Green Synthesis and Characterization of Cerium Oxide nanostructures in the presence carbohydrate sugars as a capping agent and Investigation of their Cytotoxicity on the Mesenchymal Stem Cell

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Abstract

In this study, CeO₂ nanoparticles have been prepared via microwave method from an aqueous solution containing (NH₄)₂Ce(NO₃)₆, NaOH and in the presence carbohydrate sugars as green capping agent, for the first time. The effect of three different green capping agent such as lactose, glucose, and fructose was evaluated on the size and shape of nanostructures. CeO₂ nanostructures were synthesized and characterized by x-ray diffraction (XRD) pattern, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images, X-ray energy dispersive spectroscopy (EDS), Fourier Transform Infrared (FT-IR) spectra, and Photoluminescence (PL). Also, we investigated cytotoxicity effect of CeO₂ nanoparticle on bone marrow mesenchymal stem cell. Cytotoxicity was measured after exposure of Mesenchymal stem cells with 400, 200, 100, 50, 25 and 12.5 μM of 2-6 nm size CeO₂ nanoparticles for 24h. Our results indicated that cell viability was decreased, whatever nanoparticle dose was increased.

Keywords: Green synthesis; Cytotoxicity; Cerium Oxide; Mesenchymal Stem Cell; Carbohydrate sugars; Nanostructures.

1. Introduction

Nanomaterials (such as semiconductor and magnetic materials) are attractive for many researchers, due to their unique physical and chemical properties. The great variety of nanomaterials has been widely applied to industrial fields and human-related products with the rise of nanotechnology. The nanomaterials include silver, gold, oxide nanoparticles such as cerium oxide, zinc oxide, titanium oxide, iron oxide, iron oxide-based compounds and etc [1-5]. Ceria (CeO_2) nanostructures are one of the important nanomaterials due to their great potential applications, including electrochromic devices, catalysis, cosmetics, pharmaceuticals, solar cells, gas sensors, fuel cells, oxygen pumps, phosphor/luminescence, ultraviolet ray detectors and etc [6-12]. Due to its use in cosmetics and pharmaceuticals, there is the necessary need to different their harmlessness in relation to the environment and human health [11, 12]. The properties of surface-modified CeO_2 NPs, such as the size, chemical structure of the surface, and shape are important factors for toxicity [13]. Also, in the semiconductor industries, CeO_2 nanostructures are employed as abrasives in the mechanical-chemical polishing of wafers [14]. These applications were obtained due to cerium's high thermodynamic affinity for sulfur and oxygen. CeO_2 nanostructures is a chemically stable oxide from the prominent capacity to oxygen releases or storage, because of cerium oxidation state variation between Ce(III)/Ce(IV), and unique absorption/excitation energy bands associated with their electronic structure. Although the potential effects of nanostructures on human health and generic toxicity mechanisms remain unknown, a few studies were showed toxic effects of nanoparticles [15-18]. For example, quantum dots, titanium dioxide, carbon nanomaterials, ultrafine particulate pollutants can interrupt cell function, cell membrane and DNA damage through oxidative stress and lipid peroxidation [19-21]. Many nanoparticles which are used in industries such as CeO_2 nanoparticles have not been studied systematically and their cytotoxicity potentials remain unclear.

CeO_2 nanostructures were synthesized by different methods such as hydrothermal, solvothermal, microemulsion, microwave assisted hydrothermally, coprecipitation, sol-gel and metal organic chemical vapor deposition [22-24]. In this study, we presented a simple method for preparing of CeO_2 nanoparticles using the cerium salts as a source of Ce and investigated the effect of different precursors on the synthesis of CeO_2

nanostructures by microwave method. The microwave is an electromagnetic radiation with 0.3-300 GHz frequency. Microwave irradiation as a heating approach, which has been widely used for preparing of nanosized inorganic materials [25-30]. Among conventional methods of heating, microwave heating has some advantages including more efficiency, energy saving, very rapid formation of nanostructures without serious agglomeration, narrow size distribution because of its unique reaction effects like the properties of internal and volumetric heating and consequent increase in reaction rates, also providing a uniform environment for reaction lead to avoiding thermal gradient during microwave process. [31, 32]. The type of surfactant, irradiation power, and reaction time have the important effect of the size and shape of nanostructures in the microwave method. Hence, the effect of reaction time is investigated in the synthesis of nanostructures [31, 33]. To prepared of the CeO₂ nanostructures, we used the green capping agent (carbohydrate sugars), for the first time. Also, we reported cytotoxic effects of CeO₂ nanoparticles using human cells. Nanomaterials were added in cell culture medium at different concentrations. Cytotoxicity of nanoparticles was investigated by the MTT method.

2. Materials and physical measurements

All chemical reagents were purchased from Merck. XRD patterns of products were recorded via a Philips- X' part pro, X-ray using Ni-filtered Cu K α radiation. Scanning electron microscopy image was taken via on Philips XL-30ESEM equipped with an energy X-ray spectroscopy dispersive detector, Transmission electron microscopy image was obtained on a Philips EM208 transmission electron microscope by an accelerating voltage of 100 kV. GC-2550TG (Teif Gostar Faraz Company, Iran) were used for all chemical analyses. Fourier transform infrared spectroscopy (FT-IR) spectra were taken on a Thermo Scientific Nicolet spectrophotometer in KBr pellets. The electronic spectra of the product took on a Shimadzu ultraviolet–visible scanning spectrometer (Model 2101 PC).

2.1. Preparation of CeO₂ nanoparticles

0.05 M of ammonium Ce(IV) nitrate solution was added to 0.05 M of fructose solution. Then sodium hydroxide solution was added to above solution and the pH value was adjusted to 11. Ultimately reaction was

carried out for 5 min with 300 W power in domestic microwave oven. Then, the suspension was centrifuged and washed with ethanol and distilled water in sequence. The final product was dried at 80 °C for 12h and then calcined at 500 °C for 2 hours. The reaction conditions are listed in Table 1.

2.2. Cytotoxicity assay

The cytotoxicity of the CeO₂ nanoparticles was experimented using MTT assay. This method quantified a number of viable and metabolically active cells in percentage. This method is on the principle of conversion of MTT dye into formazan crystals via cells with functional mitochondria. Human bone marrow mesenchymal stem cells were prepared from Anatomical Science Research Center of KAUMS. The cells were expanded in 25 cm² cell culture flask (SPL, Korea) with DMED (Invitrogen; USA) cell culture media that supplemented with 10% (v/v) fetal bovine serum (Invitrogen; USA), 100 IU/ml penicillin and 100 µg/ml streptomycin (both from Sigma; Germany), and placed in 5% CO₂, 37 °C temperature and 85% humidity incubator (Mettler; Germany). When cells confluent reached to 80%, cells harvested with 0.25% trypsin- EDTA (Invitrogen; USA) and seeded at the density of 5000 cell/well in 96-wells cell culture plate and placed in the incubator for 24h. Then, the pre-autoclaved dense suspension of CeO₂ nanoparticles in phosphate saline buffer was diluted with DMEM media in concentrations 400, 200, 100, 50, 25 and 12.5 µM and added to cells in 96-well plate, and incubated for 24h. Then media was replaced with 0.2 ml of MTT dye + media (0.05 mg/ml) in each well and incubated for 2-4h. Then media was removed and insoluble formazan crystals were solved in DMSO, and the absorbance was measured at 570 nm using a microplate reader (Stat Fax 2100, Awareness Technology Inc). The percentage of viable cells were estimated in comparison with controls (see Scheme 2).

3. Result and discussion

3.1 Characterization of the nanoparticles CeO₂

To investigate the effect of the calcination temperature on the phase and crystalline of the products, three temperatures 400, 500, and 600°C have been applied. The powder XRD patterns of CeO₂ nanostructures are indicated in Fig. 1. All intensive reflections can be indexed to the pure cubic phase CeO₂ (JCPDS card number 78-0694). Fig. 1(a-c) shows that by increasing the calcination temperature, the crystallinity of the product was

increased. So, the temperature is an important factor for improving the product **crystallinity**. The average crystallite diameter of the samples was calculated by Debye-Scherrer equation:

$$D = k\lambda/\beta\cos\theta$$

where D is the crystalline size, λ is the wavelength of incident X-rays, β is the broadening of diffraction line measured at half its maximum intensity, and θ is the position of the maximum of diffraction peak. The average size of crystallites calculated by Scherrer formula was between 5 to 20 nm at 400-600° C, respectively. It is clear that the obtained nanoparticles growth homogeneously with increasing the temperature.

The many surface metal oxide species can be detected by IR spectroscopy in the range of 400-4000 cm⁻¹. Since metal ions are easily caught up via carbohydrate functional groups, FT-IR spectroscopy was studied to the determination of the surface functional groups and thus characterize the purity of the CeO₂ nanoparticle. FT-IR spectra of CeO₂ nanoparticles synthesized from (NH₄)₂Ce(NO₃)₆ is shown in Fig. 2(a). In this figure, the absorption bands around at 3430.86 cm⁻¹ and 1642.35 cm⁻¹ are attributable to the ν (O-H) stretching and bending vibrations water molecules linked to CeO₂ nanostructures, respectively. The adsorption peaks belonging to vibrations involving M-O bonds (metal oxide) are mainly observed in the range of below 1000 cm⁻¹ [34-36]. Therefore, broad peak at 470.20 cm⁻¹ range corresponds to the Ce-O bond vibrations [37, 38]. An additional band at 1114.05 cm⁻¹ may be associated with the presence of residual organic species (C-O-H stretching vibration) on the CeO₂ nanoparticles surface [39]. Change the location of C-O-H bending vibration from 1154 cm⁻¹ to 1114.05 cm⁻¹ confirm the existence of electrostatic interactions between fructose chains as a green capping agent and CeO₂ nanostructures [40].

To study the product' surface structure and local elements distribution was used X-ray energy dispersive spectroscopy (EDX) analysis. The EDX of the CeO₂ nanostructures is shown in Fig. 2(b). The highly intensified peaks for Ce and O are found in the spectrum, and no impurity was detected through the EDX spectrum.

3.2. SEM and TEM studies of the nanoparticles CeO₂

The Scanning Electron Microscopy (SEM) technique was used for the analysis of the physical morphology of the surface of the products.

The reaction time has an important effect on the size and shape of nanostructures, therefore the effect of this parameter is studied in the synthesis of CeO₂ nanostructures. To investigate the effect of time, three times (5, 10, and 15 min) were conducted [41]. Fig.4 shows the morphology and structure of CeO₂ nanostructures obtained by scanning electron microscopy (SEM). SEM technique is used to consider of surface morphology of sample. The particle size achieved from SEM image was about 10 -40 nm. The results showed when the reaction time was increased, the CeO₂ nanoparticles grew rapidly and the aggregation particles were obtained (Fig. 4). Increasing the reaction time from 5 to 15 min leads to the formation of **densely** agglomerated nanoparticles with a large particle size and a decrease in homogeneity of nanoparticles (shown in Fig. 4(a-f)). The cause is that increasing of the reaction time under thermal conditions lead to growth and agglomeration of particles. In the first stage, because of the production of CeO₂ nanoparticles, the supersaturation of the system rapidly increased and the product gradually began to nucleation and growth. By prolonging the reaction time, the thermodynamic effect leads to non-uniformity at **the** surface of samples.

In order to whether the calcination temperature has any effect on the product formation conditions, three calcination temperature were investigated. Fig. 5 shows the influence of the calcinate conditions on the preparation of CeO₂ nanoparticles. It can be observed that with the increase of calcinating temperature from 500 °C (Fig. 5(a, b)) to 600 °C (Fig. 5(c, d)) and also to 700° C (Fig. 5(c, d)) for 2h, the agglomeration of nanoparticles was increased, respectively that is in agreement with the to XRD results.

The effect of green capping agent on the morphology of CeO₂ nanoparticles was investigated in the presence of fructose, glucose, and lactose (Fig. 6). The results indicated that green capping agent has a key role in determine of the morphology. The results demonstrated that in presence of glucose as a green capping agent, the particles were agglomerated (Fig. 6(a, b)), also it showed that use of the lactose decrease the agglomeration of the CeO₂ nanoparticles that it is due to their structures. The schematic of the formation mechanism of CeO₂ nanoparticles in the presence of different green capping agent is shown in Scheme 1. Particle size besides the

lactose is smaller than when glucose exists (Fig. 6(c, d)). In this study, CeO₂ nanoparticles were synthesized by fructose as a green capping agent and considered as the best morphology that further characterizations were performed on this sample.

The Transmission Electron Microscopy (TEM) analysis was studied for the confirmation of the product dimension [42]. The TEM image of CeO₂ nanostructures is showed in Fig. 7(a, b). It can be observed that the prepared sample has a particle with 2.5-6 nm diameter.

3.3. Photoluminescence analysis studies of the CeO₂ nanoparticles

Optical properties of the CeO₂ nanoparticles were investigated by using PL spectra at room temperature. Nano-semiconductors have broad absorption spectra and narrow emission spectra. For CeO₂, the highest occupied valence band exhibits O 2p character mixing with some contribution from Ce such as 4f and 5d, although the narrow band situated just above the Fermi level is due to Ce 4f states [43]. Fig. 8(a-c) shows photoluminescence spectra with one strong peak at 452, 431, and 431 nm for samples No.1, 2, and 3, respectively, that can be attributed to a high-level transition in CeO₂ nanocrystallites. The band gap of this samples determined 2.74, 2.88, and 2.88 eV, respectively, while that of bulk CeO₂ is 1.8 eV (between O 2p and Ce 4f) [43]. The samples show a blue shift emission compared to the bulk sample. The difference between the PL spectra of three samples is due to the states 4f-5d electronic transition, which has depended on the crystal structure [44]. Also, the PL characteristics of nanostructures depend on the homogeneity of the morphology of products, which corresponded with the results of the SEM images.

3.4. Cell viability and cytotoxicity assay

The cytotoxicity of CeO₂ nanoparticles on mesenchymal stem cells was evaluated via MTT assay following 24h of exposure. The results showed a dose-dependent cytotoxicity. 82% of the cells seemed to be viable up to a concentration of 12.5 µM, while 20% of the cells were viable at high concentration (400 µM) tested (Fig. 9), which was significant when compared to control. According to the study some articles on the topic, it is suggested that the dosage levels of CeO₂ nanoparticles of 12.5 to 400 µM can induce oxidative stress, increased cell membrane damage and lipid peroxidation, and reduced glutathione (GSH) levels. There is a high

correlation between reduced cell viability and increased oxidative stress (OS) level after 24h of exposure [45, 46]. Although, the mechanism of oxidative stress-induced via nanostructures is not fully understood. Based on the results of cytotoxicity of SWCNT and potential redox property of CeO₂ (Ce(III)/Ce(IV)), we offer the following chemical reactions. Reactive type like hydroxyl radical (OH[•]) and hydrogen peroxide (H₂O₂) are generated via reactions 1, 2, 3, and 4. Also, alkoxyl radicals from lipid peroxide (LOOH) can be produced by reaction 5. In reaction, A⁻_{red} related to physiological reductant, like ascorbate and thiol compounds, while A_{ox} is their oxidized states. The reactive oxygen species induced reason oxidative stress and lipid peroxidation in cells [47, 48].

4. Conclusions

In this study, CeO₂ nanoparticles were prepared by microwave method using (NH₄)₂Ce(NO₃)₆ salt as a cerium source. The results showed that the green capping agent, time and calcination temperature have the important role in the morphology and particles size. For the synthesis of this nanostructure, three different green capping agent such as lactose, glucose, and fructose was used, for the first time. The effect of these green capping agent is shown in the Scheme 1. The crystal structure of the CeO₂ nanoparticles was studied by XRD pattern and it was pure and free of impurities. Furthermore, we investigated the toxicity of CeO₂ nanoparticles on the mesenchymal stem cells. The results demonstrated that by increasing the concentration of CeO₂ nanoparticles reduce cell viability (with increasing concentrations of 12.5 μM to 400 μM, it was observed survival ability of stem cell was reduced from 82% to 20%). Also, the optical property of the CeO₂ nanoparticle was examined via photoluminescence spectroscopy and the band gap was calculated to 2.74 eV.

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Table 1: The reaction conditions

Sample No	Capping agent	Microwave Time (mine)	Decompose Time (h)	Decompose Temperature (° C)
1	Fructose	5	2	500
2	Fructose	10	2	500
3	Fructose	15	2	500
4	Fructose	5	2	600
5	Fructose	5	2	700
6	Glucose	5	2	500
7	Lactose	5	2	500

Scheme 1. Formation mechanism of CeO₂ nanoparticles in the presence of different green capping agent.

Scheme 2. Mechanism pathway of the mesenchymal stem sell (MSC) in the presence of CeO₂ nanoparticles.

Fig. 1. XRD patterns of CeO₂ calcined at temperatures a) 400, b) 500, and c) 600° C for 2h.

Fig. 2. (a) FT-IR spectra and (b) EDS pattern of the sample prepared at 180 °C for 12 h in presence of fructose as green capping agent.

Fig. 4. SEM images of (a, b) sample No. 1, (c, d) sample No. 2 and (e, f) sample No. 3, respectively.

Fig. 5. SEM images of (a, b) sample No. 2, (c, d) sample No. 3 respectively.

Fig. 6. SEM images of (a, b) sample No. 6, (c, d) sample No. 7, respectively.

Fig. 7. (a) and (b) TEM images of the as-synthesized CeO₂ nanoparticles (sample No.1)

Fig. 8. Photoluminescence (PL) spectra of nano-CeO₂ at room temperature (a) sample No.1, (b) sample No.2, and (c) sample No.3.

Fig. 9. MTT assay of MSCs treated with different concentration of CeO₂ nanoparticles (sample No.1). The graph shows the percentage viability of cells exposed to CeO₂ nanoparticles relative to control cells.