Preparation and cell imaging of nitrogen-doped graphene quantum dot conjugated indomethacin

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Abstract: The nitrogen-doped graphene quantum dot conjugated indomethacin (N-GQD-IDM) was synthesized by an amide reaction. The results of FTIR indicated that the synthesis of N-GQD-IDM was successful. It was then co-cultured with MCF-7 cells, and obvious fluorescence was observed under a laser confocal scanning microscope. With the increase of incubation time, the material accumulated significantly in the cells and the fluorescence intensity of the cells was slightly improved. This compound could be suggested as a promising fluorescent probe in cancer cell labeling.

1. Introduction

Cancer is a disease which seriously affects human health[1]. Therefore, it is important to research a fluorescent probe that specifically recognizes cancer cells. Cyclooxygenase-2(COX-2) is a key enzyme in the catalytic rate limiting step[2], which is very low in normal cell expression[3]. The expression of cyclooxygenase-2 is due to pathological changes in cancer cells. The appearance of indomethacin makes cyclooxygenase-2 easier to label. Therefore, indomethacin (IDM) can be used as a targeting group for the recognition of cancer cells by fluorescent probes. Nitrogen-doped graphene quantum dots (N-GQD) are used in various fields such as electrochemiluminescence (ECL) sensors[4-5], ion detection[6], and bioimaging[7-8]. Graphene quantum dots are not only small in size, but also have abundant functional groups on their surfaces, such as carboxyl groups and hydroxyl groups[9-10]. The advantage of smaller size makes it easier for cells to swallow them[11]. The nitrogen-doped graphene quantum dots (N-GQD) introduce an amino group due to the doping of nitrogen, which provides a reaction site with more targeting groups. In this work, N-GQD was used as a fluorescent material and indomethacin was used as a targeting group. N-GQD and indomethacin are directly conjugated with an amide bond, which saves reaction time and reduces by-product production. This fluorescent probe was then incubated with breast cancer cells for fluorescence imaging. This article also explored the effect of incubation time on cell imaging and the relationship between fluorescence intensity over time.

2. Experiment

2.1 Material

Citric acid, diethylamine, indomethacin, dimethyl sulfoxide, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) were purchased from Aladdin Industries (Shanghai, China). PBS, dulbecco’s modified eagal medium(DMEM) were purchased from Lanbao Biotechnology Co., Ltd. (Hangzhou, China). All
chemicals were of analytical grade and the water used in the experiment was deionized water.

2.2 Synthesis of nitrogen-doped graphene quantum dots (N-GQD)
Nitrogen-doped graphene quantum dots were synthesized by hydrothermal method[11-13]. Briefly, 420 mg of citric acid was dissolved in 10 ml of deionized water. Then 0.7 mL of diethylamine was slowly added during the stirring, and then stirring was continued for 2 h. The reaction solution was added to a teflon-lined stainless steel container and hydrothermally treated for 4 h at 180 °C. The reaction solution was then filtered and the filtrate was collected.

2.3 Synthesis of indomethacin and nitrogen-doped graphene quantum dot conjugates (N-GQD-IDM)
50 mg of indomethacin (IDM) was dissolved in 20 mL of dimethyl sulfoxide at room temperature and was stirred for 1 h. After the stirring was completed, 0.9 mmol of EDC and 0.9 mmol of NHS were added to the reaction solution, followed by stirring overnight. The reaction was carried out under dark conditions. Then N-GQD was added to the mixed solution and reacted for 24 h. After the reaction was completed, the dialysis bag was used for dialysis (molecular weight cutoff was 1000) and the N-GQD-IDM solution was collected.

2.4 Cell imaging
MCF-7 cells were planted in a 35 mm culture dish at a seeding density of 2×10^5 and incubated for 24 h in a humidified incubator containing 5% CO2 at 37 °C. After the cells were attached, the culture solution were replaced with 1 mL of Dulbecco's modification of Eagle's medium Dulbecco (DMEM) and 0.5 mL of material solution (1.78mg/mL). The incubation time of materials and cells was 3 h and 6 h, respectively. MCF-7 cells were washed three times with 1 mL of PBS, and the cell was imaged by confocal laser scanning microscope with a 405 nm laser.

2.5 Characterization
Micromorphology of nitrogen doped graphene quantum dots (N-GQD) were taken by transmission electron microscopy (TEM JEM-2100). The PL spectra were recorded by a Hitachi F-7000 spectrofluorimeter at different excitation wavelengths. Fourier infrared spectrum was measured with Fourier infrared spectrometer Nicolet 5700, test range 400-4000cm^-1. Fluorescence imaging was acquired by confocal laser scanning microscope FV100-IX81.

3. Results and discussions
In this paper, N-GQD was prepared by a hydrothermal process in which citric acid was used as a carbon source and diethylamine was used as a nitrogen source. N-GQD and indomethacin were then conjugated together by the action of amide bond (Scheme 1). The reaction could be fulfilled at room temperature as a result of the reaction conditions were made moderate by the action of EDC and NHS.

![Scheme 1 Schematic illustration of the synthesis of N-GQD-IDM](image-url)
Figure 1 (a) TEM image of N-GQD (b) HRTEM image of N-GQD

Figure 1a showed the microscopic morphology of nitrogen-doped graphene quantum dots (N-GQD), which could be seen to be less than 5 nm in the figure. The HRTEM image clearly showed that the lattice spacing of the nitrogen-doped graphene quantum dots (N-GQD) was 0.24 nm, which corresponded to the (1120) crystal plane of graphene. The synthesis of quantum dots was visually proved by TEM image, which laid the foundation for the next reaction.

Figure 2 FTIR spectra of nitrogen-doped graphene quantum dots (N-GQDs), nitrogen-doped graphene quantum dots conjugated to indomethacin (N-GQD-IDM) and indomethacin (IDM)

Figure 2 further confirmed that nitrogen-doped graphene quantum dots (N-GQDs) and indomethacin (IDM) were conjugated by an amide bond. The infrared spectrum of N-GQD in Figure 2 demonstrated the presence of amino groups. 3348 cm\(^{-1}\) was the stretching vibration peak of N-H. 1575 cm\(^{-1}\) was the bending vibration peak of the amine N-H. It indicates the presence of amino groups in nitrogen-doped graphene quantum dots. The infrared spectrum of N-GQD-IDM in Figure 2 demonstrated the synthesis of the product. 1708 cm\(^{-1}\) was the C=O vibration peak of the amide[15]. The carbonyl peak intensity in the N-GQD-IDM infrared spectrum was significantly enhanced compared to the carbonyl peak intensity in N-GQD. Therefore, successful synthesis of the product could be demonstrated from the infrared spectrum.
Figure 3 Fluorescence emission spectra of (a)N-GQD (b) N-GQD-IDM under different excitation wavelengths

The fluorescence intensity of N-GQD at different excitation wavelengths was shown in Figure 3a. It could be clearly seen from the figure 3a that the fluorescence intensity reached the maximum at the excitation wavelength of 360 nm and 350 nm. At different excitation wavelengths, the emission of N-GQD was around 450 nm, which proved that N-GQD had no excitation dependence. Figure 3b showed the fluorescence of N-GQD-IDM. Because of the indomethacin complex, its fluorescence emission peak is slightly red-shifted compared to the emission peak of N-GQD. At the same time, as the excitation wavelength increases (340 nm to 390 nm), the fluorescence intensity decreases.

The fluorescent labeling effect of the material N-GQD-IDM was shown in Figures 4a-f, indicating that N-GQD-IDM successfully labeled MCF-7 cells. Figure 4a-c was a confocal laser scanning microscope image of N-GQD-IDM and MCF-7 cells cultured for 3 h, and Figure 4e-f was a confocal laser scanning microscope image of 6 h incubation. Figures 4b and 4e showed that the labeled MCF-7 cells emitted light blue fluorescence. The fluorescence intensity of labeled MCF-7 cells in Figure 4e is significantly greater than in Figure 4b, indicating that the longer the incubation time of MCF-7 cells with materials, the more cells are labeled. Compared to Figure 4c, it can be clearly seen that there were black shadows in MCF-7 cells in Figure. 4f, indicating that N-GQD-IDM would accumulate in the cells, further confirming that N-GQD-IDM had a higher good biocompatibility.

Figure 4 Confocal laser scanning microscope imaging of MCF-7 cells incubated with N-GQD-IDM
(a) Merge image (b) Dark field (c) Bright field for 3 h (d) Merge image (e) Dark field (f) Bright field for 6 h by excitation at 405 nm

4. Conclusion
In this work, we synthesized nitrogen-doped graphene quantum dots by hydrothermal method using
citric acid as a carbon source and diethylamine as a nitrogen source. On this basis, the nitrogen-doped graphene quantum dots and indomethacin were amide-reacted to form a nitrogen-doped graphene quantum dot conjugated indomethacin. This compound acted as a fluorescent probe material that allowed rapid recognition of MCF-7 cells within a few hours. At the same time, the trend of N-GQD-IDM in cells was explored from the perspective of time. That is, as time increases, the material produces a certain accumulation in MCF-7 cells. It can be seen from the fluorescence imaging of the cells that the fluorescence intensity was also enhanced as the incubation time was extended. The application prospect of N-GQD-IDM was in cancer cell recognition and fluorescence imaging by confocal laser scanning microscopy.

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