SYNTHESIZE AND STABILITY ANALYSIS OF PEGYLATED NANOGRAPHENE OXIDE FOR DELIVERY OF DOXORUBICIN

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In this study, graphene oxide nanoparticle (NGO) was fabricated via modified Hummers' method and then modified with amine terminated 4-armed PEG to enhance the biocompatibility and stability of nano drug carrier. In our strategy, Doxorubicin (DOX) was used as a model drug. DOX loading onto NGO–PEG was synthesized by simply mixing with the NGO-PEG solution at pH 7 overnight. NGO, NGO-PEG and NGO-PEG-DOX was characterized by power X-ray diffraction, scanning electron microscopy, and UV spectroscopy. Stability analysis was performed with zeta potential. The experimental results showed that the zeta potential analysis values were above ±40 mV. NGO-PEG-DOX is still had very good dispersion stability even after 45 days. Therefore, this drug loaded product can be good candidate for treatment of cancer.

Keywords: *nanographene oxide (NGO) , nanostructured drug carrier, stability, DOX, PEGylated*

1. Introduction

With the nanotechnological development, advanced engineering systems are aimed to be emerge the more designed functional products for the drug, medicine and biomedical application areas [1]. The production of nanostructured drug carrier for anticancer is one the most important field of the medicine. Nanoparticles have the potential to greatly allow for high specific surface area and functional reactive groups in their molecular structures [2]. Due to their unique dimension and area properties, they have been studied in the medicine such as the application of diagnosis and treatment of cancer, targeted drug release, biosensors both in vitro and in vivo research fields. Especially in the diagnosis and treatment of cancer has gained attraction for the chemotherapy to reduce the high side effects of the current drugs that is not to be reached the targeted areas and cancer cells and destruction of healthy cells [3]. Chemotherapy is a crucial therapeutic approach for the treatment of a wide variety of cancers [4-7]. However, as chemotherapeutic agents affect all body cells in a non-specific way, they cause undesirable side effects in normal tissues and cause insufficient dosages to kill cancer cells. Therefore, targeted drug release systems are improved to overcome these challenges [8-13]. Graphene is one of the promising nanostructure for nanostructured drug carrier [14]. In this nanomaterial with sp^2 covalently bonded carbon atoms is a 2-D molecular structure perfectly arranged in a hexagonal lattice [15,16]. This aromatic structure consists the reactive regions consisting of free π electrons of carbon atoms [17].

Biomolecules can be loaded the carbon network of graphene through π-π interaction and hydrogen bonding [18]. However, graphene shows poor stability in solutions. Therefore, modified graphene such as graphene oxide (GO) and NGO are widely prefered for drug applications due to their hydrophylic nature.

GO is synthesized via chemical reduction of graphite layers [19]. It is structurally different from the graphene. GO sheets have mainly in the richness of oxygen containing groups in the surface due to ionization of carboxyl (-COOH), hdroxyl (-OH) and epoxy (-O-) groups. At the moleculer level these enriched negatively charged reactive oxygen groups interact with the electrophilic functional sides in biomolecule surface through covalent binding. Hence, electrostatic chemical interaction occurs effectively between the surface of oxygen containing groups in GO and biomolecules [20]. Another modified GO form is nano-GO which is lateral size is in the range of 20 nm-100 nm, whereas regular GO dimension is greater than 100 nm [21]. Zero-dimensional (OD) structure of NGO has higher degree of oxygen groups than conventional GO. Its small size and structure also provide the more ability of drug carrier owing to hydrogen bonding between the NGO surface and the drug molecules [22-26]. Compared to conventional drug carriers, NGO shows certain advantages with high drug loading efficiency, targeting specificity due to its abundant surface chemistry [27]. Several studies have been reported on the functionalization of GO based nano drug carriers. Wu et al. [28] proposed a strategy to reverse cancer drug resistance in DOX, a widely used anthracyclinic antibiotic, as a model anti

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cancer drug [29] to treat most types of cancer - resistant MCF-7/ADR cells by loading DOX on GO surface face via physical mixing. GO enhanced DOX accumulations in MCF-7/ADR cells causing higher cytotoxicity than free DOX. Wu et al. also analyzed the NGO-DOX system on RPMI-8226 cells [28]. The more cytotoxic effect of NGO-DOX drug on cancer cells was found as compared to pure DOX at the same concentration. Another research is on loading DOX into Zinc-clinoptilolite/GO as a hybrid nanocomposite proposed by Khatamian et al. [30]. Their results showed that DOX loaded Zn-clinoptilolite/GO nanocomposite with high loading capacity is more cytotoxicity than free DOX in the A549 cell lines. Wang et al. studied novel drug delivery system comprising nanoparticles based on galactosylated chitosan (GC)/GO/DOX for the therapeutic treatment of cancer [31]. These nanoparticles remained stable under low pH environment. For the increased the stability of synthesized nano drug carriers, stabilizing agents are used to prevent the agglomeration of GO [32]. For this, polyethylene glycol (PEG) supplies biological media and widely used because of its minimal toxicity, biocompatibility [33], protein resistance [32] and good solubility in water [34, 35] which has been approved by the the US Food and Drug Administration (FDA). The incorporation of PEG on the surface of GO could also provide the more cytotoxic effect and circulation rate in the reticuloendothelial system (RES) for in-vivo drug release [36, 37]. Zhang et al. analyzed the therapeutic effect of NGO-PEG-DOX drug hybrid system in vitro and in vivo breast cancer cells [38]. The in vivo cytotoxicity of targeting nanohybrids was significantly cytotoxic than that of non-targeting nanohybrids without weight loss and recurrence for the mouses. Additionally, the results also showed the role of the nanohybrid has reducing side effects compared as to free DOX. PEGylated NGO also promote the pharmadynamical properties of drug due to swelling of PEG and increasing hydrodynamic volume of cells. Interestingly, PEGylated drugs easily diffuses into inflammated tissue [39-41]. Several studies have that graphene has more low cytotoxicity and highly biocompatible in PEG solutions [42-44]. In their recent study, Pham et al. undertook PEG- alendronate-functionalized NGO system to enhance accumulation DOX for bone tumors [45]. In vitro study showed that this potential carrier dimished the off-target effects of DOX for treating bone metastasis in advanced breast cancer. Therefore, graphene oxide-PEG appears to be highly promising system for nanostructured drug carriers.

In this study, the nanocarrier system was synthesized in order to increase the stability of DOX that lost its stability in a short time. Here, the drug loaded product NGO-PEG-DOX was synthesized and characterized by power X-ray diffraction, scanning electron microscopy and UV spectroscopy. Stability analysis of samples was made with the zeta potential.

2. Materials And Methods

2.1. Synthesis of graphene oxide

Hummer's method was employed to synthesize GO [19, 46-48]. In a typical experiment, natural graphite flake (+100 mesh (≥75% min)) and concentrated H_2SO_4, $K_2S_2O_8$ and P_2O_5 were mixed, following which incubation of the mixture was performed at the temperature of 80˚C for the period of 6 h to ensure the preoxidation of graphite. Afterward, the product was washed using distilled water until neutral and filtered, following which it was left drying in air at ambient temperature for a night. Then, oxidation was applied to the preoxidized graphite in question by employing Hummer's method. The preoxidized graphite powder was put in concentrated H_2SO_4 at the temperature of 0˚C. The gradual addition of $KMnO_4$ was performed by stirring whereas the mixture's temperature was kept under 20˚C with ice bath. Afterward, stirring of the mixture was performed at the temperature of 35˚C for the period of 2 h, then distilled water was added, and stirring of the mixture was continued for the period of 15 min. The reaction was terminated with the addition of distilled water. Consequently, the addition of 30% H_2O_2 was performed, and the mixture's color turned to bright yellow, as reported by Kovtyukhova et al. [46]. Centrifugation of the mixture was carried out, following which it was washed using 10% HCl solution for the purpose of removing residual metal ions. Afterward, the precipitate was washed using distilled water, and its repeated centrifugation was carried out until neutral. For the purpose of exfoliating the oxidized graphite, the product was subjected to ultrasonic probe treatment at 400W for the period of 30 min, and then it was centrifuged at 13 000 rpm for the period of 30 min. The exfoliated GO was acquired in the supernatant. Repeated exfoliation of the precipitate was performed. In the end, supernatant was acquired. The acquired product was continuously dispersed in water, and its precipitation would not occur for a few months. For the purpose of acquiring NGO, cracking of GO was performed by an ultrasonic probe at 500W for the period of 120 min. SEM images were obtained for the purpose of checking the morphology of the NGO generated.

2.2. PEGylation of graphene oxide

For the purpose of PEGylation, the addition of NaOH and $ClCH_2COOH$ to NGO aqueous suspension was performed, and sonication was carried out at 500 W for the period of 3 h for the conversion of OH groups to COOH by conjugating

acetic acid moieties that result in NGO-COOH. The neutralization of the NGO-COOH solution was achieved, and it was purified as a result of rinsing repeatedly and filtrations, which produced an NGO-COOH aqueous solution that was well dispersed. The addition of EDC (*N*-(3-dimethylaminopropyl-*N*′-ethylcarbodiimide)) and NHS to the NGO-COOH suspension was performed at pH 5.6, and sonication of the mixture was carried out for the period of 5 min. Afterward, 4-arm polyethylene glycol-amine (Sigma Inc.) was added to the suspension mentioned above, and the mixture was stirred for the period of 24 h at room temperature. The final product (NGO-PEG) was acquired as a result of centrifuge filtration using Amicon 100 kDa centrifugal filters, and it was washed with DI water a few times [37, 43].

2.3. Drug loading

Doxorubicin (DOX) loading onto NGO-PEG was performed as a result of simply mixing 0.4 mg/mL of DOX and NGO-PEG solution (~0.2 mg/mL) at pH 7 for a night. The removal of unbound excess DOX was carried out by washing repeatedly and filtration using a 100 kDa filter and repeated rinsing. The resuspension of the obtained NGO-PEG-DOX complexes was performed, following which they were stored at the temperature of 4˚C [43].

2.4. Characterization

The morphology of NGO was measured by a scanning electron microscope (TESCAN MIRA3 XMU). A diffractometer (Rigaku DMAX IIIC) was used to obtain X-ray diffraction (XRD) data. A UV-Vis spectrophotometer (UV-1280, Shimadzu, Japan) was utilized for the purpose of recording the spectra of the prepared samples in the range from 200 to 800 nm, in order to homogeneously disperse the samples. A probe sonicator (Sonics & amp; materials INC, USA) at 750W power was used. The Malvern Zetasizer Nano Z was used to measure the zeta potential values of the samples.

3. Results And Discussion

A scanning electron microscopy (SEM) image (Figure 1) provides morphological information. NGO. Appearance: black powder; thickness: 3.1–6.8 nm; purity>99 wt %; lateral dimension: 15–40 μm. The EDX analysis was also performed for the synthesized NGO. As a result of the technical analysis, the metal contents of NGO were determined as follows: C-61.39%; O-36.11%; S-1.18%; P-0.38%; K-0.94%. Phosphorus, sulphur and potassium were present as a result of H_2SO_4, H_3PO_4 and $KMnO_4$ used as an oxidizing agent.

There is a significant difference between the distances between the sheets in addition to their folding and structural disruptions in graphite and its functionalized derivatives. Thus, the characterization of the graphite, NGO and

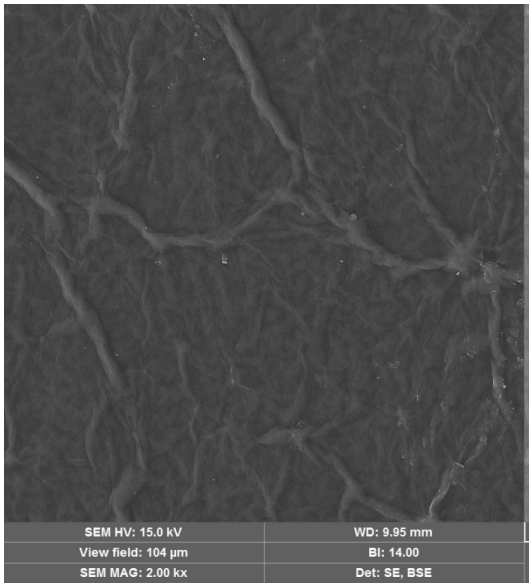

Fig. 1 - SEM image of nano graphene oxide.

carboxylated functionalized samples was performed by XRD for the purpose of more structural analysis. The chemical oxidation of the exfoliated graphite and formation of NGO were confirmed by the XRD patterns (Fig. 2). Graphite demonstrates a very strong and sharp peak at 2θ = 26.40˚, corresponding to the diffraction of the (002) plane. After graphite is oxidized to NGO, the (002) reflection of graphite disappears, and a diffraction peak at 2θ = 10.21° emerges, corresponding to the diffraction of the (001) plane, which is an indicator of the successful oxidation of graphite. A further carboxylation process in basic conditions led to a more dispersion and exfoliation of nano-sheets possibly because of adding the chloroacetic acid residue (–O–CH_2–COOH). Consequently, a considerably weaker and broader diffraction peak was displayed by the carboxylated sample at 2θ = 9.82° in comparison with NGO, which is an indicator of an increase in the interlayer spacing.

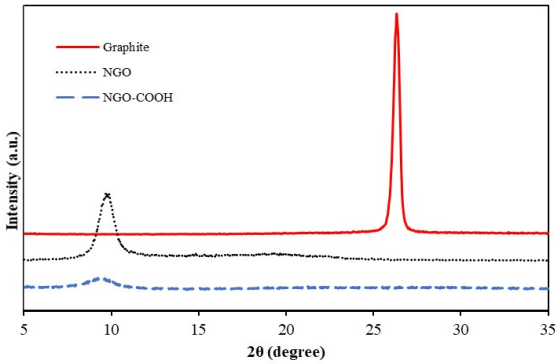

Fig. 2 - The XRD patterns of graphite, NGO and NGO-COOH.

As can be observed from the UV-vis spectrum of NGO, NGO-PEG, and NGO-PEG-DOX (Fig.3), the optical absorption of NGO-PEG in the visible and near infrared range was higher compared to NGO at the identical concentration of 0.05 mg/mL. It is possible to attribute the increase in optical absorption to the opening of epoxide groups and hydrolysis of esters on GO in basic conditions in the course of the PEGylation treatment [43]. The investigation of the drug loading behavior of NGO-PEG was performed using UV−vis spectra (Figure 3). The characteristic absorption peak of Dox (~490 nm) emerged in the NGO-PEG-DOX sample, which is an indicator that NGO-PEG-DOX conjugates were formed successfully. The efficient loading of Dox by NGO-PEG is indicated by this peak.

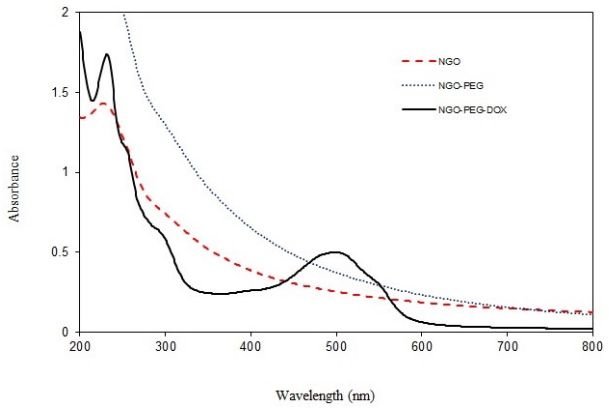

Fig. 3 - UV-vis- absorbance spectra of NGO, NGO-PEG and NGO-PEG-DOX.

Zeta potential represents the main indicator of the stability of colloidal dispersions. The degree of electrostatic repulsion between adjacent, similarly charged particles in the dispersion is shown by the magnitude of the zeta potential value. In accordance with the ASTM, it is accepted that colloid solutions having zeta potentials greater than ±40 mV have good stability [49]. In the present study, zeta potential was measured for the purpose of revealing the stability of NGO and NGO-PEG-DOX in water. As can be observed in

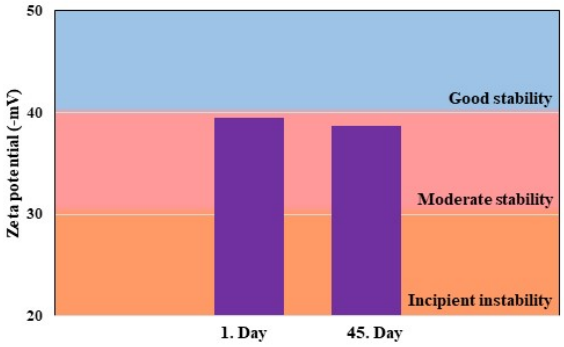

Fig. 4 - Zeta potential of NGO dispersed in water at the pH of 7.0.

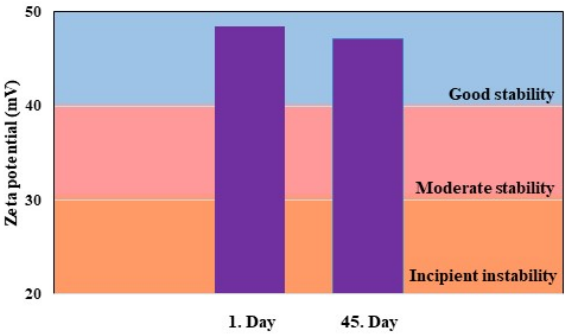

Fig. 5 - Zeta potential of NGO-PEG-DOX dispersed in water at the pH of 7.0.

Fig. 4 and Fig. 5, as a result of comparing the zeta potential value at the pH of 7.0, it was determined that NGO-PEG-DOX has in general better stability compared to NGO in water.

To explain it in detail, the zeta potential value of NGO solution is approximately -38.50 mV due to the negatively charged functional groups, including COOH, C=O, and C−OH grafted on the GO surface and providing electrostatic repulsion between GO nanoparticles, which can ensure the enlargement of their distance and avoiding of the aggregation. Moreover, an increase in the carboxyl groups of the surface, inducing high negative

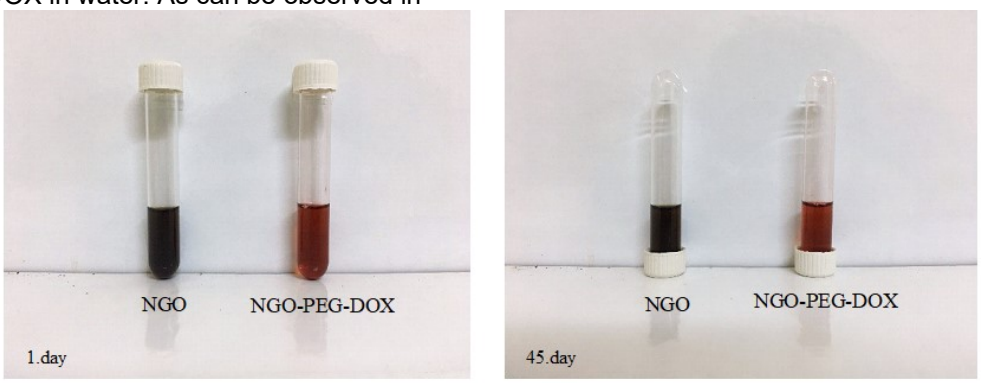

Fig. 6 - Visual observation of the dispersion of NGO and NGO-PEG-DOX in water ***(45 days later)***.

charges, will ensure that a higher number of positively charged polymers, peptides, proteins, and nanoparticles is bound to GO through electrostatic interaction. The zeta potential value of NGO-PEG-DOX system is also 48.4 mV, and after 45 days, the zeta potential shows good stability. Figure 6 depicts the visual observation of NGO and drug-loaded NGO. They were also kept at 4 ˚C. Any aggloremation and settling of DOX drug molecules were not observed after 45 days.

4. Conclusion

In this work, we reported the synthesis of the nanocarrier with good biocompatibility, physiological stability and good delivering performance of a water-insoluble anticancer drug of DOX. For this purpose, firstly multifunctional biocompatible NGO were synthesized, then the PEGylation process was performed and finally the drug was loaded. One of the most important disadvantages of cancer drugs is that they lose their stability over time. In this study, the stability of the stability drug was increased by sonication in high frequency probe sonicator and PEGylation process. According to UV and zeta potential results, long life stable NGO-PEG-DOX nanostructured drug loaded product was obtained after even 45days. Further studies on NGO-PEG-DOX system should be done especially in vivo applications due to solve various clinical problems in diagnostics and treatment of cancer area.

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