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Study of the cytotoxicity and particle action in human cancer cells of titanocene-functionalized materials with potential application against tumors

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ABSTRACT

Titanocene dichloride [Ti(η^5-C_5H_5)$_2$Cl$_2$] (**1**), has been grafted onto dehydrated hydroxyapatite (HAP), Al_2O_3 and two mesoporous silicas MSU-2 (Michigan State University Silica type 2) and HMS (Hexagonal Mesoporous Silica), to give the novel materials HAP/[Ti(η^5-C_5H_5)$_2$Cl$_2$] (**S1**) (1.01 wt.% Ti), Al_2O_3/[Ti(η^5-C_5H_5)$_2$Cl$_2$] (**S2**) (2.36 wt.% Ti), HMS/[Ti(η^5-C_5H_5)$_2$Cl$_2$] (**S3**) (0.75 wt.% Ti) and MSU-2/[Ti(η^5-C_5H_5)$_2$Cl$_2$] (**S4**) (0.74 wt.% Ti), which have been characterized by powder X-ray diffraction, X-ray fluorescence, nitrogen gas sorption, multinuclear magic angle spinning NMR spectroscopy, IR spectroscopy, thermogravimetry analysis, UV spectroscopy, scanning electronic microscopy and transmission electronic microscopy. The cytotoxicity of the titanocene-functionalized materials toward human cancer cell lines from five different histogenic origins: 8505 C (anaplastic thyroid cancer), A253 (head and neck cancer), A549 (lung carcinoma), A2780 (ovarian cancer) and DLD-1 (colon cancer) has been determined. M_{50} values (quantity of material needed to inhibit normal cell growth by 50%) and *Ti*-M_{50} values (quantity of anchored titanium needed to inhibit normal cell growth by 50%) indicate that the activity of **S1**–**S4** against studied human cancer cells depended on the surface type as well as on the cell line. In addition, studies on the titanocene release and the interaction of the materials **S1**–**S4** with DNA show that the cytotoxic activity may be due to particle action, because no release of titanium complexes has been observed in physiological conditions, while electrostatic interactions of titanocene-functionalized particles with DNA have been observed.

1. Introduction

Drug delivery systems based on biomaterials is a research field which has been developed during the last 20 years and in which nano-structured surfaces have been widely used to maximize the efficacy of several anticancer drugs [1–2].

Mesostructured materials such as MCM-41 and SBA-15 have been studied in biological matrices, observing their bioactive behavior which included in some cases an appearance of a carbonate hydroxyapatite layer that confirmed their ability to be used for medical devices [3–4]. Thus, one of the possible applications of these nanostructured materials is the fight against cancer, especially against bone tumors, due to their notable bioactivity in bone regeneration [5].

Our previous work was focused on the design and anticancer applications of novel metal-based drugs [6–8], especially of titanocene derivatives [9–11]. Inspired by the pioneering work of Köpf-Maier and coworkers on metallocene derivatives as anticancer drugs [12], the studies on the hydrolysis reactions of titanocene complexes in biological media [13], and the excellent improvements in the cytotoxicity of titanocene derivatives by the synthetic work of Tacke and colleagues [14–15], we decided to study the cytotoxic activity of different mesoporous materials functionalized with titanocene complexes.

Our previous studies in this topic have demonstrated that these materials are potentially useful as bone fillers for *in situ* treatment of bone tumors such as osteosarcoma or condroblastoma upon local implantations which may avoid recurrence of the tumor after an exhaustive work [16–18]. Thus, titanocene-functionalized MCM-41 or SBA-15 were synthesized starting from different titanocene dichloride derivatives [16,17]. In addition, mesoporous silica containing titanocene thiolate compounds with ligands with $-Si(OMe)_3$ or $-Si(OEt)_3$ moieties (in order to obtain higher titanium content on the final surface) have also been reported by our research group [18]. Our studies showed that unmodified MCM-41 or SBA-15 were not active against human cancer and inmunocompetent cells, while all the functionalized surfaces exhibited *in vitro* cytotoxic activity with this activity being higher in surfaces with higher functionalization rates [18]. This phenomenon indicated a clear dependence of the content of grafted

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titanocene complex on the final anticancer activity, showing that the cytotoxicity of these materials may be due to the release of the corresponding metallocene derivative.

These interesting results moved our interest to the study of the dependence of the surface type on the final cytotoxic activity. Thus, we decided to synthesize titanocene-functionalized silica-based materials such as MSU-2 or HMS and other functionalized non-silica based materials such as hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$) or alumina in order to observe the influence of different materials with different particle sizes, morphologies and composition on the final anticancer activity. For these purposes, hydroxyapatite and alumina, as well as MSU-2 and HMS have been used as supports, because to date, no reports have been published regarding the cytotoxicity of these materials functionalized with metal complexes.

In addition, hydroxyapatite has been chosen as a scaffold because it is an important bioactive material which has attracted a lot of attention and has been used as a bone cement, drug deliverer, toothpaste additive, dental implant and various other applications, due to its excellent osteoconductivity, biocompatibility, bioactivity, and chemical and biological similarity with the mineral constituents of human bones and teeth [19–21]. In addition, hydroxyapatite is known for its capability to bind a wide variety of molecules [22–25] and most therapeutic agents for bone diseases under physiological conditions [26–28]. On the other hand, alumina has also been used as support due to its inertness in biological systems as well as its known properties in drug delivery systems [29–30].

In this paper we describe the synthesis, functionalization with titanocene dichloride, characterization and *in vitro* anticancer tests of a wide variety of silica-based (SBA-15, HMS and MSU-2) and non-silica-based (hydroxyapatite and alumina) materials against human cancer cell lines from five different histogenic origins (8505 C — anaplastic thyroid cancer, A253 — head and neck cancer, A549 — lung carcinoma, A2780 — ovarian cancer and DLD-1 — colon cancer). In addition, the study of the release of the grafted titanocene derivative in simulated body fluids is also reported.

2. Materials and methods

2.1. General conditions

All manipulations were performed under dry nitrogen gas using standard Schlenk techniques and dry box. Solvents were distilled from the appropriate drying agents and degassed before use. Tetraethylorthosilicate (TEOS) 98% (MW = 208.33, d = 0.934 g/mL), dodecylamine (DDA) 98% (M = 185.36), poly(ethylene glycol)-blockpoly(propylene glycol)-blockpoly(ethylene glycol) (M_{av} = 5800; d = 1.019 g/mL) Tergitol® NP-9 (MW = 616.82), hydroxyapatite-nanopowder, (<200 nm particle size (BET), ≥97%) and aluminum oxide-nanopowder (<50 nm particle size (TEM)) all from Sigma-Aldrich, were used as purchased, without further purification. Water (resistance 18.2 MΩ cm) used in the preparation of materials was obtained from a Millipore Milli-Q-System (Billerica, MA, USA). [Ti(η^5-C_5H_5)$_2$Cl$_2$] was prepared according to literature procedures [31–32].

2.2. General remarks on the characterization of the materials

^{1}H MAS NMR (4 μs 90° pulse, spinning speed of 9 MHz, pulse delay 2 s) ^{13}C-CP MAS NMR (4.40 μs 90° pulse, spinning speed of 6 MHz, pulse delay 2 s), ^{29}Si MAS NMR (8 μs 90° pda, spinning speed of 6 MHz, pulse delay 10 s) ^{27}Al MAS NMR (1.5 μs 90° pulse, spinning speed of 12 MHz, pulse delay 1.5 s) and ^{31}P MAS NMR (2.5 μs 90° pda, spinning speed of 5 MHz, pulse delay 5 s) spectra, were recorded on a Varian-Infinity Plus Spectrometer at 400 MHz operating at 100.52 MHz proton frequency. X-ray diffraction (XRD) pattern of the silicas was obtained on a Philips Diffractometer model PW3040/00 X'Pert MPD/MRD at 45 KV and 40 mA, using a wavelength Cu Kα (λ = 1.5418 Å). Ti and Cl wt.% determination by X-ray fluorescence were carried out with a X-ray fluorescence spectrophotometer Philips MagiX with a X-ray source of 1 kW and a Rh anode using a helium atmosphere. The quantification method is capable of analyzing from 0.0001% to 100% titanium and chlorine.

The thermal stability of the modified mesoporous silicas was studied using a Setsys 18 A (Setaram) thermogravimetric analyzer, using a platinum crucible of 100 μL. A synthetic air atmosphere was used and the temperature increased from 25 °C to 800 °C at a speed of 10 °C per min. N_2 gas adsorption–desorption isotherms were performed using a Micromeritics TriStar 3000 analyzer. Scanning electron micrographs and morphological analysis were carried out on a XL30 ESEM Philips with an energy dispersive spectrometry system (EDS).

The samples were treated with a sputtering method with the following parameters: Sputter time 100 s, Sputter current 30 mA, film thickness 20 nm using a Sputter coater BAL-TEC SCD 005. Conventional transmission electron microscopy (TEM) was carried out on a TECNAI 20 Philips, operating at 200 kV.

2.3. Preparation of non-functionalized materials

2.3.1. Preparation of HMS

A hexagonal material (HMS) was prepared according to the methods of Onida et al. [33] and Yang et al. [34]. HMS silica was synthesized at room temperature using TEOS, DDA, ethanol and distilled water at a composition ratio of 1:0.27:6.5:36. The solution was stirred for 18 h, yielding a thick white suspension that was filtered and dried at 80 °C for 1 h. The amine was removed by heating the solid at reflux in absolute ethanol with a Soxhlet for 8 h. Finally, the remaining surfactant was removed by calcination in an atmosphere of air at 550 °C for 12 h. The surface was then dehydrated under vacuum (10^{-2} mm Hg) for 16 h at 250 °C, cooled and stored under dry nitrogen.

2.3.2. Preparation of MSU-2

A mesoporous silica of the MSU-X family (MSU-2-type) was prepared using the synthetic method reported by us [35]. MSU-2 was synthesized by a two-step process: firstly TEOS was added to a stirring 0.08 M (pH 4.8) solution of Tergitol® NP-9 in Milli-Q water, at room temperature, to obtain a milky suspension (TEOS/surfactant solution molar ratio of 8/1). The resulting suspension was then aged without agitation for 20 h to give a clear solution. In the second step, a 0.24 M sodium fluoride solution was added dropwise with stirring to the TEOS/surfactant solution to obtain a NaF/TEOS molar ratio of 0.025/1. The solutions were placed in a bath with agitation at 55 °C for 48 h. The final product was filtered off, washed with Milli-Q water and air-dried at 100 °C for 4 h. Finally, the surfactant was removed by calcination in air at 600 °C for 12 h. The surface was then dehydrated under vacuum (10^{-2} mm Hg) for 16 h at 250 °C, cooled and stored under dry nitrogen.

2.4. Preparation of modified surfaces

2.4.1. Preparation of HAP/[Ti(η^5-C_5H_5)$_2$Cl$_2$] (**S1**)

A solution of [Ti(η^5-C_5H_5)$_2$Cl$_2$] (260 mg, 1.04 mmol) (to obtain a theoretical level of 5% Ti/HAP) in toluene (100 mL) was added to dehydrated HAP (1.00 g) and the mixture was stirred overnight at 80 °C. The slurry was filtered through fritted discs and the solid residue washed with toluene (5 × 200 mL). The resultant solid was dried under vacuum at room temperature for 16 h to give a pale brown free flowing powder. UV: λ (nm) = 257 (s), 383 (m), 512 (w), 537 (w). ^{1}H MAS NMR: δ 4.24 (Cp). ^{13}C CP MAS NMR: 181.7 (Cp), 124.8 (Cp). ^{31}P MAS NMR: 2.4 (PO_4^{3-}). Ti_{exp} (wt.%) = 1.01.

2.4.2. Preparation of Al_2O_3/[Ti(η^5-C_5H_5)$_2$Cl$_2$] (**S2**)

The modified surface **S2** was prepared following the procedure described for **S1**, using a solution of [Ti(η^5-C_5H_5)$_2$Cl$_2$] (260 mg,