PREPARATION AND CHARACTERIZATION OF PALLADIUM (II) COMPLEX/CELLULOSE NANOWHISKER AS A NEW POTENT ANTICANCER PRODRUG

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Received November 17, 2016

Herein, we describe the development of a new cellulose based nanocomposite with distinctive characteristics, for utilization in different applications, particularly, in the biomedical field, as an anticancer prodrug. In this context, cellulose nanowhiskers (CNW) were prepared by acid hydrolysis of cellulose linter and used as a matrix for palladium (II) complex. The structures of the CNW, palladium complex and their new nanocomposite were characterized using FT-IR spectroscopy, UV-Vis spectroscopy and scanning electron microscopy. SEM images of freeze-dried cellulose nanocrystals showed fibrillar, network-like morphology, due to the self-assembly of the cellulose nanocrystals. In comparison with the pure CNW, the CNW/Pd complex showed higher thermal stability. Also, the 50% cytotoxic concentration (CC50) value of the Pd complex loaded nanocrystal was 203 µg/ml after a 24 h incubation time. The results showed that the CNW/Pd complex could be a promising candidate precursor for biomedical applications, such as anticancer drug delivery systems.

Keywords: cellulose nanowhisker, anticancer drug, palladium complex, bionanocomposite

INTRODUCTION

Cancer is a metabolic disease responsible for approximately 25% of the deaths in developed countries and continues to present a global challenge. Depending on the type and condition of cancers, different kinds of treatment can be applied. Chemotherapy has been identified as the major strategy for cancer treatment, which is often used together with other cancer treatment options. Surgery, radiotherapy and hyperthermia therapy are other common options.[1] Chemotherapy usually utilizes one or more chemical substances called cytotoxic neoclassic anticancer drugs. Nowadays, a large variety of anticancer chemotherapeutic drugs, such as alkylation agents, antibiotics, metal complexes, hormones, *etc*., are available and are being used against cancer.[2] Cisplatin, one of the most effective and successful anticancer drugs, has a significant chemotherapeutic potential in various kinds of human solid cancers and, due to its wide usage, has continued to attract the interest of many researchers in this field. Although cisplatin is normally utilized today, there have been numerous efforts to improve its performance, such as attempts to eliminate or reduce its side effects and its toxicity.[3] Also, there is much interest in using palladium complexes as a potential anticancer drug, due to the remarkable similarity between them and Platinum complexes from the point of view of coordination chemistry.[4] Moreover, various types of complexes based on a wide range of metals, such as ruthenium,[5] gallium,[6] rhodium, osmium,[7] copper, iron,[8] gold,[9,10] and titanium,[11] have been reported in the literature.

In addition to the type of metal used, ligands have a considerable effect on the characteristics and effectiveness of anticancer metallo-drugs, and the nature of ligands can strongly influence the stability, toxicity, bioactivity and lipophilicity of the metal complex drugs. Polysaccharides and oligosaccharides as multifunctional, green materials are excellent candidates for medicinal

chemistry. They are cost-effective, biodegradable and biocompatible ligands. Moreover, they possess many surface functional groups, generally alcoholic hydroxyl groups, which can be easily converted to various ranges of desired functional groups. However, carbohydrates in their unmodified forms are also capable of serving as ligands.[12]

Thanks to the significant advantage of carbohydrates, a great deal of research has been done to develop drug delivery systems and macromolecular prodrugs with improved therapeutic effects and/or to reduce the toxic effects of drugs. Although a broad range of natural and synthetic polymers meet the required characteristics of such systems, for example, biodegradability, biocompatibility *etc*., efforts are continuously made in search of new macromolecular prodrug carriers. Parallel to the developments in metal complex materials and their application in the biomedical filed, several other types of nanocrystals based on polysaccharides, known as starch nanocrystals, cellulose and chitin nanowhiskers and their composites have been also developed over decades by various research groups.[13] Cellulose nanowhiskers are rod-like nanoparticles that can be obtained from various plant sources, generally through the acid hydrolysis process with HCl, H_2SO_4, *etc*.[14] Like other carbohydrates, cellulose and nanocellulose[15] could be easily functionalized through the graft polymerization process with a wide range of polymers, such as polyethylene glycol,[16] polyoxazoline,[17] polycaprolactone,[18] polylactic acid[19] and poly(amidoamine) dendrimer,[20] for different fields of application, such as nanocomposites and drug delivery. Also, cellulose can be covalently decorated with drugs in its ungrafted form.[21]

In the present study, nanocrystalline cellulose, a promising nanoscale polysaccharide, was selected as a macromolecular matrix for the preparation of a palladium (II) based potent anticancer drug, due to its superior properties, such as biocompatibility, biodegradability, high surface area and hydrophilicity. Also, the Pd complex loaded cellulose nanoparticle composite prodrug was characterized using FT-IR, its thermal behavior and morphology were investigated by TGA, X-ray diffraction and SEM techniques.

EXPERIMENTAL

Materials and instruments

The cellulose material used in this study was commercial cotton linter. Sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), palladium chloride, acetone and ethanol were purchased from Sigma-Aldrich. 1,10-Phenanthroline was obtained from Merck and used as received without further purification. Other chemicals used in this study were of analytical reagent or higher purity grade.

FTIR experiments were conducted using a Perkin Elmer 65 FTIR spectrometer using KBr (1:100, w/w). The powder XRD pattern was recorded at room temperature from 4º to 70º in 2θ, using a D8 Advance instrument (Bruker AXS GmbH, Karlsruhe, Germany) with CuKα radiation (λ = 0.15406 nm). The morphology was analyzed by Scanning Electronic Microscopy in a VEGA3 TESCAN SEM under accelerated electrons with 15 kV of energy. The thermal stability was analyzed in a NETZSCH TG209 F1.

Preparation of cellulose nanocrystals

Typically, 4 g of cotton linter was added to 100 ml 2% w/v NaOH and stirred for 18 h. Then, it was neutralized and dried. This prepared cotton was treated with 70 mL of 64% w/w sulfuric acid at 55 ºC for 25 min under constant stirring. Then, it was diluted 10-fold with deionized water to quench the reaction.[22] The resulting suspension was centrifuged at 7000 rpm for 10 minutes, and centrifuging was repeated twice to precipitate the cellulose nanocrystals, while excess water and acid were removed. The obtained precipitant was dialyzed and was rinsed with purified water, until a constant, neutral pH was reached. The suspension was sonicated three times, for 60 min at 80 Hz. The cellulose nanocrystal suspension was quickly frozen by liquid nitrogen (N_2) in an ice-tray and put into a freeze-dryer to remove the solvent water. The dried product was stored in vacuum until the following characterizations.

Synthesis of Pd (II) complex

An amount of 0.354 g of $PdCl_2$ (2 mmol) was suspended in 6 ml of double-distilled water and 0.6 g NaCl (10 mmol) was added under continuous stirring and heated for 0.5 h at 40 ºC. After complete solubilization, it was cooled to room temperature. Also, a solution of 0.396 g phenanthroline (phen) (2 mmol) in ethanol was prepared and every 15 min a droplet of the Phen solution was added to the reaction with a sampler. The reaction mixture was constantly stirred at 50 ºC for 12 h. The reaction product was filtered and washed with several portions of deionized water and dried in a vacuum oven. The yield of [Pd (phen) Cl_2] was 91%.

An amount of 0.450 g of [Pd (phen) Cl_2] (0.00126 mol) was suspended in 400 ml of acetone and double-distilled water (3:1), and heated at 50 ºC. Then, 0.4284 g of AgNO3 (2*0.00126 mol) was added. The suspension was heated for 7 h at 45 ºC, then for 15 h at room temperature in the dark under constant stirring. [Pd (phen) $(H_2O)_2]^{2+}$ was obtained after filtering the reaction mixture to remove the silver chloride precipitate.

Preparation of phen-Pd complex loaded cellulose nanocrystals (Pd (phen)-NCC)

Phen-Pd complex loaded cellulose nanocrystals (Pd (phen)-NCC) were synthesized in our lab, according to the published literature with some modification.[22] Briefly, cellulose nanocrystals (150 mg) were dispersed in 20 mL of deionized water. Thereafter, the suspension was mixed with an aqueous solution of phen-Pd-(NO3)2 (which had been prepared from 0.00126 mol). The solution was stirred vigorously for 48 h at 25 ºC in the dark and under N_2 atmosphere. The obtained particles were precipitated by centrifugation and washed with deionized water to remove the unreacted palladium complex, and then verified by UV spectrometry. The obtained conjugates were named Pd (phen)-NCC.

Cytotoxic studies

Cell culture

Human colon cancer cell line HCT116 was obtained from the National Cell Bank of Iran (NCBI), Pasteur Institute of Iran. The cells were grown on DMEM medium (Sigma) supplemented with L-glutamine (2 mM), streptomycin, penicillin (5 µg/ml), and 10% heat-inactivated fetal calf serum at 37 ºC under a 5% CO_2/95% air atmosphere.

Cell viability assay

The cytotoxic or growth inhibitory activity of the Pd complex loaded in cellulose nanocrystals was measured by the MTT assay. The transformation of the yellowish MTT to purple formazan by active mitochondrial dehydrogenase of living cells has been utilized as an assay system for evaluation of cell proliferation. The HTC116 cancer cells were seeded into a 96-well plate (1×10^4 cell/ml) and were left to adhere overnight. Prior to the experiments, the cells were twice washed with phosphate-buffered saline (PBS). Then, the cancer cells were incubated with different concentrations of sterilized Pd complex loaded in cellulose nanocrystals (0-350 µg/ml) and incubated for 24 h. Four hours to the end of the incubation, 25 µL of the MTT solution was added to each cultured medium. Finally, the insoluble formazan produced was dissolved in a solution containing 10% SDS and 50% DMF (left for 2 h at 37 ºC in darkness) and optical density (OD) was read against a reagent blank with a multi-well scanning spectrophotometer (ELISA reader, Model Expert 96, Asys Hitech, Austria) at the wavelength of 570 nm. The OD value of the test groups was divided by the OD value of the untreated control and presented as the percentage of control (as 100%).

RESULTS AND DISCUSSION

The use of chemically modified cellulose and cellulose nanowhiskers in the field of bionanocomposites with improved properties is gaining increasing importance not only because they are cheap, but also mainly because the polysaccharide portion of the product is biodegradable. Cellulose nanowhiskers have been generally used as nanofillers in nanocomposites.[23,24] However, in this study, cellulose nanowhiskers were used as a green biocompatible polymer matrix.

FT-IR and UV-Vis spectroscopy

The FTIR spectra of cellulose nanowhiskers (CNW), palladium phenanthroline complex and cellulose nanocrystal/palladium phenanthroline, as well as the difference spectrum of the composite and cellulose nanowhisker (composite-cellulose nanowhisker), are illustrated in Figure 1. Evidently, the IR spectrum of cellulose nanocrystals shows an intense broad peak in the range of 3300-3400 cm^{-1}, which is ascribed to the stretching vibration of the hydroxyl groups of glycosidic moieties of the cellulose nanocrystals. The C–O–C pyranose ring skeletal vibration is located at 1064 cm^{-1} and the peak appearing at 897 cm^{-1} is related to the β-glycosidic linkages. The OH vibrations for the primary and secondary hydroxyl bending are located at 1437 and 1300 cm^{-1}, while the band in the 2800 to 3000 cm^{-1} range is due to the presence of aliphatic CH groups. Generally, in the spectrum of the phenanthroline complex, there are bands in four frequency regions, namely between 600-900 cm^{-1}, between 1100-1300 cm^{-1}, between 1400-1650 cm^{-1} and about 3100 cm^{-1}, corresponding to out-of-plane vibration of aromatic C-H, in-plane vibration of aromatic C-H groups, stretching vibrations of C=C and C=N double bonds, and =C-H stretching vibrations, respectively.[25] The peaks in the fourth frequency region are observable in Figure 1b. Also, Figure 1c shows the FT-IR spectrum of the cellulose nanowhisker/palladium phenanthroline complex. The spectrum of the nanocomposite is similar to that of the cellulose nanowhisker, probably due to the low content of palladium complex, as well as to an overlapping of the peaks in several regions.

Thus, for better comparison, the corresponding difference spectrum of the composite and cellulose nanowhisker (composite-cellulose nanowhisker) has been shown in Figure 1d. The difference spectrum clearly shows peaks in four frequency regions related to the phenanthroline complex (3100, 1250, 1500 and 800 cm^{-1}). The presence of these bands, along with the characteristic bands of CNW, in the final product's spectrum suggests that the palladium complex has been successfully loaded into the CNWs matrix.

Also, the electronic absorption spectrum of the Pd phenanthroline complex in water as solvent is illustrated in Figure 2. The spectrum consists of two main bands: a broad band with maximum absorbance at λ = 221 nm and a narrower one, located at λ = 272 nm, which are related to the π-π* electron transfer of phenanthroline. In fact, similar peaks are observable for the free ligand. This indicates that the Pd (II) coordination does not influence significantly the π–π* transitions of the free ligand.

TGA

Thermogravimetric analysis was conducted to evaluate the thermal stability of cellulose linter, cellulose nanowhisker and palladium phenanthroline complex/cellulose nanowhisker. The thermal decomposition profiles of cellulose, cellulose nanocrystal and palladium complex/cellulose nanocrystal are presented in Figure 3.

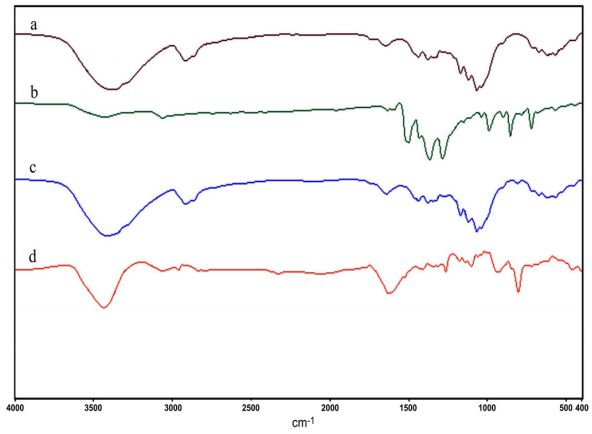

Figure 1: FT-IR spectra of CNW (a), Pd phenanthroline complex (b), and cellulose nanowhisker/Pd phenanthroline complex (c), as well as difference spectrum of the composite and cellulose nanowhisker (composite-cellulose nanowhisker) (d)

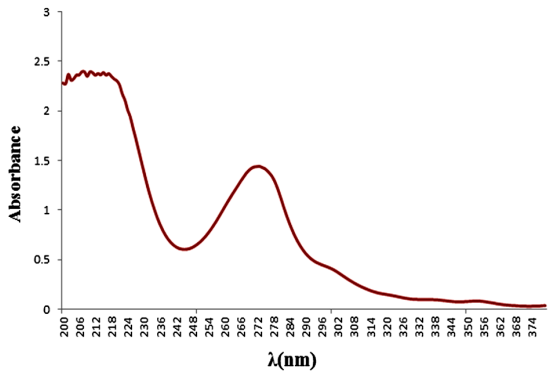

Figure 2: UV-visible absorption spectrum of palladium phenanthroline complex

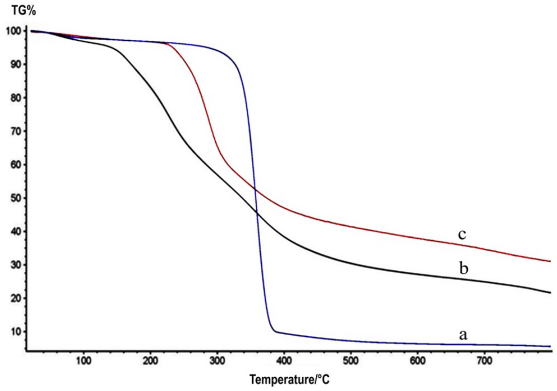

Figure 3: TGA curves of cellulose (a), cellulose nanowhisker (b) and CNW/palladium phenanthroline complex

As clearly shown in Figure 3, cellulose linter undergoes single-stage decomposition. Cellulose linter decomposes mainly through dehydration, depolymerization and glucosan formation. The dehydration process usually takes place in the temperature range of 100-300 ºC, which leads to the formation of 1,4 anhydroglucopyranoside. Depolymerization takes place around 360 ºC through chain scission at 1,4 glucosidic bonds. Generally, at higher temperatures, pyrolysis leads to the production of materials with lower molecular weights. The weight loss below the decomposition temperature is due to loss of the absorbed moisture. However, CNW shows two decomposition stages with increasing temperature, which were confirmed by derivative TGA curves by the appearance of two maximum peaks. The first one was at the temperature of 224 ºC, and was related to the onset of cellulose decomposition, while the second, at 365 ºC, was ascribed to the carbonization of cellulose nanoparticles. The weight loss before the whisker decomposition, as reported by other researchers, could be due to the degradation of the remaining amorphous part of the microcrystalline cellulose.[26] On the other hand, cellulose nanocrystal decomposition begins from 224 ºC to 365 ºC. As shown in Figure 3, the onset decomposition temperature of the palladium complex modified CNW was higher than that of the pure cellulose nanocrystal and the highest peak was at 294 ºC, which suggested the thermal stability of the cellulose nanocrystal was improved by the incorporation of the palladium phenanthroline complex.

Scanning electron microscopy

Figure 4a presents the SEM image of untreated cotton linter. This image clearly shows the shape and size of individual macrofibrils in the cotton linters. These well-separated macrofibrils have diameters of about 7 µm. An SEM image of the obtained cellulose nanocrystals is shown in Figure 4b. A very dilute aqueous suspension of cellulose nanocrystals was used for morphological investigation. For sample preparation, the aqueous suspension of CNC was quickly frozen by adding liquid nitrogen, and then freeze-dried. Surprisingly, the SEM images of freeze-dried sample showed fibrillar, network-like morphology, which is completely different from the expected morphology of cellulose nanowhiskers. However, such interesting morphology has been reported for freeze-dried rice straw CNC.[27] The observed morphology is probably due to the self-assembly of the CNCs into mainly wide ribbon-like fibers with clusters randomly strung together. These ribbons had greatly varied widths, mostly from 60 nm to nearly 90 nm, with the majority centering at around 70 nm. An SEM image of the palladium complex/cellulose nanocrystal is presented in Figure 4c. As shown in this image, after incorporation of the palladium complex, the assemblies of cellulose nanowhiskers disrupted and more individual cellulose nanowhiskers with diameters of about 10 nm were observable.

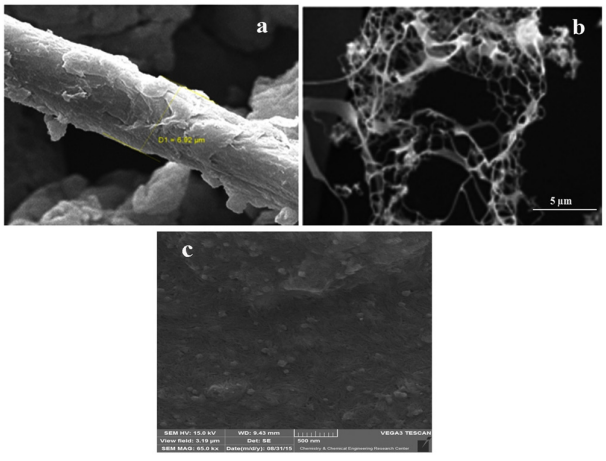

Figure 4: SEM images of cotton linter (a), cellulose nanowhisker (b) and palladium complex/cellulose nanocrystal (c)

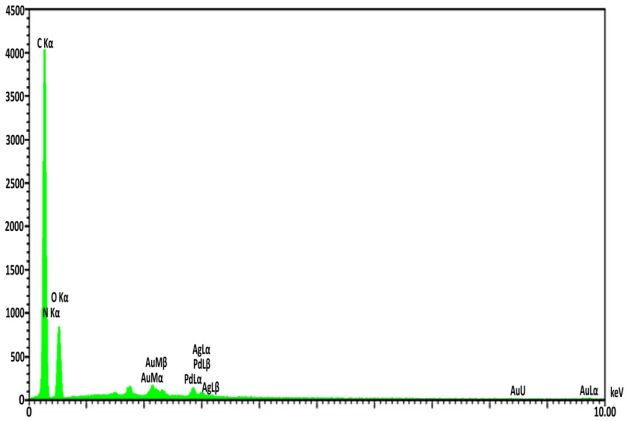

Figure 5: EDX spectrograms of CNW/palladium phenanthroline complex

Table 1
Chemical composition of CNW/Pd complex determined by EDX

Element	Line	W%	A%
C	Ka	62.08	75.40
N	Ka	2.06	2.15
O	Ka	22.64	20.65
Pd	La	9.97	1.37
Ag	La	3.24	0.44
		100.00	100.00

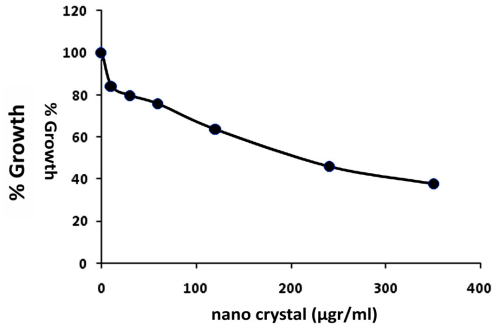

Figure 6: Effects of Pd loaded cellulose nanocrystals on the growth of human colon cancer cell line HCT116

The chemical composition of the treated CNW/Pd complex was also assessed by EDX analysis and the results are presented in Figure 5. As shown in the EDX spectrum of the CNW/Pd complex and the detailed information in Table 1, the final product contains C, O, Pd and N, which confirms the loading of a remarkable amount of Pd complex onto the cellulosic nanowhisker matrix. Besides, the amount of loaded Pd onto the cellulose nanowhisker matrix was of about 9.57% weight percent.

Cytotoxicity studies

In order to assay the anticancer activity of the Pd complex encapsulated into cellulose nanocrystals, cell viability studies of the nanocrystals were carried out against the human colon carcinoma cell line HCT116.

In this experiment, HCT116 cells were incubated in the absence and presence of various concentrations of Pd complex encapsulated into cellulose nanocrystals (from 0-350 µg/ml) at 37 °C for 24 h incubation time. The 50% cytotoxic concentration (CC50) for the designed Pd complex encapsulated into cellulose nanocrystals was calculated from the results of Figure 6. As shown in Figure 6, the cell growth decreased when increasing the concentration of Pd complex encapsulated into cellulose nanocrystals and the response was demonstrated to be dose-dependent. Also, the value of CC50 for the nanocrystals was calculated as 203 µg/ml after 24 h incubation time.

CONCLUSION

Cellulose nanowhiskers were prepared through acid hydrolysis and used as a macromolecular support to assist in the fabrication of a palladium complex/cellulose nanowhisker composite. Surprisingly, after freeze-drying, the resulting cellulose nanowhiskers showed web-like morphology, probably due to the self-assembly of individual cellulose nanowhiskers. In the next step, the palladium phenanthroline complex and its composite with cellulose nanowhiskers, as a carbohydrate polymer matrix, were prepared. After each step, the resulting materials were fully characterized. The results of the above investigation revealed that the palladium phenanthroline complex was formed on the surface of the CNW. Also, thermogravimetric analysis indicated that the palladium phenanthroline/CNW was much more thermally stable than the CNWs. The obtained Pd (II) complex/cellulose nanowhisker could be helpful in the development of various nanomaterials with great promise for advanced functional applications, such as drug delivery systems.

ACKNOWLEDGMENTS: The authors wish to gratefully acknowledge the support of Lorestan University.

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