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Cytotoxicity of PEGylated graphene oxide on lymphoma cells

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Abstract. Graphene oxide (GO) is a hotspot, especially in the field of biomedical. However, the clinical application of GO is still faces a lot of challenges. In order to improve the solubility and biocompatibility of GO, polyethylene glycol (PEG) was grafted on the surface of graphene oxide by amide reaction. PEGylated graphene oxide (PEG-GO) was characterized using Fourier transform infrared spectroscopy (FTIR). The stability of PEG-GO detected in different solutions. Raji cell was selected as a lymphoma cell model to study the cytotoxicity of PEG-GO. Cell viability was detected using the Cell Counting Kit-8 assay. Cells were treated with different concentrations (10-100 μg/mL) of PEG-GO at different time points (6, 12, and 24 h). The FTIR spectrum of PEG-GO indicated that polyethylene glycol was successfully grafted onto GO. PEG-GO had excellent stability in all solutions. Cells treated with PEG-GO (10-100 μg/mL) for 24 hours had survival rates were over 80%. These results demonstrate that PEG-GO had an excellent dispersion in biological solutions and the toxicity of PEG-GO to lymphoma cells was low. The paper may provide cytological evidence for the application of PEG-GO in medicine.

Keywords: PEGylated graphene oxide, lymphoma cells, cytotoxicity, CCK-8

1. Introduction

Graphene is a kind of two-dimensional (2-D) carbon nanostructure of single-layer [1,2]. In recent years, graphene, and its derivatives, attract great interest of some researchers in biomedical applications. Previous studies have shown that graphene oxide (GO) acts as a nanocarrier, and therefore may load with many drugs such as camptothecin (CPT), 7-ethyl-10-hydroxycamptothecin (SN38) and doxorubicin (DOX) [3–5]. As one derivative of graphene, GO is normally hydrophilic due to a large number of functional groups such as the hydroxyl group, the carboxyl group, the carbonyl group and the epoxy functional group [6]. However, GO may generally aggregate in the salt solution, cell culture medium or other biological solutions [7,8]. Thus, the solubility of GO in solvent needs to be improved for further biological application.

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Polyethylene glycol (PEG) has an excellent biocompatibility and hydrophilicity, which has been widely applied in drug delivery systems such as liposomes [9], micelles [10,11]. Moreover, due to its advantage of non-immunogenic and its good adaptation in vivo [12], some researchers have conducted the work of modifying GO with PEG [3,13–15]. PEG-GO may be prepared by many kinds of methods such as covalent approach [16], amide reaction [3] and noncovalent approach [17]. Dai et al prepared PEG-GO sheet that was used in vitro drug delivery and the physiological solution of biological imaging [3,13], their results showed that PEG-GO had good dispersion, stability and could load with various water-insoluble drugs. Liu et al [14] also prepared PEG-GO that had efficient passive tumor targeting ability. However, there are very few reports about the cytotoxicity of PEG-GO on tumor cells, especially on human lymphoma cells (Raji cells).

The aim of our present work is to improve the stability of PEG-GO in different solutions as well as its cytotoxicity on lymphoma cells. In the paper, PEG-GO was prepared using amine-terminated polyethylene glycol (NH_2-PEG-NH_2) through amidation with GO. After PEG-GO was characterized by FTIR, the cytotoxicity of PEG-GO on lymphoma cells was studied by using CCK-8 assay. This paper may provide cytological evidence for the application of PEG-GO in medicine.

2. Materials and methods

2.1. Materials and reagents

Expanded graphite was purchased from Qingdao Henglide Graphite Co, Ltd; All chemicals and reagents (analytical reagents) were obtained from Sinopharm Chemical Reagent Co. Ltd; EDC and NHS were purchased from Beijing Ruitai bio, Ltd; The dialysis bags (MWCO=100 Da) were purchased from Spectrum Laboratories Inc; NH_2-PEG-NH_2 was obtained from ShangHai Yarebio, Co, Ltd; DOX was purchased from Zhejiang *His un* Pharmaceutical Co, Ltd; RPMI-1640 medium was purchased from Hyclone USA; Fetal bovine serum was obtained from Gibco (Gaithersburg, USA); CCK-8 assay kits were purchased from Dojindo Laboratories (Kyushu, Japan).

2.2. Preparation of graphene oxide

Graphene oxide (GO) was prepared from expandable graphite using the modified Hummers method [18,19]. The expandable graphite (1 g) was dissolved in 46 ml H_2SO_4 (98 wet %) with stirring in the ice bath. Next, $NaNO_3$ (1 g) and $KMNO_4$ (6 g) were stirred into the mixture and the temperature kept between 10°C to 15°C.The temperature of the mixture was subsequently raised to 35°C and the mixture was continuously stirred for 30 min. Distilled water was slowly added to the mixture. The reaction temperature was rapidly raised to 95°C, and H_2O_2 (30%) was added to the mixture. Finally, the mixture was centrifuged and washed with HCl (5%) and deionized water until neutral got GO. TEM was used to characterize the morphology and structure of GO.

2.3. Synthesis of PEGylated graphene oxide and characterization

PEG-GO was prepared through an amide reaction of amine-terminated polyethylene glycol (NH_2-PEG-NH_2) and GO [3,20]. In order to prepare PEG-GO, the GO solution (2 mg/mL) was bath-sonicated for 3h at the room temperature. NaOH (0.6 g) and $ClCH_2COOH$ (0.5 g) were added to distilled water. This solution was added to 5 ml of GO (2 mg/mL) and bath-sonicated for 3 hours. The

reaction mixture was centrifuged at 10,000 rpm for 15min, and washed with 5% HCl and deionized water for several times until the pH read close to neutral in order to obtain GO-COOH. The GO-COOH aqueous solution was bath-sonicated for 1 h. NH_2-PEG-NH_2 was added to the GO-COOH suspension and the mixture was stirred for 3 min. EDC was added to the above suspension and the solution was stirred overnight. PEG-GO was successful prepared [21]. Dialysis of the product was repeated for 48 hours to remove the unreacted PEG through dialysis bags. Functional groups of PEG-GO were analyzed by Fourier transform infrared spectroscopy (FTIR) spectra.

2.4. Stability of GO/PEG-GO

The same volumes of distilled water, 0.9% NaCl, phosphate buffer saline (PBS) and the RPMI-1640 medium were added to the same concentration of GO and PEG-GO. The stabilities of the solutions were observed after centrifugation at 10,000 rpm for 5 minutes.

2.5. Cytotoxicity detection of PEG-GO by CCK-8 assay

The cytotoxicity of GO-PEG on human lymphoma cells was assessed by the CCK-8 assay. Raji cells were seeded in cell culture flasks containing RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin in 5% CO_2 at 37°C in a humidified incubator. Cells were passaged every 2-3 days. The logarithmic growth phase cells were used in the research. Cell viability was detected with the CCK-8 assay. Raji cells were seeded in 96-well plates (Corning Technologies, Corning, NY, USA) at a density of 1.0×10^4 cells/well. Cells were cultured with different concentrations of PEG-GO (10, 20, 40, 50, 100 μg/mL) and were incubated for varying periods of time (6, 12, 24h). After the specified treatment, 10 μL of CCK-8 was added to every well. The plate was incubated for 4 hours at 37°C. Moreover, in order to prove if the PEG would interfere with GO activity, cells were treated with GO for comparison with the PEG-GO treated cells. The absorbance was recorded by microplate reader at 450nm.

3. Statistical analysis

All experimental data was expressed as mean ± standard deviation (mean ± SD). Comparison between two groups was analyzed using Student's t-test or one-way analysis of variance (ANOVA). A value of $P \leq 0.05$ was considered statistically significant.

4. Results and discussion

4.1. Characterization of GO/PEG-GO

TEM was used to characterize the morphological structure of GO. The surface of GO was smooth and the lateral size of GO was about 500 nm (Figure 1). FTIR spectrum of PEG-GO showed that peaks at 2860 cm^{-1} and 1650 cm^{-1} which indicated the existence of (-CH_2-) and (NH-CO) groups. Contradictingly, these peaks do not exist in the undecorated graphene oxide. Therfore, The results proved that PEG was successfully grafted onto GO (Figure 2).

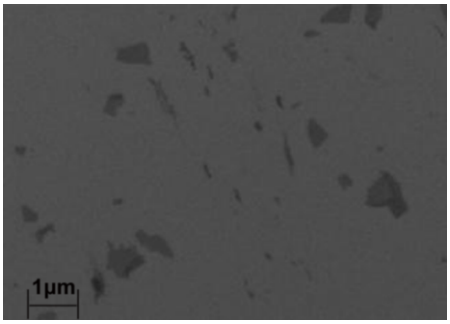

Fig. 1. TEM image of GO.

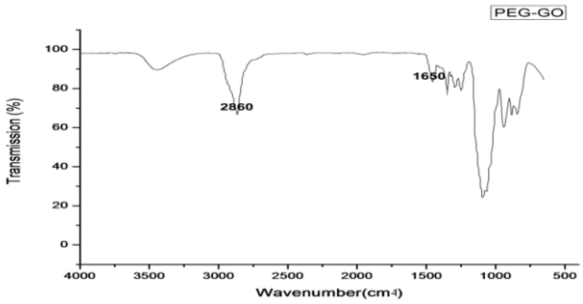

Fig. 2. FTIR image of PEG-GO: Peak at 2860 cm-1 and 1650 cm-1 that indicated the existence of (-CH_2-) and (NH-CO) groups.

4.2. Stability of GO/PEG-GO

In order to study the stability of PEG-GO, GO and PEG-GO were added to different solutions (distilled water, 0.9% NaCl, PBS and the RPMI-1640 medium), respectively. The results showed that GO could dissolve in water but aggregated in 0.9% NaCl, PBS and the RPMI-1640 medium (Figure 3A). PEG-GO exhibited excellent stability in all above solutions (Figure 3B). The results are consistent with previously reported literatures [22–24]. This data indicates that PEG-GO has good solubility due to a lot of functional groups such as hydroxyl and carboxyl on the surface of GO and good hydrophilicity of PEG. PEG-GO can enhance the water-solubility of GO as well as its stability in the biological solutions.

4.3. Cell morphology

Cell morphology was observed through inverted microscope. Raji cells were seeded at 1×10^5 cells/well in 6-well plates. Different concentrations of PEG-GO were added to each well. The cell morphology after incubation for 24 hours was observed. Untreated lymphoma cells were large and round. Lymphoma cells treated with PEG-GO had morphological changes, such as shrinkage. Particularly, if the concentration of PEG-GO was 100 μg/mL, the cell membrane would shrink (Figure 4).

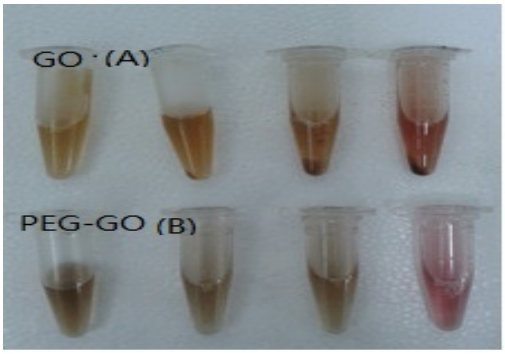

Fig. 3. The stability of GO/PEG-GO in biological solutions. GO (A) and PEG-GO (B) in various biological solutions. GO dissolved in water but aggregated in PBS, 0.9% NaCl and cell medium. PEG-GO was stable in all above solutions.

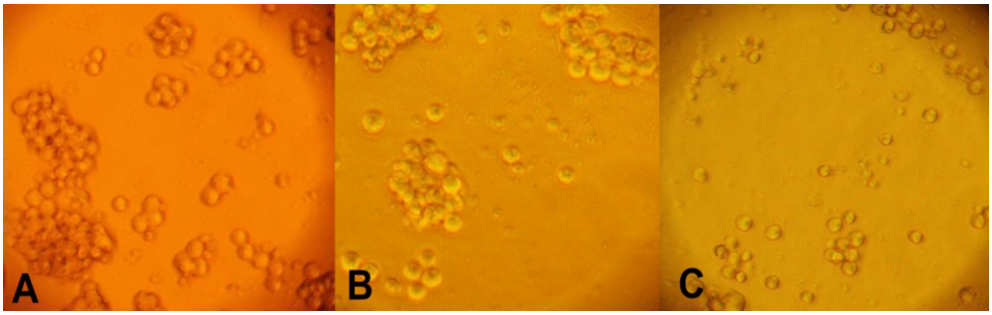

Fig. 4. Morphology of Raji cells treated with different concentrations of PEG-GO for 24h. A. without PEG-GO; B. 50 μg/mL PEG-GO; C. 100 μg/mL PEG-GO (100X).

4.4. Cytotoxicity of PEG-GO

The cell viability was assessed using the CCK-8 assay. Cells were treated with PEG-GO (10-100 μg/mL) for 6 hours, and then the cell viability was decreased from 98.97% to 96.12%. When cells treated with PEG-GO for 12 hours at the same concentration, the cell viability decreased to 88.8% from 97.41%. Cells treated with PEG-GO (10, 20, 40, 50, 100 μg/mL) for 24 hours, decreased in cell viability from 97.05% to 81.97% ($p < 0.05$) after the PEG-GO (100 μg/mL) treatment for 24 hours.

The results indicate that the cytotoxicity of PEG-GO was time-dependent and dose-dependent. In addition, after PEG-GO treatment (0-100 μg/mL) for 24 hours, the cells' survival rates were still over 80% (Figure 5). Cells were treated with GO/PEG-GO(0-100μg/ml) for 24 hours, and the survival rates show no different between GO and PEG-GO ($p > 0.05$) (Figure 6). The results demonstrate the PEG would not interfere with GO activity, which might show that PEG-GO is of low toxicity to lymphoma cells.

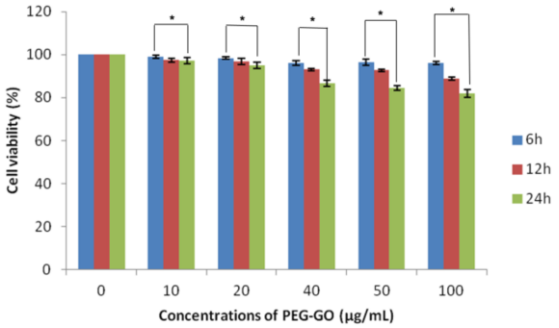

Fig. 5. The cell viability of Raji cells with different PEG-GO (10-100 μg/ml) concentrations at various time points (6, 12, 24 h). Data are presented as mean ±SD (n=6 per group). t=2.1965, *$p < 0.05$ for two particular groups (6h and 24h).

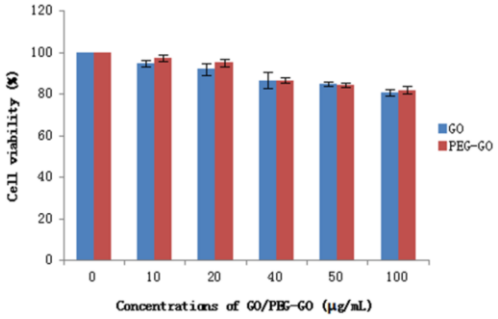

Fig. 6. The cell viability of Raji cells with GO/PEG-GO (10-100 μg/mL) at the same concentrations for 24h. Data are presented as mean ±SD (n=6 per group), $p > 0.05$ for two particular groups.

5. Conclusion

The purpose of our present work is to improve the stability of PEG-GO in different solutions and to determine its cytotoxicity of it on human lymphoma cells. In this experiment, PEG-GO is successfully prepared and shows good solubility in aqueous solution, saline solutions, and cell culture medium. GO can be dissolved in water but aggregates in PBS, 0.9% NaCl and cell medium. Cytotoxicity of PEG-GO on lymphoma cell indicates that PEG-GO has a certain time-dependent and dose-dependent response. In addition, after treated with PEG-GO (100 μg/mL) for 24 hours, the cells' survival rates were still over 80%. These results suggest that PEG-GO is of low toxicity to lymphoma cells. Moreover, the PEG would not interfere with GO activity. These results provide a theoretical basis for the clinical application of PEG-GO in medicine as a nanocarrier from the cellular level.

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