

Cytotoxic effect of an alumina matrix composite with APTS-functionalized carbon nanotubes in Vero cells

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RESEARCH

ABSTRACT

Alumina (Al_2O_3) is a hard, highly biocompatible and low friction ceramic material of multiple biomedical applications, but mechanically weak and showing poor flexibility. To circumvent these limitations, it could be complexed as composite materials with carbon nanotubes (CNTs). However, CNTs display cytotoxic effects. In this work, the cytotoxic effect of a composite of Al_2O_3 and CNTs functionalized with 3-aminopropyl-triethoxysilane (APTS; APTS, 125-5000 $\mu\text{g}/\text{mL}$) was evaluated in Vero cells. Cell viability was determined by MTT assay and Hoechst staining, 72 h after treatment. Additionally, the hemolytic capacity of the nanomaterial was evaluated in human red blood cells. The lowest cytotoxicity values were attained at 1.5 % CNTs by MTT, and 1.0 % by Hoechst staining. Nanometric Al_2O_3 -CNT showed the highest cytotoxicity as compared with the micrometric composite. The APTS-functionalized composite was cytotoxic starting at 500 $\mu\text{g}/\text{mL}$ and higher concentrations, with less than 50 % cell viability attained at 5000 $\mu\text{g}/\text{mL}$. Cells treated with Al_2O_3 -functionalized CNTs showed significantly lower viability as compared to those treated with Al_2O_3 -functionalized CNTs at concentrations above 500 $\mu\text{g}/\text{mL}$ ($p < 0.05$). Overall, the Al_2O_3 -CNTs nanocomposite did not significantly affect Vero cells viability at lower concentrations (below 1000 $\mu\text{g}/\text{mL}$). Moreover, none of the treatments showed hemolytic activity, implying that the higher cytotoxic effect was associated to the nanometric scale of Al_2O_3 -CNTs composite, CNTs percentage and APTS functionalization, but not affecting directly the integrity of the red blood cells membrane. So far, this is the first *in vitro* approach on the evaluation of Al_2O_3 with CNTs material and APTS functionalization in Vero cells.

Keywords: aluminum oxide, carbon nanotubes, APTS functionalization, composite materials, cytotoxicity test

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RESUMEN

Evaluación del efecto citotóxico de un nanomaterial compuesto de alúmina y nanotubos de carbono funcionalizado con APTS en células Vero. La alúmina (Al_2O_3) es un compuesto cerámico que destaca resalta por su alta biocompatibilidad, dureza y bajos coeficientes de fricción, a pesar de su pobre resistencia mecánica y su baja flexibilidad. Para superar estas limitaciones, se acomplejó tanto a escala micro como nanométrica con nanotubos de carbono (CNTs), y se evaluó el efecto citotóxico que pudiera derivar del uso de los CNT. Estos se funcionalizaron con 3-Aminopropil-trietoxisilano (APTS; 125-5000 $\mu\text{g}/\text{mL}$), y la citotoxicidad del compuesto se evaluó en células Vero mediante ensayo MTT y tinción de Hoechst, 72 h después del tratamiento, junto a la capacidad hemolítica del nanomaterial en glóbulos rojos humanos. El compuesto de alúmina nanométrica fue más citotóxico, con los menores valores de citotoxicidad detectados al 1.5 % de CNTs mediante MTT y 1.0 % mediante tinción de Hoechst. El compuesto de Al_2O_3 nanométrica-CNTs funcionalizados afectó la viabilidad celular a 500 $\mu\text{g}/\text{mL}$ o más, con menos del 50 % a 5000 $\mu\text{g}/\text{mL}$, e igualmente la afectó en comparación con el nanocompuesto sin APTS a partir de 500 $\mu\text{g}/\text{mL}$ ($p < 0.05$). Ningún tratamiento causó hemólisis. Esto indicó que el nanocompuesto Al_2O_3 nanométrica-CNTs no afectó significativamente la viabilidad de las células Vero a concentraciones inferiores a 1000 $\mu\text{g}/\text{mL}$, y que la citotoxicidad se debió al tamaño del material, el porcentaje de CNTs y su funcionalización con APTS, no así a la afectación directa de la integridad de la membrana celular en eritrocitos humanos. Este es el primer reporte sobre la evaluación de la alúmina acomplejada con CNTs funcionalizados con APTS en células Vero.

Palabras clave: óxido de aluminio, nanotubos de carbono, funcionalización con APTS, materiales compuestos, test de citotoxicidad

Introduction

Ceramic materials have been of great interest for the development of composite biomaterials to be used as scaffolds or implants for bone tissue regeneration [1, 2]. Their potential application in

biomedicine is determined in part by their chemical composition, which includes ions commonly found in the physiological milieu (e.g., calcium, potassium, magnesium and sodium, among others) or of limited

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toxicity for body tissues (e.g., zirconium and aluminum)[3, 4].

In fact, alumina or aluminum oxide (Al_2O_3) is a ceramic material widely used in orthopedics, such as femur head, hip prosthesis, acetabular caps and tibial plates in artificial knees [5]. Similarly, it is applied in ophthalmology to produce artificial corneas or keratoprosthesis, and porous orbital implants, also showing great potential in tissue engineering for dental implants and bone regeneration scaffolds [6-8]. Several studies have focus on the biological properties of alumina *in vitro* (osteoblasts, osteosarcoma cells, mesenchymal stem cells and bone marrow cells) and *in vivo*, evidencing its biocompatibility by favoring cell proliferation, adhesion and differentiation [9-12]. Nevertheless, in spite of its biocompatibility, alumina is prone to fracture, this weakness limiting its application on implants that get into contact with the supporting bone tissue, as happens in artificial joints, plates and bone repair scaffolds, and the cause for its limited use for non-pressure sites [13, 14]. Therefore, in order to improve its mechanical properties, several composite materials have been developed by reinforcing alumina with hydroxyapatite, zirconium and carbon nanotubes (CNTs)[8, 9].

Carbon nanotubes bear physicochemical properties that remarkably improve the mechanical properties of ceramic materials, including alumina [15, 16]. It has been recently demonstrated that the synthesis of a natural alumina composite material containing nanotubes significantly improve its properties as tension, strength, and resistance to flexion and fracture, all these providing a structural material of high performance [13, 17]. However, the compatibility of every nanocomposite with the target biological systems has to be studied prior to its application as biomaterial. In fact, several *in vitro* investigations have indicated that CNTs tend to be cytotoxic and genotoxic in general, with some controversial reports of their stimulatory effect on cell proliferation [1, 2, 18]. Abundant scientific literature refers the biological behavior of CNTs, showing that its cytotoxic effects could vary depending on their size, structure and functionalization method [12-14, 17, 19, 20]. But the biological effects of alumina-containing CNTs have not been tested in detail for their effects on human health. In fact, some authors have stated that chemical functionalization of the surface or the outer layers of CNTs could improve their solubility and dispersion, thereby decreasing in part the undesired biological effects [1, 2, 18, 21], in spite of the few reports available on chemically-functionalized CNTs.

Since CNTs improve the mechanical properties of alumina, this increases its potential for application in tissue engineering. Results published by Ogihara et al. on the biocompatibility of an alumina matrix-CNT composite showed that the nanocomposite displayed a resistance to fracture and biocompatibility with the bone tissue comparable to that of alumina, but the direct effect of the material on cell proliferation was not determined [22]. Hence, in this work, the cytotoxic and hemolytic effect was determined for a nanomaterial composite of alumina and CNTs functionalized with (3-Aminopropyl)triethoxysilane (APTS).

Materials and methods

Cell culture and composite materials

Vero cells (ATCC CCL-81) were used to characterize the *in vitro* biological effect. Briefly, cells were cultured in RPMI 1640 medium (Lonza; Switzerland) supplemented with bovine fetal serum (Gibco) and 1 % penicillin/streptomycin (Gibco), and incubated at 37 °C under a 5 % CO_2 atmosphere.

Previously synthesized micrometric alumina-CNTs and nanometric alumina-CNTs materials were used [23]. The composite was generated by mixing commercial micrometric alumina (Ereckos) or nanometric synthesized alumina [24], with multiwalled CNTs (Nanostructured & Amorphous Materials Inc.; 95 % purity, 40-60 nm in diameter and 1-2 μm in length) at either 0.5, 1.0 or 15 % ratios, respectively. CNTs were functionalized by subsequent mixing of CNTs at 12.5 % (w) in 87.5 % ethanol, sonication for 2 h and stirring at 200 rpm. In the following, the APTS reagent (2.25 μM ; Sigma-Aldrich) was added at a temperature in the range 50-70 °C for 4 h under magnetic stirring at the same speed. Then, CNTs were washed with distilled water and alcohol to eliminate the excess APTS in the surface of the material, and the final CNTs preparation were recovered in filter paper and dried out for 24 h at 100 °C.

The obtained composite materials were sterilized by ultraviolet radiation for 1 h and sterility was corroborated by putting a sample of the material in rich culture media and incubated for 7 days at 37 °C under a 5 % CO_2 atmosphere, and it was periodically monitored for any sign of contaminant microbial growth. Once the sterility of the composites was checked, solutions were prepared for each composite at 10 mg/mL in RPMI 1640 medium and aged for a week under constant stirring at 20 rpm in the dark. All the assays were run in triplicates in at least two independent experiments.

MTT colorimetric assay

The effect of the different materials on the cell viability was determined by the MTT colorimetric assay. For that purpose, 50 000 cells were seeded per well in 24-well plates and the different composites added 24 h later. Cells were treated with the aged medium containing either micrometric- or nanometric alumina-CNTs at the 0.5, 1.0 or 1.5 % ratios, respectively, in concentrations ranging 125-5000 $\mu\text{g}/\text{mL}$, with and without APTS. The treatment was repeated every 24 h (ASTM Guidelines 1499-09), for an overall treatment period of 72 h. Controls included: untreated cells, cells treated either with each alumina without CNTs, APTS (2.25 μM), potassium permanganate at cytotoxic concentration (400 μM) and carbon nanotubes (1.85-75 $\mu\text{g}/\text{mL}$). Afterwards, the MTT reagent ((Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; 200 $\mu\text{g}/\text{mL}$) was added in RPMI 1640 medium at 5% FBS and cells were further incubated at 37 °C for 3 h. Then, the generated formazan crystals were solubilized in 100 % DMSO and absorbance at 560 nm ($\text{Abs}_{560\text{nm}}$) was measured in a GloMax®-Multi Detection System plate reader (Promega). The absorbance of untreated cells was regarded as 100 % cell viability

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to calculate the cytotoxicity of the tested materials by the formula:

$$\text{Viability (\%)} = \frac{\text{Absorbance}_{\text{material}}}{\text{Absorbance}_{\text{negative control}}} \times 100$$

Hoechst's nuclei staining

MTT results were compared in Vero cells once treated with each composite for 72 h. Then, medium was removed and cells were washed twice with 1× isotonic phosphate-buffered saline (PBS) solution, fixed with 4 % paraformaldehyde and stained with a Hoechst 33258 (Life Technologies) solution at 2 µg/mL. Finally, cells were observed by using a Nikon Eclipse Ti inverted fluorescence microscope. Images were obtained with the following conditions: exposure for 600 ms, 2.0× gain, dynamic contrast, and source intensity value of 4. For each treatment, three microscopic fields were averaged, and the mean intensity fluorescence (MIF) determined for each image with the ImageJ software (NIH, USA).

Hemolysis assay

Human whole blood was used for the hemolysis assay, provided by healthy volunteers with prior informed consent. Blood samples were obtained by venipuncture using Vacutainer® tubes with heparin. Red blood cells were separated by centrifugation at 2000 rpm for 10 min and washed five times with isotonic PBS 1× solution (Gibco). Cells were tested diluted 1:10 in PBS 1× by mixing them at 1:4 ratios with each treatment solution and further incubated at 37 °C for 2 h with either PBS 1× (negative control), RPMI 1640 culture medium supplemented with APTS and each APTS-functionalized composite material at cytotoxic concentrations (1-5 mg/mL). Afterwards, cells were centrifuged at 1000 rpm for 2 min. Hemolysis was evaluated by measuring the Abs_{560nm} in a GloMax®-Multi Detection System plate reader (Promega, USA).

Statistical analyses

Data obtained in triplicate from two replicas of each experiment were analyzed with the GraphPad Prism 6 software; parametric two-tailed variance analysis (ANOVA) was done for multiple comparisons. Differences between treatments were regarded as statistically significant for $p < 0.05$ by the Tukey's multiple comparison test.

Results

The alumina composite material (Al₂O₃-CNT) was first compared at both nanometric and micrometric scale in the 125-5000 µg/mL concentration range. The micrometric-scale composite decreased cell viability starting at 1000 µg/mL as determined by the MTT assay, while the nanometric started at 500 µg/mL (Figure 1). Similarly, nanometric alumina decreased cell viability starting at 2500 µg/mL, while micrometric alumina showed no cytotoxicity at any of the assayed concentrations. Cell viability results of at 2.500 and 5.000 µg/mL, the nanometric Al₂O₃-CNT composite was less toxic than alumina alone. It was inferred the size of the material influenced on the cytotoxicity

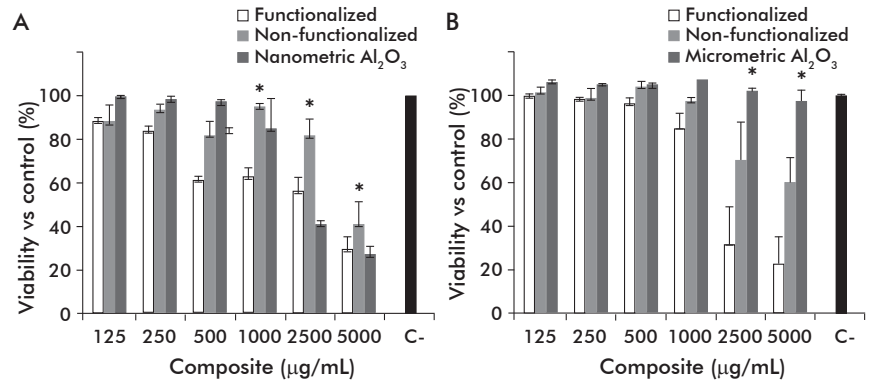


Figure 1. Comparison of the cytotoxic effect of alumina-carbon nanotubes (Al₂O₃-CNTs) composites on Vero cells, attending to its functionalization with (3-Aminopropyl)triethoxysilane (APTS), at different composite concentrations. A) nanometric alumina. B) micrometric alumina. CNTs were tested at a 1.5 % concentration. Viability was assessed by the MTT assay, 72 h after the incubation of Vero cells with either compound. C-: Negative control, cells without treatment. A two-tailed ANOVA parametric test was run and differences were detected by the Tukey's test ($p < 0.05$).

observed, since the composite material analyzed was prepared with 1.5 % CNTs and it as established in previous reports that CNTs doses below 1.0 % in nanometric alumina did not significantly affect cell viability [15]. This is further supported by the lack of cytotoxicity in the control condition of micrometric alumina, while the nanometric one reduced cell viability below 50 % at 2500 µg/mL and higher concentrations.

Therefore, the nanometric scale alumina composite was selected for subsequent testing, that scale relevant for nanomaterial engineering approaches [16], and assuming that micrometric alumina with high CNTs concentrations (1.5 %) could affect even more the bioavailability and the dispersion of micrometric alumina in cell culture, what could complicate the interpretation of viability results. Similarly, the functionalized Al₂O₃-CNT composite showed cytotoxic effects either at nanometric or micrometric scale in the range starting at 500 and 2500 µg/mL concentrations, respectively.

The cytotoxic effect of the nanometric functionalized nanomaterial was analyzed at different CNTs concentrations to determine the effect of these lasts on cell viability, as shown in figure 2 A and B. There were statistically significant differences in cell viability when cells were treated with the different CNTs percentages for the nanometric non-functionalized Al₂O₃-CNT material at 2500 and 5000 µg/mL. Noteworthy, the highest CNTs concentration tested (1.5 %), showed the lowest cytotoxicity values, this result consistent among the MTT assays using that CNTs concentration. Additional testing is required to explain this result. Moreover, at the 5000 µg/mL concentration, cell viability was less than 50 % (Figure 2A). Similarly, cells treated with the APTS-functionalized nanomaterial showed statistically significant differences at 500, 1000 and 5000 µg/mL at CNTs concentrations of 0.5 and 1.5 %, with cell viability below 50 % at 5000 µg/mL Al₂O₃-CNT APTS composite for CNTs at 1.0 and 1.5 % (Figure 2B). No CNTs-dependent differences were observed at 2500 µg/mL, but there was a general tendency to increase cytotoxicity by increasing the concentration of the nanocomposite.

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In summary, the results evidenced that CNTs at 1.0 and 1.5 % significantly affected cell viability and that the APTS-functionalized Al_2O_3 -CNT nanometric matrix tended to produce a cytotoxic effect as compared with the non-functionalized matrix.

Cell proliferation in culture was comparatively evaluated in the presence of the functionalized material against the non-functionalized one by Hoechst fluorescence staining at 125-5000 $\mu\text{g/mL}$ composites concentrations. The mean fluorescence intensity (MFI) was quantified from cell culture images taken at 40 \times , by using the ImageJ software, on three optical fields of Hoechst-stained cells evaluated for each treatment. A tendency was observed for the functionalized material to show lower MFI values (Figure 3A), in agreement with previous results indicating a tendency of the functionalized material to be less toxic than the non-functionalized one. In fact, the Al_2O_3 -CNT functionalized matrix showed statistically significant differences at the three CNTs concentrations tested (0.5, 1.0 and 1.5 %) and all the matrix concentrations except at 5000 $\mu\text{g/mL}$, with cell viability values below 50 % (Figure 3B). In contrast, notable differences were only observed for the non-functionalized material at 0.5 and 1.5 % CNTs (data not shown). At the same time, these results indicate that the higher the CNTs concentrations, the higher the cytotoxic effect, particularly at 1 % since cell viability was even lower than that of the positive control.

To try to characterize a possible cytotoxic mechanism related to the interaction of the composite with or the damage to biological membranes, a hemolysis assay was run in human red blood cells (Figure 4). Both, neither Al_2O_3 -CNT nanocomposites, APTS-functionalized or not, and non-functionalized nor CNTs showed any hemolytic effect at the concentrations tested. These results suggest that the cytotoxic effect of the APTS-functionalized Al_2O_3 -CNT composite observed is independent of the direct interaction of the composite with biological membranes.

Discussion

The cytotoxic effect of a nanomaterial composed of alumina and CNTs functionalized with APTS was investigated. Alumina is commonly used as scaffold material in bone regeneration, due to its advantageous porous nature aiding cells to colonize and growth [25]. It has been reported that alumina is highly biocompatible in *in vitro* models. For instance, Karlsson *et al.* studied the interaction of primary human osteoblasts with nanoporous alumina, finding a proliferation curve comparable to that of the control [25]. Similar results have been obtained in various studies testing different cell lines [26-28].

Particularly for micrometric alumina, our results demonstrate that it does not have a cytotoxic effect in Vero cells, since viability was similar to that of the negative control at all the concentrations. In contrast, nanometric alumina did show cytotoxicity at the highest concentrations tested (2500 and 5000 $\mu\text{g/mL}$), what could be related to a better dispersion, according to microscopy images observations (data not shown), thereby contributing to a better interaction with the cell monolayer due to an increased surface/volume

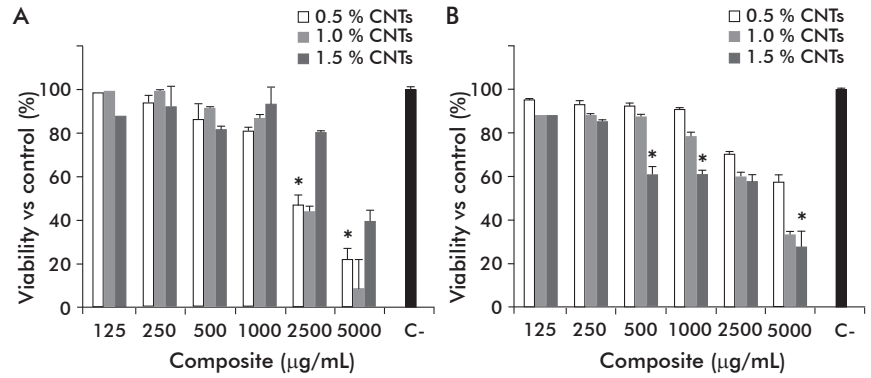


Figure 2. Evaluation of cytotoxicity of nanometric alumina-carbon nanotubes (Al_2O_3 -CNTs) composites on Vero cells, at different composite concentrations. A) Composite with CNTs functionalized with (3-Aminopropyl)triethoxysilane (APTS). B) Non-functionalized composite. Cell viability was assessed by the MTT assay, 72 h after incubation with the given composite. Three CNTs concentrations were tested: 0.5, 1.0 and 1.5 %. C-: Negative control, cells without treatment. A two-tailed ANOVA parametric test was run and differences were detected by the Tukey's test ($p < 0.05$).

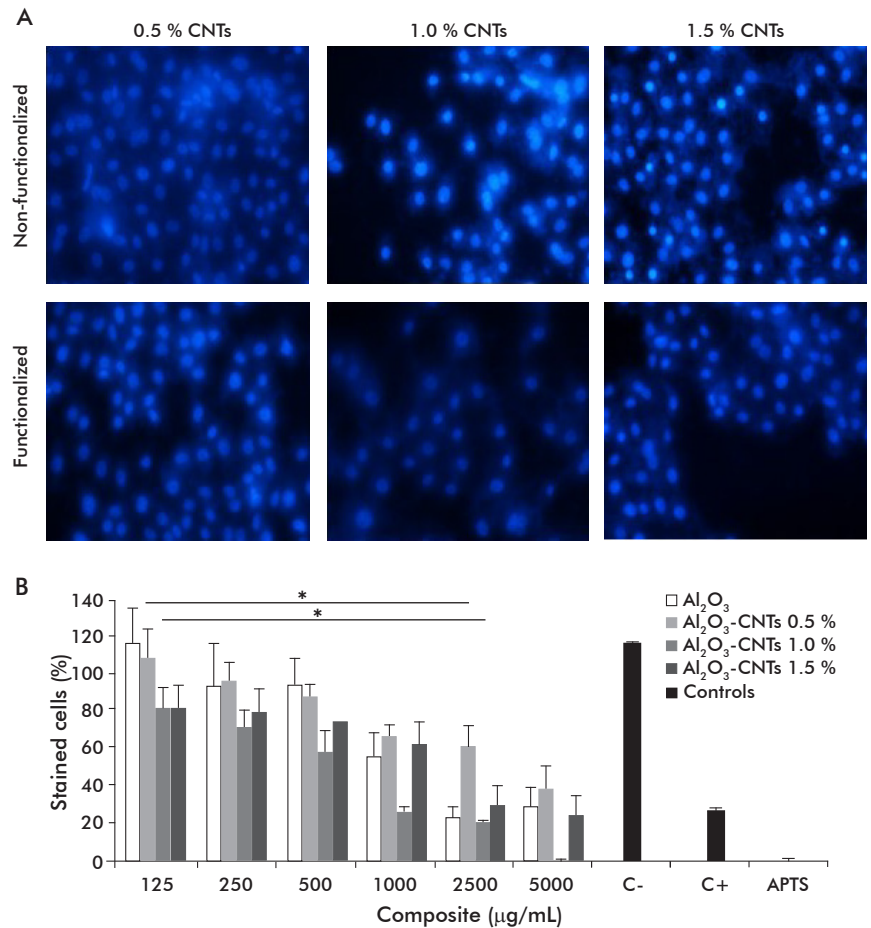


Figure 3. Evaluation of cytotoxicity of nanometric alumina-carbon nanotubes (Al_2O_3 -CNTs) composites, CNTs functionalized or not with (3-Aminopropyl)triethoxysilane (APTS) and at different CNTs concentrations (0.5, 1.0 and 1.5 %). A) Representative fluorescence microscopy images of Vero cells stained with Hoechst 33258, 72 h after treatment with Al_2O_3 -CNTs composites either functionalized or not. Images were taken at 40 \times magnification. B) Percentage of live cells after incubation with the different composite treatments, as compared to the untreated controls, quantified in respect to the mean fluorescence intensity. C-: Negative control, untreated cells. C+: Positive control, cells treated with 400 μM KMnO_4 . APTS: Treatment with 0.2% (v/v) APTS. A two-tailed ANOVA parametric test was run and differences were detected by the Tukey's test ($p < 0.05$) among composite concentrations in the range 125-2500 $\mu\text{g/mL}$, containing 0.5 or 1.0 % CNTs.

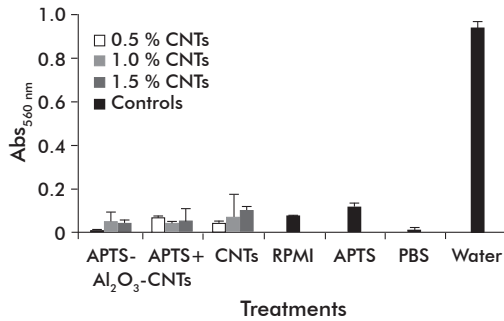


Figure 4. Hemolytic effect of a nanometric alumina-carbon nanotubes (Al_2O_3 -CNTs) composite, containing 1.5 % CNTs functionalized or not with (3-Aminopropyl)triethoxysilane (APTS). The nanocomposite was tested at 1000, 2500 and 5000 $\mu\text{g}/\text{mL}$. Hemolysis was evaluated by measuring the hemoglobin released to the culture medium, its absorbance determined at 560 nm ($\text{Abs}_{560\text{nm}}$) in culture supernatants obtained after treatment. As negative control, human red blood cells were incubated under isotonic conditions in RPMI culture medium and phosphate-buffered saline (PBS) 1x, respectively. As positive control, cells were incubated under hypotonic conditions (sterile distilled water). APTS- Control APTS treatment at 2%, v/v. CNTs were also tested at 1.5% as control.

ratio [15]. Moreover, treatments with the Al_2O_3 -CNT composite evidenced a decrease in cell viability at concentrations of 500 $\mu\text{g}/\text{mL}$ or higher, this effect more pronounced in the APTS-functionalized Al_2O_3 -CNT. Previous studies demonstrated that non-refined CNTs could be cytotoxic both *in vitro* and *in vivo*, mainly due to the presence of transition metals, while chemically-functionalized CNTs showed a relatively increased cell viability [29, 30], possibly related to a lower cytotoxic effect of the APTS chemical agent as compared to non-refined CNTs.

As widely known, CNTs are tubular structures formed by graphite layer nanometric in diameter and up to 1-mm long, making of them the strongest and lighter fibers available, being ideal to improve the mechanical properties of materials used to produce synthetic tissues [10, 24, 31-33]. Nevertheless, they are not soluble in organic solvents, their surface properties having to be enhanced by functionalization to increase their dispersion in biological media while decreasing their toxic effects.

Therefore, in this work, the APTS was used for chemical functionalization of the nanometric alumina particles, to enhance the adhesion interface of the particles with the CNTs by coupling to APTS, and subsequently, the dispersion of the nanomaterial [10, 24, 31-33]. This was previously tested both for nanometric and micrometric alumina by electron microscopy, evidencing a deficient dispersion of the material. Afterwards, the alumina composite agglomerated in the culture media at the different CNTs concentrations tested. Hence, assays were run with different surfactants (CHAPS, SDS, Triton X-100, Tween 20 and others) to try to circumvent this limitation. Since no significant changes were detected in the materials, they were subjected to aging for 7 days in RPMI culture media according to the ASTM 1499-09 method, to obtain a more homogeneous distribution. This was qualitatively corroborated by light microscopy, what caused a better dispersion of the nanometric material

in culture, and a higher effect on cell viability when compared to the micrometric material.

Interestingly, our results indicate that the nanometric and micrometric alumina composites at 1.5 % of CNTs and functionalized with APTS induce a higher cytotoxicity as compared to the non-functionalized materials. Moreover, the decrease in cell viability was more pronounced for nanometric alumina with CNTs in the 125-1000 $\mu\text{g}/\text{mL}$ range. In this sense, when Vero cells were treated with APTS, there was a drastic decrease in more than 90 % of cells stained, 72 h after treatment. In fact, other authors have reported that functionalization decreases cell cytotoxicity. Wang et al. evaluated the biological effect of the surface modification of nanohydroxyapatite with APTS [34], finding that APTS-modified nanoparticles with APTS up to 100/ mL were more cytotoxic on mice fibroblasts than the unmodified ones. There has been mentioned that the positive charges of the APTS amine groups could be responsible for the cytotoxicity seen, by facilitating their interaction with the phosphate groups of membrane lipids, leading to cell death. In this sense, the cytotoxicity of the APTS functionalization could be decreased by neutralizing the charges of the amino groups at the APTS surface by adding methyl groups or by negating them by adding carboxyl groups [10, 24, 31-34].

In contrast, when the APTS-functionalized Al_2O_3 -CNT composite was evaluated as related to cell lysis or not, it was observed that treatments with the composite material and the APTS agent had no hemolytic effect on human red blood cells. Therefore, it could be inferred that there are other mechanisms involved in the cytotoxicity of this composite material, for instance through the activation of cell death signal transduction pathways. There was previously reported that CNTs are able to permeate the cell membrane and induce apoptosis, this mechanism possibly related to the apoptosis intrinsic pathway [10, 24, 31-33]. So far, there have been described three CNTs-dependent cell death mechanisms: i) increased production of reactive oxygen species (ROS) by free radicals formation within the cell, leading to the accumulation of peroxidation products and the exhaustion of cellular antioxidants, ultimately activating proapoptotic factors [10, 24, 31-33]; ii) increased intracellular calcium concentrations, triggering the mitochondrial apoptotic pathway [35, 36]; and iii) the alteration of the mitochondrial membrane potential, what leads to a CNTs-mediated apoptosis activation mechanism [10, 24, 31-33, 37]. Therefore, further research is required to elucidate the mechanisms involved in the cytotoxic effects seen in the *in vitro* model tested.

Varying and contradictory results were also found in experiments analyzing the toxicity of CNTs, mainly due to impurities, the type of functionalization and the cell lines tested [35, 36, 38, 39]. At the same time, several studies employ the MTT assay to determine the cytotoxicity of CNTs and alumina [10, 24, 31-33], as used in our study to evaluate cell viability. We observed that the APTS-functionalized CNTs and nanometric alumina cytotoxicity increased proportionally to the amount of CNTs and the concentration of the material. Subsequently, the viability results obtained by the MTT assay for the functionalized composite

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materials were further corroborated by Hoechst staining, the highest number of stained cells achieved at 0.5 % CNTs as compared to 1.0 or 1.5 %, respectively. This indicated a dose-dependent effect, with similar results at these two concentrations.

In the single study found, analyzing the material proposed in this work, cylindrical test tubes (2.0 mm in diameter and 6.0 mm long) were implanted in the defect area in rabbits, this material found to be as biocompatible as alumina, with the nanocomposite proving useful for future applications in biomedicine, specifically for tissue engineering. Noteworthy, the material tested in our research was particulated and not forming compact structures, since the test was aimed to analyze its cytotoxic effects on cultured cells while interacting with them in the culture medium.

Additionally, we tested Vero cells, a cell line of epithelial origin cells with fibroblastic-like morphology and properties, commonly used for biocompatibility evaluations. Even when we tested the nanomaterial at concentrations higher than those previously reported, the control condition using micrometric alumina alone did not evidenced cytotoxic effects, further validating the concentration range used in Vero cells and does not limit the results obtained. Nevertheless, it would be adequate to evaluate the effect of the nanocomposite in other cell lines, including some of human origin with properties similar to those of the tissues where it is intended to be used the nanocomposite. Moreover, we tested the nanomaterial at 50 000 cells per well, based on the previous standardization of the optimal number of cells required to guarantee an exponential kinetics during the 72 h of the test. This required since there were no detectable biological effect with the

nanomaterial at shorter incubation periods. It would be also pertinent to evaluate the long term cytotoxicity of the material and its full biocompatibility in cell adhesion, apoptosis induction and genotoxicity assays.

Other tests could also be run to characterize the mechanical properties of the functionalized composite material (supporting its use as scaffold for bone tissue repair), the distribution of CNTs in the composite matrix by scanning electron microscopy or by other more sensitive methods such as infrared spectroscopy or RAMAN spectroscopy (providing the typical bands corresponding to carbon nanotubes; bands D and G). It is worth to mention that the observed cytotoxic effect of the nanomaterial could be advantageously used for anti-neoplastic treatments, since the nanoparticles can be engineered to specifically interact with malignant cells and to act as anti-proliferative compound on malignant tumor cells. Moreover, there are ongoing investigations using CNTs for the efficient delivery of anticancer drugs [40-43].

In summary, and so far as we know, this is the first work analyzing the effect of an alumina and CNTs functionalized with APTS on cell proliferation, in spite of the abovementioned recommendations to corroborate the findings in other cell lines and to carry out other experiments to evaluate its biocompatibility, particularly for bone regeneration *in vivo*.

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