Synthesis and characterization of Zinc (II)-loaded Zeolite / Graphene oxide nanocomposite
as a new drug carrier

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Abstract

Current research has focused on the preparation of Zinc-clinoptilolite/Graphene Oxide (Zn-Clin/GO) hybrid nanostructure and investigating its biocompatibility for the first time. As prepared samples were characterized by X-ray diffraction (XRD), Scanning electron microscopy (SEM), Thermo gravimetric analysis (TGA) and Fourier transform infrared (FT-IR). In order to use it as a drug carrier two important factors were investigated: cytocompatibility of nanocomposites and their drug loading capacity. The results showed that the prepared nanocomposite is cytocompatible and its high loading capacity and slow release performance for Doxorubicin (DOX), as a cancer drug, proved that it can be used as a drug carrier. At last in-vitro toxicity of DOX loaded nanocomposite was compared with pure DOX.

Keywords:

Drug carrier system, biocompatibility, Doxorubicin, in vitro cytotoxicity, Clinoptilolite

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1. Introduction

Graphene, the thinnest known material, is a sheet of two-dimensional (2D) sp^2 -hybridized carbon atoms [1]. It has special properties which make it suitable for many applications. Its high specific surface area, exceptional electrical and thermal conductivity, 97.7% light transmittance, high strength and tunable mechanical properties makes it suitable respectively in absorbents for organic molecules[2], electric devices and biosensors[3,4], Magnetic Resonance Imaging (MRI) and biomedical imaging [5,6] and medical implants and scaffolds used in tissue engineering [7]. Graphene oxide (GO) is oxidized form of graphene that has carboxylic, hydroxide and epoxy groups. It is used more than graphene in biomedical applications. But there are some concerns about toxicity of graphene and its derivatives. Recent researches have shown that different forms of graphene will affect differently on cells. A lot of factors are important, some of them are rigidity, lateral dimensions and etc. [8,9] Additionally, large surface area of graphene, π - π stacking and electrostatic or hydrophobic interactions of graphene, hydrophilic interaction of graphene oxide allows it to achieve high drug loading capacity. Polyethylene glycol (PEG)-functionalized nanoscale graphene oxide (NGO) sheets loaded with a camptothecin (CPT) analog, SN38 was synthesized [10], which showed high cytotoxicity in HCT-116 cells, 1000 times more than CPT. In the other study PEG-NGO was used to load DOX [11]. This led to a series of studies on using of graphene based materials for loading of drugs such as DOX [11-21]. For example Wu et al. [22] proposed a strategy to reverse cancer drug resistance in DOX-resistant MCF-7/ADR cells by loading DOX on graphene oxide surface via physical mixing. GO enhanced DOX accumulations in MCF-7/ADR cells causing higher cytotoxicity than free DOX. Composites of GO maybe divided to two main groups: pH sensitive [21, 23-25] and thermal sensitive [26]. Graphene-based materials have been conjugated with a number of natural organic biopolymers like gelatin and

chitosan as functionalizing agents for drug delivery applications [17, 27, and 28]. Natural biopolymers are biocompatible, biodegradable and have low immunogenicity which can greatly reduce the toxic effects of graphene. So preparing safe composites has got a lot of interest. Here we use natural inorganic polymer such as zeolites.

Zeolites, porous aluminosilicates, have found many uses because of their special architecture. They have been used widely as absorbants, catalysts, and detergent builders [29,30]. Recently their uses in biomedical applications have attracted a lot of attention. For example they have been used in biosensors [31], magnetic resonance imaging [32] and drug delivery systems [33]. Clinoptilolite (Clin) is known as the most prevalent natural zeolite all over the world, which is biocompatible and has been used as substrate in controlled drug delivery. Jevtic et al. [34] used surfactant-modified Clin as a salicylate carrier. This composite displayed antibacterial activity against *E. coli* and *S. aureus*. Rahimi et al. [35] reported that Clin can absorb fat-soluble vitamins and because of its buffer property can protect vitamins against acidic pH. Recently Tonder et al. [36] suggested Clin as a carrier for pH-controlled oral delivery aspirin. To the best of our knowledge, there are few studies carried out on zeolite/GO nanocomposites [37-39]. As mentioned before, different forms of graphene will affect on cells differently and preparing safe composites of it has got a lot of interest so adding zeolite not only improves its biocompatibility but also doesn't decrease surface area of nanocomposite so much because of high surface area of zeolite. In order to combine zeolite and graphene He et al. synthesized Cu-Zeolite A/graphene nanocomposite [37], they used Cu^{2+} as coordinating cation. In previous work, our group used Cu-Zeolite A/graphene nanocomposite as an absorbant in the removal of arsenic from contaminated water [38].

Herein we synthesized Zn-Clin/GO nanocomposite as an in vitro drug carrier system for the anticancer drug, DOX.

Then its drug loading capacity was evaluated and its cytotoxicity has been studied using methyl thiazolyl tetrazolium (MTT) assay.

2. Experimental

2.1 Material

Graphite, 37% HCl, 98% H₂SO₄, 30% H₂O₂, KMnO₄, NaNO₃, NaCl, Zn(acetate)₂·2H₂O, NaBH₄ (all obtain from Merck), natural Iranian zeolite known as Clinoptilolite (Clin) (Si/Al = 9.53) were used.

2.2 Preparation of Zn-Clin

Clin (0.5g) was first suspended in aqueous NaCl solution (5mL, 3M) and stirred for 24h. Then the resulted powder was separated and washed with water by using a centrifuge for several times at 2000rpm and dried at 60 °C. Then the resulted powder was added to an aqueous zinc (II) acetate solution (25 mL, 1M) and stirred for 24h, so its cations exchanged with Zn²⁺. This powder was separated and washed with water by using a centrifuge for several times at 2000rpm and dried at 60 °C.

2.3 Synthesis of Zn-Clin/GO nanocomposite

GO was synthesized using widely reported modified Hummers method starting from graphite [40]. Then GO (0.05g) and Zn-Clin (0.05g) were dispersed in 30 mL distilled water, using ultrasonic treatment for 1 hour and then NaBH₄ (0.001g) was added into the suspension. This suspension was transferred to a microwave Teflon reactor (scheme 1) and treated for 20 min at 300 W. After cooling to room temperature, obtained residue was separated, washed with distilled water, dried at 60°C and gray composite of Zn-Clin/GO was obtained. This composite is named Zn-Clin/GO (M).

In the other method, GO and Zn-Clin (mass ratio of 1:1) were dispersed in water by using ultrasonic irradiation, then NaBH₄ was added and treated under reflux at 80 °C for 8 hours. Then obtained powder was filtered, washed with distilled water, dried at 60 °C and is called Zn-Clin/GO (R).

Scheme 1

2.4 Measurements

X-ray diffraction patterns (XRD) were collected using a Siemens D500 diffractometer with Cu K α radiation ($\lambda = 1.5418$ Å and $2\theta = 4-80^\circ$) at room temperature. FT-IR spectra were obtained with a Bruker Tensor 27 Fourier Transform Infrared spectrometer with the KBr pellet technique. Scanning electron microscope (Philips XL30) equipped with energy dispersive X-ray (EDX) facility was used to capture SEM images and to perform elemental analysis. The SEM sample was gold coated prior to examination and SEM was operated at 5 kV while EDX analysis was performed at 15 kV. Thermal gravimetric (TG) was conducted under N₂ atmosphere in the temperature range of 30–800 °C at a heating rate of 5 °C min⁻¹.

2.5 Preparation of drug loaded carriers

100 mg of carriers (Clin, Zn-Clin, GO and Zn-Clin/GO (R)) were impregnated in 20 mL of doxorubicin (50 ppm) in water under constant stirring at room temperature for 24 h. Samples were taken out at given time intervals and the solution was centrifuged at 1500 rpm for 5 min and then filtered to remove the carrier particles completely. The supernatant was collected and analyzed with a Shimadzu UV-240 spectrophotometer at 485 nm (λ_{max} of DOX). After a certain adsorption time, there were no further changes in the concentration of DOX in the liquid phase, and it was

assumed that the loading capacity of the particles had been reached. Residues were collected and dried and in-vitro toxicity of obtained powders were examined using MTT assay.

2.6 Investigation of nanocomposite cytotoxicity (MTT assay)

A549 alveolar adenocarcinoma cells (9×10^3 cells/well) were incubated in 96-well plates each containing 200 μ L of supplemented cell culture media for 24 hours at 37°C and 5% CO₂. The cells were divided in 4 groups in quadruplicates: blank, Zn-Clin/GO nanocomposite (different concentrations: 0.1, 0.5 and 1 mg/mL) were treated. After an incubation periods of 24 and 48 h, the spent media were removed and the plate wells were washed with Phosphate-buffered solution. Briefly, 50 μ L of 2 mg/mL MTT and 150 μ L culture medium of was added to each well. The cells were incubated at 37°C and 5% CO₂ for 4 hours and then the media was discarded and dimethyl sulfoxide and Sorenson buffer was added to each well as solubilizer buffer. Finally, absorbance was read using an ELISA plate reader (BioTeck, Bad Friedrichshall, Germany) at 570 nm wavelength.

3. Results and discussion

3.1 Characterization of Zn-Clin/GO nanocomposite

Using a coordinating cation is necessary because it is impossible to prepare a uniform and stable composite without using a coordinating cation. As mentioned in our previous report [41], our group prepared a similar nanocomposite using Cu²⁺ as coordinating cation. In this paper we used Zn²⁺ cations because in this case the prepared nanocomposite was more homogenized, uniform and stable and Zn²⁺ cation is safe, already presented in human body, cofactor in some enzymes and has a crucial role in regulating how neurons communicate with one another.

Here two different methods were used to synthesis of nanocomposites. Figure 1 shows images of Zn-Clin, Zn-Clin/GO (R) and Zn-Clin/GO (M) powders. Zn-Clin is a white powder whereas GO is dark brown and Zn-Clin/GO (M) nanocomposite was not uniform and the white particles of zeolite were separated from black RGO and two different phases was clearly observed. But the Zn-Clin (R) nanocomposite was completely uniform and stable.

Figure 1

In order to characterize the obtained composites, their XRD patterns (figure 2) were analyzed. By using microwave reactor, the white particles of zeolite were separated from black RGO and two different phases was clearly observed so two phases were separated and their XRD patterns relieved that the light one is Clin (figure 2A) and the dark phase is graphene oxide which is reduced (figure 2B). In XRD pattern Zn-Clin/GO (R) (figure 2C), all of Clinoptilolite peaks were clearly observed and it was highly crystalized and the peak at $2\theta=11.33$ is related to GO. So in the following steps, Zn-Clin/GO (R) nanocomposite was chosen to be used.

Figure 2

For synthesis of composite, a minimum reduction in functional groups of GO is needed. It is reported that NaBH_4 reduces epoxy and alcoholic groups on the surface on GO but carboxylic groups remains. These carboxylic groups can react with $-\text{OH}$ groups on the surface of zeolite so that a stable composite can be synthesized. Now the question is why still it is named GO, not reduced GO. Reduced GO is a form of graphene in which most of functional groups of GO is removed. XRD pattern of this nanocomposite showed the peak of GO. It means that most of functional groups of GO is still remains in the experimental condition.

Figure 3a shows FT-IR spectra of GO. The peaks at 3444 cm^{-1} , 1739 cm^{-1} , 1045 cm^{-1} and 1222 cm^{-1} , respectively attribute to O-H stretching vibration, C=O stretching vibration, to vibration of C–O (epoxy) and vibration of C–O (alkoxy). The peaks 1630 cm^{-1} and 1419 cm^{-1} attribute to C=C stretching vibration [41]. Figure 3b shows FT-IR spectra of Zn-Clin/GO (R). The peaks 464 cm^{-1} , 606 cm^{-1} and 1066 cm^{-1} are respectively related to vibrations of TO_4 and T-O in Zn-Clin. The peak 1600 cm^{-1} can be related to vibration of C-O the O–H stretching vibration peak at 3422 cm^{-1} [42]. Figure 3c shows FT-IR spectra of DOX loaded Zn-Clin/GO nanocomposite. All previous peaks were observed. Moreover the peaks at 2899 cm^{-1} and 2822 cm^{-1} signify primary $-\text{NH}_2$ group which exists in DOX structure. Also the C–N stretch is at 1338 cm^{-1} and the peak at 1549 cm^{-1} can be related to N–H bend.

Figure 3

Figure 4 showed SEM images of Zn-Clin and Zn-Clin/GO nanocomposite. It is obvious that cubical Zn-Clin particles were randomly placed on graphene plates and graphene acted like a substrate for Zn-Clin particles. Thin films of graphene with thickness of 20-30 nm is clearly observed. It should be mentioned that the graphene plane which is shown in the image is almost curved.

Figure 4

The EDX diagram confirmed that about 40% of composite were C which presented GO and it approved that the Zn-Clin/GO nanocomposite were prepared successfully. It has 1.27% Zn due to cation exchange of Clin, about 17.2% Si and Al because they are main metals in structure of Clin

and 41.5% Oxygen. Elemental analysis was performed to measure percentage of Zn in the compounds. It showed that Zn-Clin contains around 3% Zn and Zn-Clin/GO nanocomposite (ratio of Zn-Clin: GO is 1:1) has almost 1.35% Zn, as it was predicted.

The thermal stability of Zn-Clin/GO nanocomposite was measured using TGA in a N₂ atmosphere. About GO, it has been reported that, because of evaporation of adsorbed water and due to the removal of the oxygen-containing functional groups respectively, GO has a 21% mass loss below 200 °C, a rapid 20% mass loss from 200 to 250 °C and about 50% of graphene remains below 600 °C. According to references, Graphite does not show any mass loss between room temperature and 600 °C [43]. As shown in Figure 5, TGA curve of Zn-Clin/GO nanocomposite has three steps, about 12.74% mass loss below 160 °C which is because of evaporation of adsorbed water, about 8.95% from 160 to 220 °C due to removal of oxygen-containing functional groups and 13 % mass between 220 and 600 °C. On the other hand, Clin has a 24% mass loss because of evaporation of adsorbed water. Overall it has 35% mass loss up to 600 °C. Considering Clin content as x and GO content as y (in Zn-Clin/Go nanocomposite), we will have two relations as below:

$$x + y = 1$$

$$0.24x + 0.5y = 0.35$$

Solving these two relations, it can be calculated that about 57% of nanocomposite is Clin and 42% of nanocomposite is graphene oxide. So instead of mass ratio of 50:50 Zn-Clin to GO, 57:42 is right.

Figure 5

3.2 Investigating drug loading capacity and drug release behaviors of nanomaterials

In figure 6 loading capacity of DOX by different nanomaterials was compared. As mentioned before zeolites are porous materials contains OH groups and can absorb or be a host to DOX molecules. Loading efficiency of Clin and Zn-Clin for DOX is 70% in 120 and 90minutes, respectively. In the case of Zn-Clin, Zn cations can coordinate to Heteroatoms (O in ketone, ester, carboxylic acid and alkali groups and N in amine groups) in structure of drug and facilitates drug loading.

On the other hand, GO owning a High specific surface area, π - π stacking and electrostatic interactions of graphene can be a good candidate to achieve high drug loading [7]. And the test relieved that 78% of drug can be loaded within 30 minutes. So it is expectable that Zn-Clin/GO nanocomposite would have a high drug loading capacity. As shown in figure 7, before 30 minutes 90% of DOX was loaded. So this nanocomposite had the highest loading efficiency among compared nanostructures and can be a better choice as a drug carrier.

Figure 6

Drug release profiles of nanomaterials at neutral and acidic pH (pH=5.4, acetate buffers) are shown in figure 7 and 8, respectively. It can be seen that, even after washing, about 90% of the initial doxorubicin was still adsorbed in the Zn-Clin/GO nanocomposite, and it was progressively released by desorption and diffusion to the buffer solution (pH=7), respectively, 11% (4.29 ppm), 25.5% (8.9 ppm) and 21% (9.45 ppm) of the doxorubicin loaded on the GO, Zn-Clin and Zn-Clin/GO nanocomposite was released in 24 h. But in the lower pH, similar to the cancer cell

(pH=5.4), more desorption of the drug (Zn-Clin/GO) was occurred (46% of loaded DOX) in 24h. Results are summarized in table1.

Figure 7

Figure 8

Table 1

3.3 Cytotoxicity of nanomaterials

The cytotoxicity of the Zn-Clin/GO nanocomposite were investigated in the A549 cell line. Figure 9 shows the relative cell viability ($[C_r/C_0]$ 100%) vs. different concentration of DOX and nanocomposites, determined by the MTT assay. Here, C_0 is the viable cell numbers of the control sample, and C_r is the viable cell numbers treated with the nanomaterials. The error bars are the calculated standard deviation. According to the Sun et al. [11] slight reductions of cell viability only for extremely high GO concentrations (>0.1 mg/mL) was observed, so here the relative viabilities (%) of cells treated with 0.1, 0.16 & 0.36 mg/mL of Zn-Clin, GO, Zn-Clin/GO nanocomposite, DOX and DOX loaded Zn-Clin/GO nanocomposite are illustrated in figure 7 and 8 after 24 and 48 hours incubation, respectively. The results indicated that GO and Zn-Clin are not toxic even in high concentrations as well as Zn-Clin/GO nanocomposite. GO and Zn-Clin/GO are completely cytocompatible below 0.1 mg/mL and 0.16 mg/mL, respectively. According to the DOX diagram (figure 9), by increasing DOX concentration from 50 to 150 ppm, cell viability was decreased up to 25% (150 ppm).

However in the case of DOX loaded Zn-Clin/GO which contains 45 ppm DOX (90% loading), compare with free DOX (50 ppm) only 22% of cells survived which this viability is adapted with 150 ppm DOX. So by using drug loading system low concentration of drug (DOX) will be used. It gives this idea that the nanocomposite can release DOX in cell medium and import more DOX into cells.

Figure9

Figure10

4. Conclusion

In this paper we have introduced Zn-Clin/GO nanocomposite as a new drug carrier with high loading capacity. It was synthesized using two different methods, microwave assisted hydrothermal method and reflux method. The nanocomposite synthesized using reflux method was homogeneous and stable so it was used as carrier. Moreover Zn-Clin/GO nanocomposite exhibited no toxic effect to cells especially at concentrations below 160 mg/mL. Loading capacity of Zn-Clin, GO and Zn-Clin/GO nanocomposites for DOX are respectively, 70% in 120 min, 80% in 30 min and 90% in 30 min. MTT assay for DOX loaded Zn-Clin/GO nanocomposite relieved that DOX loaded nanocomposite exhibited more cytotoxicity than free DOX so it can release DOX in cell medium and import more DOX into cells. So the nanocomposite can be a good choice as a drug delivery system. This paper presents a simple approach to prepare a new carrier with great potential in cancer therapy.

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Table captions:

Table 1. Release results of DOX from nanomaterials in 24h and 48h

Figures captions:

Scheme 1. Microwave Teflon reactor

Figure 1. Images of Zn-Clin/GO (M) and Zn-Clin/GO (R) powders. As seen in the picture Zn-Clin/GO (M) was not a uniform powder and could be easily separated to Zn-Clin and RGO.

Figure 2. XRD patterns of the: (A) Clin, (B) RGO and (C) Zn-Clin/GO (R)

Figure 3. FT-IR spectra of: (a) GO, (b) Zn-Clin/GO (R), (c) DOX loaded Zn-Clin/GO

Figure 4. SEM images of Zn-Clin and Zn-Clin/GO (R) and EDX diagram of Zn-Clin/GO (R)

Figure 5. TGA curve of Zn-Clin/GO nanocomposite

Figure 6. Loading capacity of DOX by Clin, Zn-Clin, GO and Zn-Clin/GO

Figure 7. Release profile of GO, Zn-Clin and Zn-Clin/GO at pH=7

Figure 8. Release profile of GO, Zn-Clin and Zn-Clin/GO at pH=5.4

Figure 9. In vitro cell toxicity experiments. Relative viabilities of A549 alveolar adenocarcinoma cells after being incubated with various concentrations of: (A) GO, (B) Zn-Clin, (C) Zn-Clin/GO, (D) free DOX and (E) DOX loaded Zn-Clin/GO nanocomposite for 24 h.

Figure 10. In vitro cell toxicity experiments. Relative viabilities of A549 alveolar adenocarcinoma cells after being incubated with various concentrations of: (A) GO, (B) Zn-Clin, (C) Zn-Clin/GO, (D) free DOX and (E) DOX loaded Zn-Clin/GO nanocomposite for 48 h

Figures:

Scheme 1. Microwave Teflon reactor

Figure 1. Images of Zn-Clin/GO (M) and Zn-Clin/GO (R) powders. As seen in the picture Zn-Clin/GO (M) was not a uniform powder and could be easily separated to Zn-Clin and RGO.

Figure 2. XRD patterns of the: (A) Clin, (B) RGO and (C) Zn-Clin/GO (R)

Figure 3. FT-IR spectra of: (a) GO, (b) Zn-Clin/GO (R), (c) DOX loaded Zn-Clin/GO

Elt	Line	Int	W%
C	Ka	334.0	39.63
O	Ka	661.1	41.46
Al	Ka	229.1	2.58
Si	Ka	1345.8	14.71
K	Ka	14.6	0.27
Ca	Ka	4.6	0.09
Zn	Ka	7.8	1.27

Figure 4. SEM images of Zn-Clin and Zn-Clin/GO (R) and EDX diagram of Zn-Clin/GO (R)

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Tables:

Table 1. Release results of DOX from carriers in 24h and 48h

Samples	<i>pH=7</i>	<i>pH=5.4</i>
<i>GO</i>	<i>(11% : 4.3 ppm) 24h</i> <i>(14% : 5.4 ppm) 48h</i>	<i>(26.5% : 10.3 ppm) 24h</i> <i>(34.51% : 13.5 ppm) 48h</i>
<i>Zn-Clin</i>	<i>(28.5% : 10 ppm) 24h</i> <i>(38.5% : 13.5 ppm) 48h</i>	<i>(68.5% : 24 ppm) 24h</i> <i>(83% : 29 ppm) 48h</i>
<i>Zn-Clin/GO</i>	<i>(21% : 9.5 ppm) 24h</i> <i>(24.5% : 11 ppm) 48h</i>	<i>(46% : 20.7 ppm) 24h</i> <i>(60.5% : 27 ppm) 48h</i>