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Impregnation of silver nanoparticles into bacterial cellulose: Green synthesis and cytotoxicity

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Abstract: Bacterial cellulose was produced by using Acetobacteroxydans (ATCC 15163). Bacterial cellulose is an interesting material for using as wound dressing since it provide moist environment to a wound resulting in better wound healing but it has not antibacterial activity, which is one of critical skin barrier functions in wound healing. To overcome such deficiency, we developed a novel method to synthesize and impregnate silver nano particles on the surface of bacterial cellulose by using polyvinyl alcohol to form nano porous matrix and reduce the cytotoxicity of the prepared silver nanoparticles compared with those prepared by chemical reduction. Polyvinyl alcohol used to reduce the absorbed silver ion (Ag+) on the surface of bacterial cellulose to the metallic silver nanoparticles. (Ag0). Silver nanoparticles displayed the optical absorption band around 420 nm. The formation of silver nanoparticles was also evidenced by the X-ray diffraction, TEM, SEM and EDX. Cytotoxicity of the prepared silver nano particles prepared by PVA evaluated Vs. those produced by chemical methods.
Keywords: Acetobacter oxidants; Bacterial cellulose; Silver nanoparticle; cytotoxicity Green synthesis.

1. Introduction:

Bacterial cellulose (BC), produced by Acetobacterxylinum, is biocompatible and biodegradable polymer[(1)]. It has been used in bio medical application, such as tissue engineering, artificial blood vessels, wound healing and wound dressings, due to its unique properties including high mechanical properties, high water absorbance, high porosity and unique nanostructure[(2-5)]. So that it is an interesting material for using as a wound dressing since it can control wound exudates and can provide moist environment to a wound resulting in better wound healing. Nevertheless, it has no antimicrobial activity to prevent wound infection[(6)].

Silver metal and its ions have antimicrobial activity on board spectrum on Gram-positive and Gram-negativemicroorganisms. There are several mechanism to explain silver ions antibacterial; it interact with the thiol groups of enzyme and proteins that are important for the bacterial respiration and the transport of important substance across the cell membrane and within the cell and silver ions are bound to the bacterial cell wall and outer bacterial cell, altering the function of the bacterial cell membrane thus silver metal and its compounds were the effective preventing infection of the wound[(7)].

Silver metal was slowly changed to silver ions under our physiological system and interact with bacterial cells, thus silver ions will not be so high enough to cause normal human cells damage. Silver nanoparticles have a high specific surface area and a high fraction of surface atoms that lead to high antimicrobial activity compared to bulk silver metal[(8)].

Polymer nanocomposite containing metal nanoparticles, prepared by mechanical mixing of a polymer with metal nanoparticles, via the in situ polymerization of a monomer in the presence of metal nanoparticles, or the in situ reduction of metal salts or complexes in a polymer. These polymer nanocomposites have attracted attention, due to their unique optical, electrical, catalytic properties and biomedical properties [(7)].

Glucose used to reduce Silver nitrate to silver nanoparticles in the presence of Poly(vinyl alcohol) (PVA). Glucose is a kind of soft reducer and is widely used in biomedical process and PVA act as a good host material and poly reduces for metal and semiconductor, because of its good thermostability and chemical resistance[(9-13)].

Many in vitro studies has been done to show the cytotoxicity of AgNPs mainly due to oxidative stress So that the binding of AgNPs with some natural polymers e.g. cellulose, chitin, chitosan may reduce its cytotoxicity[(14, 15)].

The main goal of this study is to achieve the cytotoxicity of silver nanoparticles impregnated into bacterial cellulose, using water as an environmentally being solvent and PVA as reducing and capping agent at the same time to reduce that cytotoxicity of silver nanoparticles. PVA/glucose system was then used to reduce the absorbed silver ion (Ag^+) inside of bacterial cellulose to metallic silver nanoparticles (Ag^0)[(16)]. Then the prepared nanoparticles was characterized and the cytotoxicity of the silver nanoparticle impregnated into bacterial cellulose was evaluated compared with these prepared by chemical reduction methods.

2. Experimental:

2.1. Materials:

Bacterial cellulose prepared in our laboratory. Calcium carbonate and analytical grade silver nitrate were purchased from Fisher Scientific. Polyvinyl alcohol (M.W. 115,000, Polymerization 1700-1800, viscosity 25-32 and hydrolysis (mole%): 98-99) was purchased from Alpha achemika, India. Analytical grade sodium hydroxide anhydrate pellet and sodium chloride were purchased from Aldrich Chemical. Analytical grade glacial acetic acid was purchased from CSL Chemical. Ethanol was commercial grade and used without further purification.

2.2. Isolation and purification of produced bacterial cellulose:

The isolation and purification is the important step in the production of any cellulose product. This process is aimed to remove essentially all of undesired residual and other chemical bound to cellulose as well as impurities occurring during processing and converting.

There are two stages in the purification process, firstly BC was washed in 4 wt % KOH solution for one day and then washed with 6 wt % K2CO3 also for 1 day and it had known as the two step purification process.

2.3. Impregnation of silver nanoparticles into bacterial cellulose

Silver nanoparticles were impregnated into bacterial cellulose by immersing bacterial cellulose pellicles in 0.001M, 0.01M and 0.1M aqueous AgNO3for 1 h, followed by rinsing with ethanol for ca. 30 s. After then the silver ion-saturated bacterial cellulose pellicles were reduced in 1%, 3% and 6% of the aqueous PVA in the presence of glucose to overcome the silver oxidationfor 72 hrs.

Table 1 shows the experimental conditions for impregnation of silver nanoparticles into bacterial cellulose.

2.4. Characterization

- UV–vis absorption spectra of PVA-BC nanocomposites containing AgNPs were recorded using a Spectronic UV–Vis spectrometer (Model: Genesys 2, USA).
- The morphology of silver nanoparticles impregnated onto bacterial cellulose was investigated using JEOL,TEM-2100 Electron microscope and scan viewed in a JEOL JXA-840 electron probe microanalyzer, Japan.

- Fourier transform infrared (FT-IR) spectra of the all samples were obtained using a Nexus 670 FT-IR spectrophotometer (Lelet Co., USA). The spectra of sample in the range of 4000-800 cm-1 are investigated.
- X-ray diffraction studies wereconducted using an X-ray diffractometer (D2 Phaser, Bruker AXS, Germany) operating at 30 kV and 10 mA. The diffraction patternswere recorded using Cu-K _ radiation and the film samples wereanalyzed at a 2 range, 1–50.

2.5. Assay for cytotoxicity

2.5.1. Cell culture:

Culture was maintained in Dulbecco's Modified Eagle's medium (DMEM) medium (in case of A549), and supplemented with 10% foetal bovine serum at 37 ^{o}C in 5 %CO2 and 95% humidity, cells were sub-cultured using trypsin versene 0.15 %. Notable, skin normal human cell line (BJ-1) " Immortalizednormal foreskin fibroblast cell line "was obtained from Karolinska Center, Department of Oncology andPathology, Karolinska Institute and Hospital, Stockholm, Sweden. Other cell lines "were obtained fromVacsera (Giza, Egypt).

2.5.2. Cell viability assay

After about 24 h of seeding 20000 cells per well in case of A-549 cells per well (in 96 well plates), the medium was changed to serum-free medium containing a final concentration of the extracts of 100 µg/ml in triplicates. The cells were treated for 24 h. 100 µg/ml doxorubicin was used as positive control and 0.5 % DMSO was used as negative control. Cell viability was determined using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as described by Mosmann 1983 with minor modifications [(17)].

The equation used for calculation of percentage cytotoxicity: (1- (Av(x) / (Av(NC)))* 100

Where Av: average, X: absorbance of sample well measured at 595 nm with reference 690 nm, NC: absorbance of negative control measured at 595 nm with reference 690.

2.5.3. Determination of IC_{50} values

In case of highly active extracts possessing ≥ 75 % cytotoxicity on different cancer cell lines and human normal cell line, different concentrations were prepared for dose response studies. The results were used to calculate the IC_{50} values of each extract using probit analysis and utilizing the SPSS computer program (SPSS for windows, statistical analysis software package / version 9 / 1989 SPSS Inc., Chicago, USA).

3. Results and Discussion:

The reduction of silver ions in aqueous solutions generally yields colloidal silver with particle diameter of several nanometers. These particles have specific optical properties indicated by the presence of intense absorption band at 380nm caused by collective excitation of all the free electrons in the particles [(18)].

The optical properties of silver were studied using PVA of different concentration. Metal atoms formed by PVA and glucose tend to coalesce into oligomers which themselves progressively grow into larger clusters. However, the coalescence must be limited by adding a polymeric molecule acting as a cluster stabilizer.

Functional groups with high affinity for the metal ensure the anchoring of the molecule at the cluster surface while the polymeric chain protects the cluster from coalescing with the next one through electrostatic repulsion or steric hindrance.

PVA was selected as a capping agent. When AgNO3 is mixed with poly vinyl alcohol solution, the electropositivity of Ag+ ions allow it to bind withPVAthrough electrostatic interactions, because the electron-rich oxygen atoms of the hydroxyl groups of poly vinyl alcohol. These types of interactions cause a change of the color of the PVA colloidal solution upon the addition of AgNO3, from coluorless to pale yellow, yellow and brownish yellow in the presence of glucose.

Silver ions/PVA undergo redox system where the hydroxyl groups of PVA oxidized to carbonyl groups and the silver ions reduced to elemental silver. In addition, the added glucose supports the reduction power of PVA by offering more active sites for oxidation through its free aldehyde groups.Similar observations were reported previously for natural polymers such as starch, hydroxypropyl cellulose, and carboxymethyl cellulose [(19, 20)].

At the same time, the structure of bacterial cellulose has non-woven structure and many pores. So that when it immersed inthe aqueous $AgNO_3$, silver ions could penetrated into bacterial cellulose though their pores. The absorbed Ag^+ were bound to bacterial cellulose micro fibrils probably via electrostatic interactions, because the electron-rich oxygen atoms of polar hydroxyl and ether groups of bacterial cellulose are expected to interact with electropositive transition metal cations [(21)].

So that poly vinyl alcohol reduce $AgNO_3$ into Ag^+ in the presence of glucose and they could form with bacterial cellulose together sandwich among Ag nanoparticles which decreasesthe Ag nanoparticles cytotoxicity which the main target of this study.Reduction of silver nitrate into Ag nanoparticlesin aqueous PVA/bacterial cellulose hybrid solution monitored by changing its coluorless solution to yellow. Finally, the lyophilized silver nanoparticle-impregnated bacterial cellulose was dried by the freeze-drying method to maintain the original structure of bacterial cellulose [(6)].

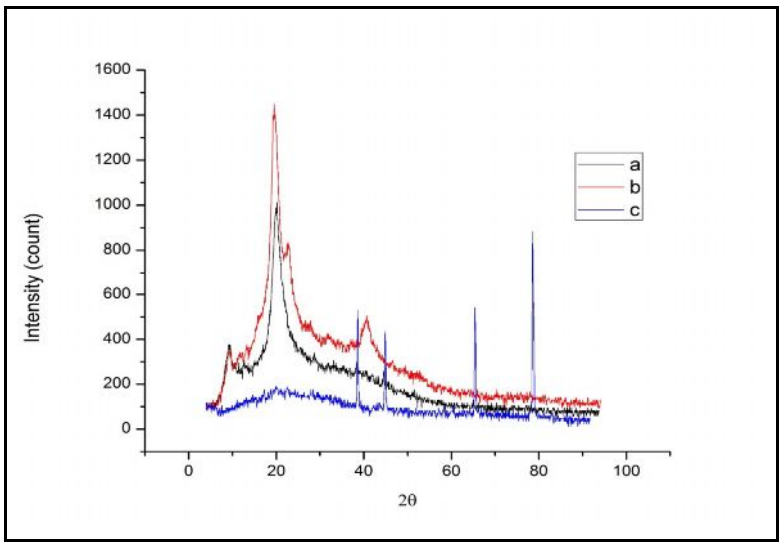

Figure. 1. XRD pattern of (a) poly vinyl alcohol (PVA), (b) bacterial cellulose (BC), and c silver nanoparticle-impregnated bacterial cellulose

The X-ray diffraction (XRD) was used to examine the crystal structure of metal nanoparticles that used to confirm the formation of silver nanoparticles. Figure 1 show X-ray diffraction pattern of poly vinyl alcohol, bacterial cellulose and silver nanoparticle-impregnated bacterial cellulose.

Figure 1a shows characteristic peaks at 10.7, 19.3 and 22.5 which corresponding to typical XRD peaks for PVA. Figure 1b illustrates characteristic peaks at 22.1 and 42.0 which corresponding to typical XRD peaks for bacterial cellulose (BC). Also, figure 1c show the XRD pattern of metallic silver nanoparticles that impregnated inside of bacterial cellulose which show characteristic four peaks at 38.1, 44.3, 64.4 and 78.0 that prove the structure of Ag nanoparticles impregnated into bacterial cellulose [(22-24)].

UV absorption use to monitor the formation of silver nanoparticles by using PVA/BC hybrid matrix with various concentrations from $AgNO_3$ (0.1 M, 0.001 M and 0.0001 M); PVA (1g/100ml, 3g/100ml and 6g/100ml) and bacterial cellulose (BC) (0.1g, 0.2g and 0.3g) to obtain the optimum conditions for obtaining homogeneous nanoparticle have minimumcytotoxic effect on human being.

The color of freeze-dried silver nanoparticle-impregnated bacterial cellulose gradually changed from a bright yellow to a brown yellow with increasing molar concentration of $AgNO_3$ from 0.001 M to 0.01 M to 0.1 M at constant PVA concentration (3g/100ml water) and BC weight (0.2 g). In addition, its colour changes from a bright yellow to a brown yellow with increasing weight percent concentration of PVA from 1g/100ml to 3g/100ml to 6g/100ml at constant AgNO3 concentration (0.001M) and BCweight (0.2g). the colour of silver nanoparticle-impregnated bacterial cellulose give yellow only with increasing the weight of BC from 0.1g to 0.3g to 0.3g.

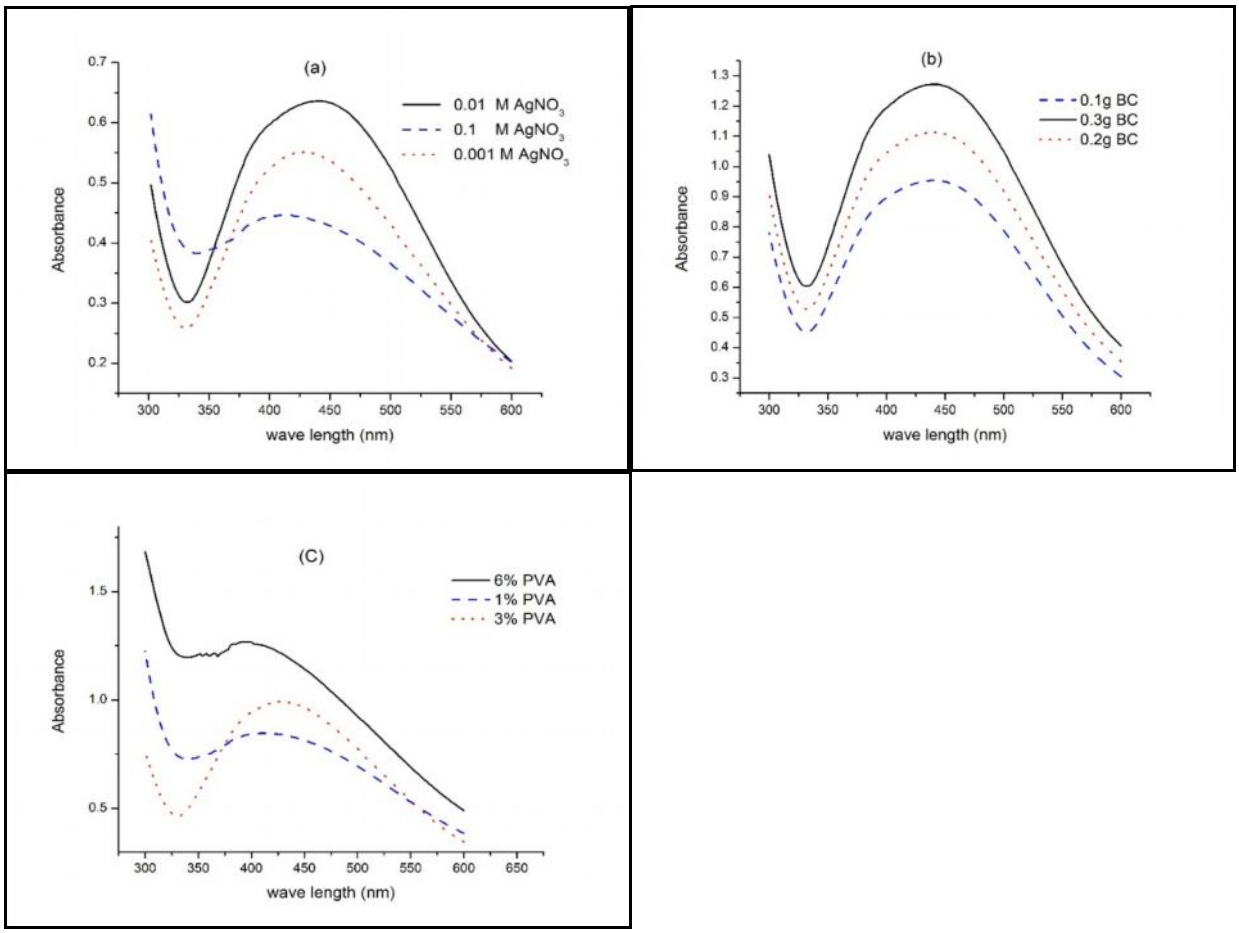

Figure. 2. UV Absorption spectra of silver nanoparticle-impregnated bacterial cellulose prepared from the PVA, AgNO3 at different reaction condition

The. Figure2 shows the optical absorption spectra of freeze-dried silver nanoparticle-impregnated bacterial cellulose, typical absorption of metallic silver nanoparticles. This is due to the surface plasmon resonance (SPR) of conducting electron (or free electron) on the surface of silver nanoparticles **(25)**. Moreover, freeze-dried silver nanoparticleimpregnated bacterial cellulose was a brown yellow color, due to the intense band around the excitation of SPR[(26)].

Figure 2a shows the changing in $AgNO_3$ molar concentration (0.001M to 0.01M to 0.1M)with constant PVA, (3g/100ml) and BC, (0.2 g), $AgNO_3$ molar concentration of 0.001M show homogeneous narrow absorption band is located at 420 nm. No absorption was observed at wavelengths longer than 500 nm. This implied that the small silver nanoparticles with the narrow size distribution were formed. The absorption band underwent a red-shift to 428 nm and was slightly broadened at the $AgNO_3$ molar concentration of 0.1M. The absorption band also underwent a red-shift to 442 nm and become much broadened at the $AgNO_3$ molar concentration of 0.01M. The red-shift and broadening of absorption band are possible to show the increase in a particle size and size distribution[(27)].

The results shown in figure 2b illustrate that there no change in band broadness with changing of BC weight (0.1g to 0.2g to 0.3g).

The same results obtained in figure 2c by changing the concentration of PVA from 1g/100ml to 3g/100ml to 6g/100ml at constant $AgNO_3$, (0.001M) and BC, (0.2g); the homogeneous narrow absorption band is located shown at 3g/100ml PVA concentration while both 1% and 6% PVA weight concentration show more and much broaden bands.

From these results, we found that the optimum conditions for obtaining silver nanoparticle-impregnated bacterial cellulose are 0.001 M AgNO3, 3g/100ml PVA, and 0.2g BC.

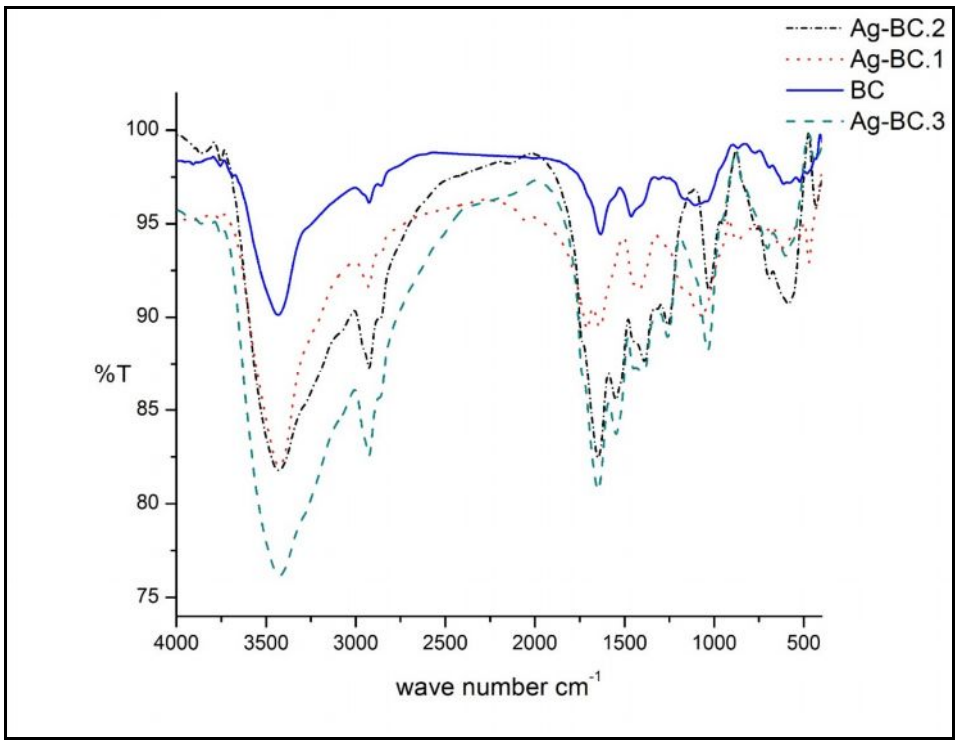

Figure 3 FT-IR spectra of the bacterial cellulose, silver nanoparticle-impregnated bacterial cellulose prepared from the PVA, AgNO3 at different reaction condition: Ag-BC.1, Ag-BC.2 and Ag-BC.3 corresponding ration 1:1, 1:10 and 1:100 respectively

Figure3 shows the FT-IR spectra of bacterial cellulose and bacterial cellulose–silver nanocomposites obtained at different reaction conditions as shown in the figure. Obviously, all the FT-IR spectra of bacterial cellulose–silver nanocompositesdisplay the typical bands of bacterial cellulose. For the bacterial cellulose–silvernanocomposites, the band centered at around 3428 cm^{-1} can beattributed to the stretching vibration of hydroxyl group. The band atabout 2925 cm^{-1} is assigned to the C–H group; the band at around1389 cm^{-1} is corresponded to the C–H bending mode. The absorptionband at 1031 cm^{-1} is ascribed to C–O–C stretching mode fromthe glucosidic units. The peak at 949 $cm^{-1}$was related to the C–H rocking vibration of cellulose [28-30]. It is important to note that the bacterial cellulose bands more intense than bacterial cellulose impregnated silvernanoparticles bands itself due to the effect of nano silver impregnated in it surface.

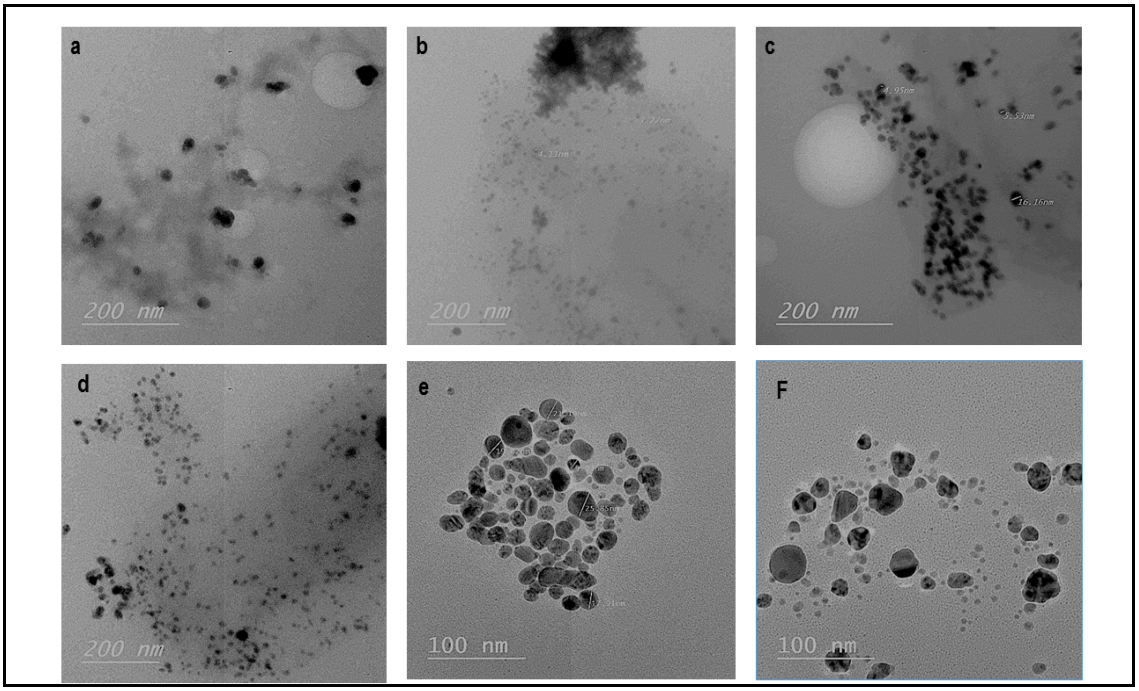

Figure 4. TEM images of silver nanoparticle-impregnated bacterial cellulose prepared from the PVA:$AgNO_3$ molar ratio of 1:1 (a and b), 10:1 (c and d) and 100:1 (e and f)

These conclusions are confirmed by TEM observations. Which shows the morphology of hybrid nanocomposites of silver nanoparticle-impregnated bacterial cellulose shown in Figure4a and b, irregular shape of silver nanoparticles with the large size and the wide size distribution were obtained at the higher $AgNO_3$ concentration (0.1M). When the concentration of $AgNO_3$ decreased, the particle size decreased.as shown in Figure4c and d. The well dispersed and regular spherical silver nanoparticles were obtained at the concentration of $AgNO_3$(0.001M). The particle size is much smaller as shown in Figure4e and f. Therefore, the size and size distribution of silver nanoparticles can be controlled by adjusting the molar $AgNO_3$.

Mechanism of reduction of Ag nanoparticles using glucose and PVA:

Moreover, the $AgNO_3$ molar concentration influenced the depth of silver nanoparticles inside of bacterial cellulose which resulted from the cation concentration gradient between the absorbed Ag^+ inside bacterial cellulose and the glucose of the aqueous PVAoutside bacterial cellulose during the reduction of silver nanoparticles.

At the $AgNO_3$ molar concentration of 0.01M, the concentration of the absorbed Ag^+ inside bacterial cellulose was equal to that of the glucoseoutside bacterial cellulose. When the Ag^+ at the surface of bacterial cellulose were reduced to form the silver nanoparticles, the cation concentration gradient was occurred and the some deeper Ag^+ penetrated to the surface and formed nanoparticles. Thus, the silver nanoparticles were formed only at the surface of bacterial cellulose and there are some absorbed Ag^+ inside bacterial cellulose pellicle.

At the lower $AgNO_3$ molar concentration of 0.01M (0.001M), silver nanoparticles which formed inside bacterial cellulose were deeper, respectively. Because the concentration of absorbed Ag^+ inside bacterial cellulose is much lower than concentration of glucose of the aqueous PVAthus, the cation concentration gradient occurred, then glucose in the aqueous PVApenetrate into bacterial cellulose pellicles, not Ag^+ penetrate out.After that Ag^+ were reduced and formed nanoparticles inside of bacterial cellulose pellicle.

At higher $AgNO_3$ molar concentration of 0.01M (0.1M),the concentration of absorbed Ag^+ inside bacterial cellulose pellicle is much more than the concentration of glucose in the aqueous PVA.Thus the cation concentration gradient occurred, then absorbed Ag^+ inside bacterial cellulose pellicle penetrate out and form nanoparticles in the solution, not at the surface of bacterial cellulose pellicle, that can be observed by the color change of a clear solution of aqueous PVAto a brown solution [(6)].

3.1. Cytotoxicity of AgNPs suspensions:

AgNPs suspensions are toxic [(14)]. To study the effect of silver ions concentration presentin AgNPs suspensions on their toxicity, A549 cellswere treated for 24 h with three different batches of AgNP suspension, which contained the same concentration of AgNPs (0.5-2.0 µg/ml) in three Ag NP types. Type 1 is AgNPs prepared by chemical reduction, type 2 is AgNPs prepared by PVA and glucose and type 3AgNPs prepared by PVA/glucose/bacterial cellulose as shown in figure 6. The cell viability was measuredby the MTT assay. As expected, the AgNPs suspension with chemical reduction showed higher toxicity, then that prepared by PVA/glucose followed by that prepared by PVA/glucose/bacterial cellulose. This suggests that the toxicity of AgNPs suspensions strongly dimensioned by the presence of bacterial cellulose due to its impregnated on its surface.

MTT assay used to measure the cell viabilityexpressed in the decrease in mitochondrial activity (figure 5). A reduction in mitochondrial function of A549 cells exposed to the three AgNPs types prepared as mentioned before.As expected, AgNPs suspension prepared with PVA/glucose/BC less toxic than that prepared with PVA/glucose which less than that prepared by chemical reduction due to the PVA/glucose/BC matrix produce AgNPs with lowest silver ion fraction exposed to the vial cell which reflect the safest one and, lowest toxic effects on these cells[(31-33)]. So that the toxicity of AgNPs suspensions directly depends on the exposed silver ion content to the vial cells.

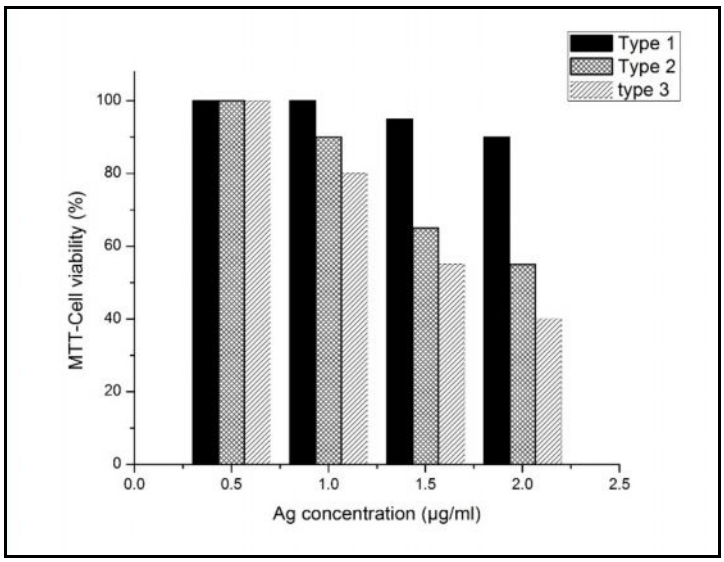

Figure 5. MTT assays for the 3 types of AgNPs suspensions and cell viability

Figure 6 shows that the IC_{50} of silver nanoparticles prepared by chemical reduction less than that of silver nitrate i.e. its more toxic. But its toxicity reduces in the presence of PVA and PVA/BC so that the presence of BC make Ag NP to be safer. In this study, Ag^+ decreased mitochondrial activity more than AgNPs with almost two fold difference in IC_{50}values as shown in figure 6, which agreed with previous studies of many researcher [(29,32-34)].

But it is increased than the other two types by using matrices from PVA/glucose and PVA/glucose/BC which give the more safely on towards A549 viable cell lines due to the impregnation of AgNPs on bacterial cellulose which decrease the exposed Ag^+ to these cells without any effects on their antibacterial and others biological activities.

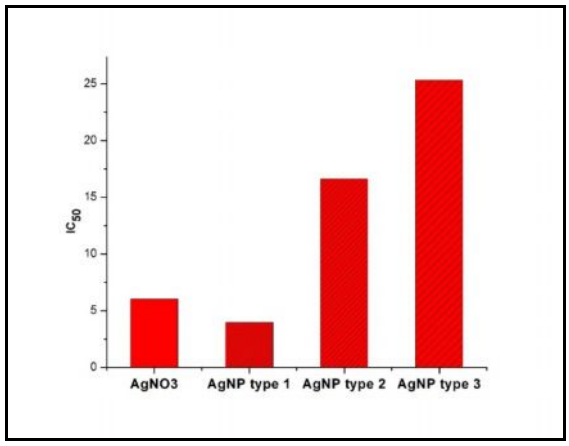

Figure 6 IC50 for A549 cell line after exposing to AgNO3, AgNPs type 1, AgNPs type2 and AgNPs type3 (for 24 h)

Where type 1= AgNPs prepared by chemical reduction, type 2= AgNPs prepared by PVA and glucose and type 3=AgNPs prepared by PVA/glucose/bacterial cellulose

4. Conclusion

- We succeeded to prepare Silver nanoparticles by three different methods: chemical reduction, PVA/glucose reduction and PVA/glucose/BC reduction.
- The size and size distribution are controllable by adjusting the molar ratio of PVA: AgNO3.
- At the optimum conditions, well dispersed and regular spherical silver nanoparticles were obtained with size ranges within 15-35nm particle size.
- The AgNPs were characterized by using several tools e.g. X-ray diffraction, UV absorption, FT-IR spectroscopy and TEM imaging.
- The cytotoxicity of these AgNPs were evaluated by using MTT assay and IC_{50} values to show the effect of AgNPs preparation method on the cytotoxicity of these nanoparticles on A549 viable cells.
- Ag^+ decreased mitochondrial activity more than AgNPs prepared by chemical reduction but it is less than that prepared by PVA/glucose and PVA/glucose/BC matrices.
- The free Ag^+ in AgNPs suspensions play an important role in its cytotoxicity towards a viable A549 cells. The toxic effects of AgNPs related to the release of Ag^+.
- These properties are essential for manufacturing of silver nanocomposites, with minimal toxicity compared with these prepared by chemical reduction by using bacterial cellulose and PVA composite.

Acknowledgements

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