenic (Saos) were obtained from Pasteur Institute of Iran (Tehran) and the fibroblast cell line was derived from newborn human foreskin by non-enzymatic method at Amirkola children's hospital (Babol, Iran).



Scheme 1. Synthetic path for Cell.Com preparation.

Synthesis of TiO_2 -grafted cellulose bio-nanocomposite (Cell.Com)

The Cell.Com was successfully synthesized according to the synthetic path shown in scheme 1. Briefly, the Cell.Com was prepared by reacting alkyne terminated TiO_2 nanoparticles (Ti/GPS/PA) with azide terminated cellulose (Cell- N_3) via click reaction. The cellulose azide was achieved by the

functionalization of microcrystalline cellulose (MCC) with *p*-toluene sulfonyl chloride (TS-Cl) followed by reacting with sodium azide (NaN₃). TiO₂ nanoparticles were treated consecutively with 3-glycidoxypropyltrimethoxysilane (GPS) and propargylamine (PA) to generate Ti/GPS/PA. The synthesized bio-nanocomposite was characterized in detail and reported in our previous paper [23].

Biological evaluation

Antibacterial activity study

The Kirby-Bauer disk diffusion method was used to investigate the antibacterial activity of Cell.Com [24]. The tablets of Cell.Com (0.1 g, diameter: 13 mm) were placed on agar dishes infected with bacteria. The Cell.Com antibacterial effect was evaluated against gram negative bacteria: *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*); and gram positive bacteria: *Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis*). The incubated bacteria in nutrient Mueller-Hinton broth media at 37 °C for 24 h, led to bacterial concentration in the seeding culture of about 10⁸ colony forming units (CFU)/mL. Muller-Hinton agar was inoculated from the standardized cultures of the test organisms which then spread uniformly throughout the whole media. Afterwards, the prepared tablets of the Cell.Com were put on the upper layer of seeded agar plate in at 37 °C incubator for 24 h. Commercially available discs of gentamicin (10 mg per disc) and chloramphenicol (30 mg per disc) antibiotics were used as positive controls to evaluate the antibacterial activity of Cell. Com. The antibacterial efficiency was estimated from clear zone inhibition diameter (mm) around the samples after 24 h incubation. All samples/standards were run in triplicate.

Cytotoxicity study

Cytotoxicity assay is a test for analyzing the cytotoxic effects of compounds on the living organism via the cell viability assessment. The cytotoxicity effect is the simplest and earliest in vitro technique for biocompatibility evaluation of materials. MTT cytotoxicity assay is a standard method for the screening of cytotoxicity effect of materials. So, the in vitro Cell.Com cytotoxicity was investigated against cervical cancer (HeLa), breast carcinoma (MCF-7), sarcoma osteogenic (Saos) and fibroblast (Fib) cells by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay [25]. This method is based on the formation of formazan crystals by the reduction of the MTT tetrazolium salt [26]. The cells (8×10³ cells/well onto 96-well) were cultured in complete growth medium [Dulbecco's modified eagle's medium (DMEM; PAA cat: E15-883), 10% fetal bovine serum (FBS; PAA cat: A15-15) and 1% antibiotics (penicillin and streptomycin; PAA, cat: p11-010)] and then incubated at 37 °C in an atmosphere of 5% CO₂ for 24 h to allow cell attachment. Then cells were treated with the Cell.Com with different concentrations for 24 and 72 h

along with replacing of the cell culture medium by a fresh medium and further incubated for 24 h. The cells without any treatment were served as a control and grown on each plate under the same conditions as treated cells. After incubation, the medium was removed, washed with PBS and then MTT reagent (50 mL; 5 mg L⁻¹ in PBS) was added to each well and further incubated for 4 h at 37 °C. The absorbance of formazan in DMSO (Optical density, OD) was measured at 570 nm by microplate reader (Rayto analyzer, CHINA) to determine the %cell viability. The cell viability is calculated from the following Eq. (1) [27]:

$$\% \text{Cell Viability} = \frac{\text{Experimental OD}}{\text{Control OD}} \times 100 \quad (1)$$

Statistical analyses

All experiments were carried out in triplicates and the data were presented as mean ± SD. The statistical significance was determined using the two-sided student's T-test at a p-value < 0.05.

Results and discussion

Antibacterial activity evaluation

Recently, infections caused by multidrug-resistant microorganisms have reached critical levels. So, the synthesis of polymeric nanoparticles is being evaluated for the expansion of new and effective antimicrobial agents. Currently, antimicrobial polymeric materials have been investigated as potential new antibiotics to struggle with the ascension of infectious diseases [28]. The in vitro antibacterial efficiency of Cell.Com was evaluated against gram positive: *B. subtilis* and *S. aureus*, and gram negative: *P. aeruginosa* and *E. coli* bacteria. The antibacterial properties of Cell.Com are shown in Fig. 1 and the results of bacterial inhibition are compared with chloramphenicol and gentamicin antibiotics as controls in Figure 2. As it can be seen, the Cell Com exhibits antibacterial effect against both gram positive and gram negative bacteria. It is well known that crucial step for disinfection of bacteria is to destroy the bacterial outer wall and membrane [8]. Microorganisms have different outer membrane [29], for example, the cell wall of *E. coli* (15-20 nm) is thinner than *S. aureus* (20-80 nm) [8, 30] which are easily destroyed. Among the three moieties in the Cell.Com structure (TiO₂, triazole and cellulose), microcrystalline cellulose does not display any antibacterial effect [31], and on the basis of reported antibacterial activity of the TiO₂NPs [32, 33] and triazole ring [34, 35], it is assumed that the observed antibacterial effect of Cell.Com is related to synergic effects of the TiO₂ and triazole moieties. However, it is proposed that the oxygen and nitrogen atoms on the surface of Cell.Com initially interact with bacteria to capture them [29], then the nano-sized particles of TiO₂ could create pores in cell walls of bacteria resulting in enhanced

permeability, thus causing DNA damage and cell death [9, 36, 37]. Besides, 1,2,3-triazole rings with remarkable antimicrobial activity could act further as DNA cleaving agents [38].

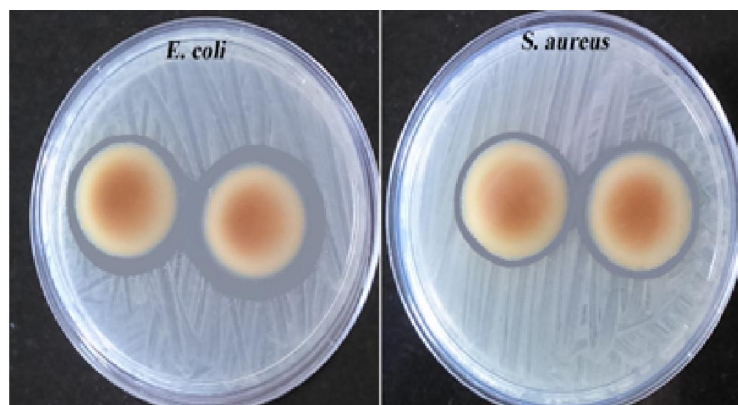


Figure 1. The inhibition zone of Cell.Com against different bacteria.

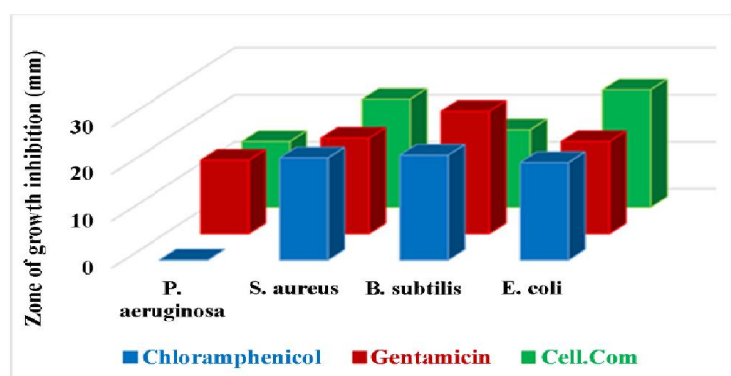


Figure 2. The antibacterial activity comparison.

Cytotoxicity evaluation

Cancer is the second most common cause of death. After breast cancer, cervical cancer is the most abundant cancer in women. To evaluate the cytotoxicity of Cell.Com, MTT assay was performed in normal cell (human foreskin fibroblast) and cancer cells (HeLa, MCF-7 and Saos). Cell viability was estimated at 24 and 72 h under different concentrations of Cell.Com ($31.25-500 \text{ mg L}^{-1}$) (Figure 3). Obtained results indicate that the Cell.Com does not have any cytotoxic effects against fibroblasts and cancer cells over the studied range of concentrations at least during first 24 h. After 72 hours, while the Cell.Com showed significant inhibitory effects on cancer cell lines, it didn't have any cytotoxic effect against normal fibroblasts. This finding might be a favorable property of the Cell.Com with high biocompatibility with normal fibroblasts and cytotoxic effects on cancer cell lines. The cytotoxic effect of Cell.Com may be due to the presence of triazole linker, that it is known to possess very interesting biological activities, including anticancer effect [39].

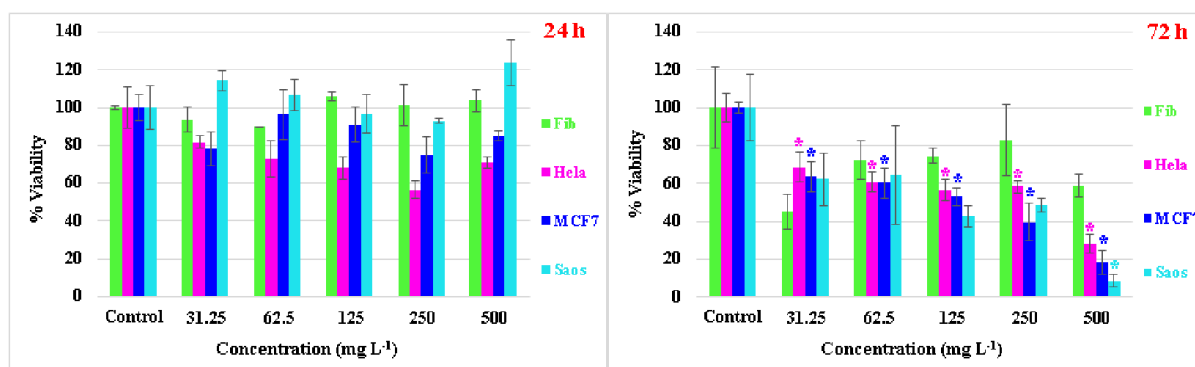


Figure 3. The cell viability of different studied cell lines after exposure to increasing concentrations of the Cell.Com after 24 and 72 h using the MTT assay.

Conclusion

It is concluded that TiO₂-grafted cellulose nanocomposite (Cell.Com) containing titanium dioxide nanoparticles and 1,2,3-triazole rings exhibits wide range of antibacterial activity against both gram-positive and gram negative bacteria. Furthermore, the cytotoxicity test indicated that the Cell.Com has higher toxicity against cancer cells than the normal cell especially after 72 h of exposure. Although more biological tests are needed to explore the possible mechanism of cytotoxic effect of Cell.Com, but triazole ring moiety in the Cell.Com structure might have an outstanding role in creating of cytotoxic effect. The promising in vitro biocompatibility of Cell.Com with fibroblast cell is a useful property in designing new products to ensure the safety of the end-users exposure. However, the cytotoxic effect of this bio-nanocomposite observed at concentrations higher than 31 mg L⁻¹, despite low potency, might be considered for further anticancer studies.

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