Copper complexes loaded on nanostructured TiO_2 materials as citotoxic agents of cancer cells.

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ABSTRACT

Copper complexes (CC) were loaded on a functionalized titania with the purpose to determine their anticancer properties. Each CC was incorporated to titania during the hydrolysis step of the titanium butoxide precursor using the sol-gel procedure. The cytotoxic properties of both CC alone as well as those loaded on titania were determined using the RG2 rat glioblastoma cell line. We used UV-VIS and FTIR spectroscopy, and N_2 adsorption-desorption technique for the sample characterization. An "in vitro" release test was developed as well. The spectroscopy studies indicate that CC did not undergo any structural change during the synthesis procedure. The textural properties of titania were affected by the incorporation of the CC molecules. The classical first order kinetics were observed from the release profiles. A substantial death of RG2 cell was observed when they were treated with low concentrations of CC alone and Cu-TiO_2 materials compared with Cis-Pt, TiO_2 and RG2 non treated cell.

Keywords: copper complexes, functionalized TiO_2, CC release, cell death, RG2 cell line.

1 INTRODUCTION

Glioblastoma Multiform (GBM), a grade IV astrocytoma, is the most common and lethal brain tumor in adult humans [1]. This type of tumor tends to grow rapidly and the average survival of patients with this condition does not exceed 15 months regardless of treatment choice. Surgery and chemotherapy alone or their combination with radiotherapy are the standard treatments for patients with GBM, however, at present, these treatments cannot cure this type of cancer. Due to this, the scientists have dedicated an enormous effort in find new strategies to eradicate these tumors. Drug release using nanomaterials as vehicles arises as a potential alternative to the treatment of various kinds of nervous system diseases [2].

Numerous platinum (Pt) analogs have been evaluated in preclinical and clinical studies but only cisplatin, oxaliplatin and carboplatin have been approved for clinical use. The heavy metal compounds exert their anti-neoplastic effect by binding to DNA [3]. Cisplatin combined with an external beam radiotherapy has resulted beneficial in the survival of patients with GBM [4]. However, its clinical use has been limited because it causes severe adverse reactions such as renal toxicity and gastrointestinal toxicity, among others.

Copper complexes have emerged as another kind of inorganic drugs for cancer treatment. A number of Cu (II) complexes have exhibited cytotoxic activity through cell death by apoptosis or enzyme inhibition [5]. Such complexes containing bases as ligands, which are effective in reducing tumor size, delay metastasis and significantly increase the survival of the patients. It is likely that copper complexes interact with enzymes and inhibit vital cell functions, rather than interact with DNA and induce crosslinking.

In this work, we studied the potential properties of two copper complexes as anti-cancer agents alone and when they were released from the functionalized titania. The general purpose is to obtain new strategies to GBM treatment.

2 MATERIALS AND METHODS

The copper complexes used for this study were ammonium tetrachlorocuprate (II) and copper (II) chloride. The amounts used were calculated to obtain the molar ratios of water:alkoxide 16:1 and ethanol:alkoxide 8:1. The amount used of each copper complex was 10 % mol in titania. Surface titania was functionalized with sulphate, phosphate and amine groups using as precursors sulfuric acid, phosphoric acid and γ-aminobutyric acid (GABA).

2.1 Sample preparation

TiO_2. One gram of GABA was dissolved in a mix of water/ethanol and refluxed at 70 °C under constant stirring. Next, five drops of sulfuric and five drops of phosphoric acid were added to the previous solution. Then, titanium n-butoxide was added slowly during four hours. Later, the

whole mixture was maintained at 70 °C with stirring for 24 hours. Finally, the water and alcohol excess was removed and the gel was dried at 70 °C for two days.

TiO_2-Cu. Solution 1 was prepared by mixing of water, ethanol and the copper salt, while solution 2 of water and GABA was prepared. Both solutions were mixed and refluxed at 70 °C under stirring. Five drops of sulfuric acid as well as phosphoric acid were added. Then, titanium n-butoxide was dripped slowly during four hours. The total mixture was maintained at 70 °C with stirring for 24 hours. Finally, the water and alcohol were removed and the gel was dried at 70 °C for two days.

2.2 Sample characterization

TiO_2 and Cu/TiO_2 samples were characterized by different physicochemical techniques: for FTIR-spectroscopy studies the samples were mixed with KBr and measured in an IRAffinity-1 Shimadzu spectrometer. The N_2 adsorption-desorption analysis was developed on a Belsorpt II-BEL equipment, prior the N_2 adsorption step the samples were treated at 70 °C for 24 h. The UV spectra were recorded in a reflectance diffuse mode using a S-3100 SCINCO spectrometer.

2.3 "In vitro" tests

Cell Viability. The cytotoxic properties of both pure complexes as well as those loaded on titania were determined using the rat glioblastoma RG2 cell line. RG2 cells were exposed to copper complexes, Cu/TiO_2, TiO_2 and Cis-Pt materials at different concentrations (15.75-1000 µg/ml) at 37º C under sterile conditions. 24 hour post treatment of the cell viability was tested by MTT assay, which measures mitochondrial activity.

Copper Complex release. One tablet of 20 mg was immersed in 25 ml of water and at predetermined times an aliquot of 4 ml was removed for its measurement by ultraviolet spectroscopy at 205 nm for $CuCl_2/TiO_2$ and 200 nm for $Cu(NH_4)_2Cl_4/TiO_2$, respectively. After the measurement, the aliquot was returned to the release solution. A calibration curve was used to determinate the CC released.

3 RESULTS AND DISCUSSION

Figure 1 shows the infrared spectra for TiO_2, $Cu(NH_4)_2Cl_4$, and $Cu(NH_4)_2Cl_4/TiO_2$ samples. Titania spectrum contains different signals (identified with blue asterisk) derived manly from GABA molecules. The most interesting signals are those located at 3353 cm^{-1} and 3195 cm^{-1} due to stretching vibrations of O-H groups from GABA molecules, Ti-OH superficial groups on titania and adsorbed water. Other band is located at 3195 cm^{-1} that corresponds to N-H stretching vibrations of amine groups of GABA molecule. Three signals located at 2959, 2939 and 2873 cm^{-1} are attributed to asymmetric modes of vibration of C-H bonds in the three $–CH_2-$ groups of GABA. Others important signals are those located at 1634 and 1534 cm^{-1}, which correspond to deformation vibrations of C-H bonds and N-H bonds of GABA molecules, respectively. The signals at 1446 and 1370 cm^{-1} are associated with the stretching vibrations of sulphate groups present on the surface of titania. Below 1000 cm^{-1} a wide band is observed that corresponds to Ti-O bonds of titania.

The infrared spectrum of $(NH_4)_2CuC1_4$ shows several bands derived principally from different combination modes of NH_4^+ ions [6]. The band at 3347 cm^{-1} is attributed to the interaction of water-copper bonds within CC. The bands at 3165, 3047 and 2817 cm^{-1} arise from combination modes of N-H stretching vibrations. The signal located between 2500-1750 cm^{-1} is attributed to combination of bending + librational modes. A strong band at 1575 cm^{-1} (with a shoulder at 1690 cm^{-1}) could be assigned to either stretching asymmetry or bending + librational modes of NH_4^+. The antisymmetric bending fundamental mode occurs as an intense absorption at about 1412 cm^{-1}. The spectrum of the CC-titania is a mixture of signals from TiO_2, GABA, and $Cu(NH_4)_2Cl_4$. Not to repeat all the bands, we marked the signals derived from the copper complex with the red asterisks and the signals derived from GABA - with the blue asterisks. For $CuCl_2$ alone and loaded on titania a similar study was developed (data are not shown). We can conclude that the copper complexes do not undergo the structural change when are added to titania. The UV spectra (data are not shown) are in accordance with these results.

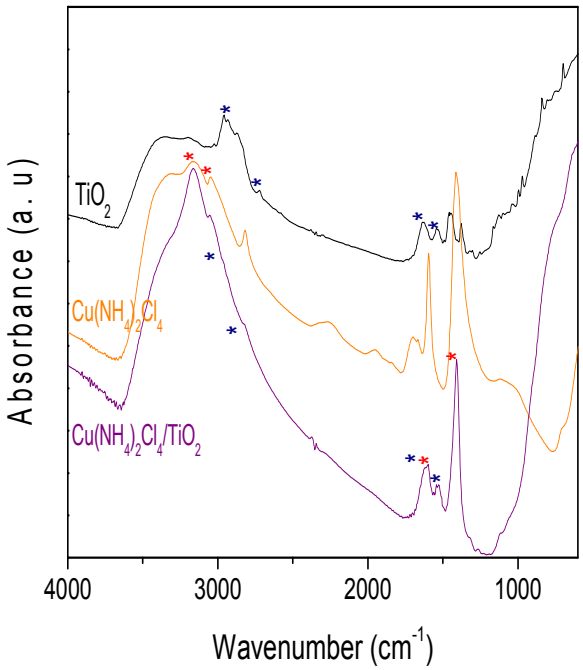

Figure 1: Infrared spectra of TiO_2, $Cu(NH_4)_2Cl_4$ and $Cu(NH4)_2Cl_4/TiO_2$ samples.

The N_2 adsorption-desorption isotherms of TiO_2, $CuCl_2/TiO_2$ and $Cu(NH_4)_2Cl_2/TiO_2$ materials are shown in figure 2. All isotherms are of the type IV in accordance with IUPAC classification [6]. Its type is obtained from mesoporous solids (2 nm <pore size <50 nm). Only the samples containing any copper complex developed a small hysteresis loop, which is associated with the secondary process of capillary condensation, which results in the complete filling of the mesopores at P/P_0 <1. These hysteresis loops have been recognized according to IUPAC classification as type H3 [7]. The principal information derived from this hysteresis is that usually the solids have a very wide distribution of pore sizes. In contrast, the N_2 adsorption-desorption isotherm of TiO_2 does not exhibit a hysteresis loop, which implies that the nitrogen desorption procedure follows the same path as tha nitrogen adsorption process. This observation suggests a probable existence of pores with cylindrical form.

The BET surface areas (S_{BET}), pore diameter (D_p) and pore volume (V_p) are given in table 1. There is an effect on the textural properties of titania by the copper complex added to its mesoporous structure. The surface area for TiO_2 was 515 m^2/g and it decreases with the copper complex addition to 226 and 205 m^2/g for $CuCl_2/TiO_2$ and $Cu(NH_4)_2Cl_4/TiO_2$, respectively. We attributed this decrease of the area to the fact that CC molecules occupy a part of the surface area of titania. The pore size of TiO_2 is 2.40 nm, while the pore sizes for $CuCl_2/TiO_2$ and $Cu(NH_4)_2Cl_2/TiO_2$ are of 4.97 and 4.12 nm, respectively. This increase is probably due to the fact that CC compound induces the formation of particles larger that those formed on titania alone, causing the formation of larger pores. The pore volume decreases slightly from TiO_2 to those materials containing Cu/TiO_2. This is attributed to the fact that CC molecules are deposited into the pores without their total occlusion.

Sample	**S_{BET} (m^2/g)**	**D_p (nm)**	**V_p (cc/g)**
TiO_2	515	2.40	0.3080
$CuCl_2/TiO_2$	226	4.97	0.2821
$Cu(NH_4)_2Cl_4/TiO_2$	205	4.12	0.2677

Table 1: Textural properties of TiO_2, $CuCl_2/TiO_2$ and $Cu(NH_4)_2Cl_4/TiO_2$ materials, where S_{BET} is the specific surface area, D_p is the average pore diameter, and V_p is the total pore volume.

The delivery profiles of the copper complexes (data are not shown) released from titania indicate that there are two different stages of liberation. During the first sixteen hours a fast CC release is observed. After this point the CC release is slow till the end (approximately forty-nine hours).

In the first stage the CC molecules from the external surface of titania are released. While in the second step the copper molecules are released from the pores through a diffusion process.

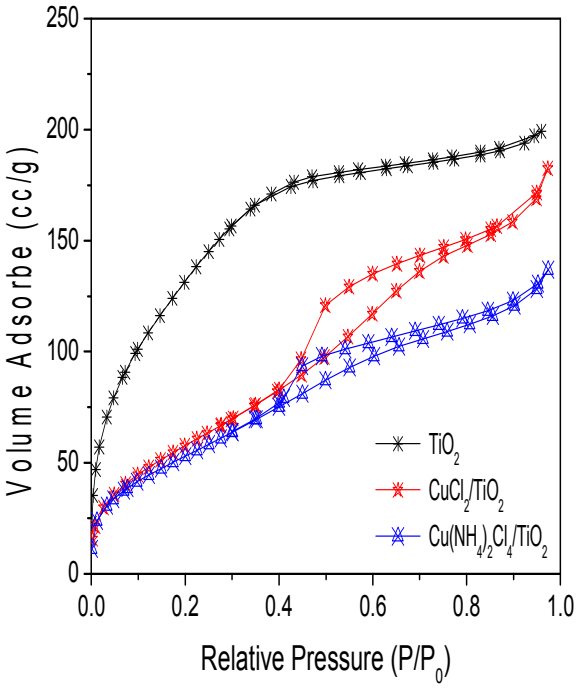

Figure 2: N_2 adsorption-desorption isotherms of TiO_2, $CuCl_2/TiO_2$ and $Cu(NH_4)_2Cl_4/TiO_2$ materials.

Cytotoxic response to titania, copper complexes and those loaded on titania was assessed in a glioma rat cell line (RG2) and compared with the cytotoxic effect of the most reported inorganic compound Cis-Pt. Figure 3 gives a correlation between the cytotoxic activity of $CuCl_2$, $CuCl_2/TiO_2$, $Cu(NH_4)_2Cl_4$, $Cu(NH_4)_2Cl_4/TiO_2$, TiO_2 and Cis-Pt content and the cell viability. The dose response curve showed that $CuCl_2$ and $Cu(NH_4)_2Cl_2$ had similar toxic effect. Low concentrations of both compounds were required to exert its toxic effect. Approximately 70 % of the cells died at dose of 31.2 µg/ml. The $Cu(NH_4)_2Cl_4/TiO_2$ material had a sustained toxic effect until the dose of 250 µg/ml where 88 % of cells died. The $CuCl_2/TiO_2$ material had less toxic effect, since a dose of 1000 µg/ml was necessary to obtain the same toxic effect as that of the previous material.

The copper complexes alone at low concentrations had more toxic effect than Cis-Pt, approximately eight times more than Cis-Pt was necessary to obtain 30% cell viability. The $Cu(NH_4)_2Cl_2/TiO_2$ has similar toxic effect to that of Cis-Pt but $Cu(Cl_2)/TiO_2$ has less toxic effect than Cis-Pt. On the other hand, copper complexes alone had more toxic activity than that when they were loaded on titania, however, it is important to note that these complexes were

released in a sustained form from the titania network. This implies that the administration of these copper compounds can decrease in a controlled way the side effect caused by the traditional administration (commonly oral) of a drug. It is also necessary to determine the toxic effect "in vivo" when these copper complexes are administered in the blood stream.

Titania material did not have toxic activity when it was compared with the control (untreated) cell. We observed a similar behavior when it was incubated with C6 cell line [8], indicating, again, a great biocompatibility of titania when it is functionalized.

There was no effect of the ligand on toxic activity of the copper complexes. Previously, we observed a similar effect when C6 cell line (a glioma rat cell) was treated with copper acetate [8].

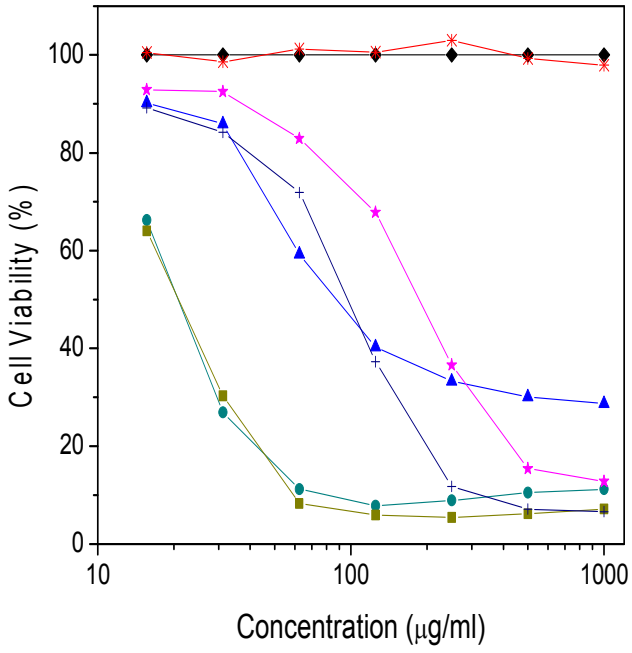

Figure 3: Cytotoxic effect of TiO_2 (✱), $CuCl_2$ (●), $CuCl_2/TiO_2$(★), $Cu(NH_4)_2Cl_4$ (■) $Cu(NH_4)_2Cl_2/TiO_2$ (+) and Cis-Pt (▲) on a RG2 cell. Control [untreated cell] (♦).

4 CONCLUSIONS

1) The structure of copper complexes does not undergo changes during its incorporation into the titania matrix.

2) The textural properties of TiO_2 were affected by the copper complex addition. The surface area decreases because CC molecules occupy approximately 40 % of its total surface area.

3) Two different stages of copper release were obtained: during the first stage the molecules from the external surface were released quickly to the aqueous medium.

4) A substantial cell death was found when the cell were treated with the copper complex alone at low concentrations, while a sustained cell death was observed when they were treated with the CC loaded into titania.

5) Copper complexes alone had more toxic activity than Cis-Pt compound, but the latter had similarly toxic effect as the copper complexes loaded on titania.

6) Functionalized titania is highly biocompatible with RG2 cell.

7) The "*in vitro*" tests suggest that copper complexes alone and loaded on titania can inhibit the growth of RG2 glioblastoma cells. Therefore, these preliminary results show potential properties of cupper complexes as anticancer agents for the GBM treatment.

5 ACKNOWELEDGMENT

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