Microwave-assisted and one-step synthesis of PEG passivated fluorescent carbon dots from gelatin as an efficient nanocarrier for methotrexate delivery

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**ABSTRACT**

A green and simple process for preparing the polyethylene glycol passivated fluorescent carbon dots (CDs-PEG) have been studied by a microwave pyrolysis method, using gelatin and PEG as starting materials. This method is very effective for development of carbon-based quantum dots from gelatin with high quantum yield (QY). The synthesized CDs-PEG were found to emit blue photoluminescence (PL) with a maximum QY of 34%. At the following research, we investigated the effect of the presence of PEG on PL intensity, and the result showed that CDs-PEG becomes stronger PL properties than pure CDs from gelatin. The synthesized CDs-PEG were characterized by FTIR, TEM, UV–vis, PL, zeta potential and XRD analyses. The anticancer performance of developed CDs-PEG was evaluated by in vitro tests such as MTT assay and fluorescence microscopy analyses. The examination of CDs-PEG as an anti-cancer drug nanocarrier for methotrexate (MTX) illustrated a better antitumor efficacy than free MTX due to its enhanced nuclear delivery in vitro, which resulting in highly effective tumour growth inhibition and improving targeted cancer therapy in clinical medicine.

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**Introduction**

Fluorescent carbon dots (CDs), as one of the most promising carbon nanomaterials, have gained an impressive interest due to their unique optical and electronic properties [1–5]. In comparison with traditional organic dyes and inorganic quantum dots (QDs), CDs are more superior due to a) biocompatibility, b) low toxicity, c) chemical solubility, d) aqueous solubility and e) easy surface modification [6–8]. Their unique properties are being used in various applications including catalysts, optoelectronic devices, bioimaging, biosensing and drug delivery [9–12]. Biocompatible and highly nontoxic fluorescent CDs are generally obtained from synthetic or natural polymeric materials [13]. Among natural polymers, gelatin due to its good pharmacological characteristics (as excellent biocompatibility and degradability to non-toxic products) and low cost, could be an excellent candidate for the synthesis of CDs [14,15]. Commonly techniques have been used to synthesis CDs to include electrochemical synthesis [16,17], chemical oxidation [18], laser ablation of graphite [19], ultrasonic methods [20], hydrothermal [21,22], solvothermal [23] and microwave-assisted pyrolysis [24,25]. However, most of these techniques may involve a) complex reactions, b) time-consuming processes, c) post-treatment procedures and d) an expensive carbon source, which limit the applications of CDs. So, the facile, rapid and environmentally friendly viable synthetic ways for the production of high-quality fluorescent CDs are in high demand. The microwave-assisted method was an effective way for obtained nanoparticles (NPs) [26–29]. Among these conventional methods, the microwave mediated synthesis indications many preferable benefits, such as rapid reaction rate, controllable conditions and non-hazardous [30]. However, the microwave irradiation generally produces CDs with lower quantum yields.
Thus, to overcome this disadvantage, surface passivation scheme has been used to increase the fluorescence properties of CDs and also to make them appropriate for biological applications [31]. Among the various organic molecules served as a CDs surface passivation, polyethylene glycol (PEG) has been more concerned, due to excellent biocompatibility, non-toxicity and hydrophilic properties [32–36]. Conjugating PEG onto the surface of CDs (PEGylation) would be an ideal approach to improving the biocompatibility and avoid recognition and clearance of the CDs by the reticuloendothelial system (RES) which increases the circulation halftime of CDs [37,38]. Methotrexate (MTX) has been one of the most commonly applied therapeutic agents in the treatment of lymphomas, breast cancer, choriocarcinoma and lung cancer. MTX is capable to enter cells via a similar transportation system to folic acid (FA), due to the structural similarity of MTX with FA and presence of FA receptor as targeting ligand on the surface of the cancer cells [39,40]. In this study, the polyethylene glycol passivated CD (CDs-PEG) with stronger optical-absorbing ability were synthesized by economical and facile microwave pyrolysis method using gelatin as the carbon source and PEG as the passivator. Then, the CDs-PEG was loaded with MTX, to prepare the MTX/CDs-PEG complexes as an efficient drug delivery nanosystem. The obtained MTX/CDs-PEG were thoroughly characterized by various microscopic and spectroscopic techniques. Finally, the biocompatibility and cytotoxicity effects of the developed nanocarrier were carried out via the evaluating survival rate of human breast epithelial adenocarcinoma cell line (MCF-7) by the MTT assay.

**Experimental**

**Materials and method**

Gelatin and PEG-4000 (PEG-4000) were purchased from Merck (Darmstadt, Germany), deionized water was used in all of the experiments where required. UV–vis absorption of the aqueous solutions of CDs-PEG was recorded using a UV-2550 UV–vis spectrophotometer (Shimadzu, Kyoto, Japan). The photoluminescence (PL) emission measurements were recorded using a fluorescence spectrofluorometric, LS45 (PerkinElmer, Waltham, MA). Transmission electron microscope (TEM) image was captured by a CM120 Philips (Magdeburg, Germany) with an accelerating voltage of 100 kV. The surface groups on CDs-PEG were determined with a Fourier transform infrared (FT-IR) spectrometer, Model Tensor 27 (Bruker Instruments, Karlsruhe, Germany) using KBr pellets. The crystal structure of CDs was determined by X-ray diffraction, D-500 XRD (Siemens, Munich, Germany) using Cu-Kα radiation at 35 kV. The average diameter and zeta potentials of the NPs were determined by photo-correlation spectroscopy (Zetasizer ZS, Malvern Instruments, Malvern, UK). The microwave-assisted carbonization reaction was done in a microwave oven with a tunable output power ranging from 100 to 900 W and a 2450-MHz microwave frequency, 1250 W, NN-SD797S Genius (Panasonic, Kadoma, Japan). The QY was calculated by the previously established method [6].

**Preparation of CDs-PEG**

As presented in Scheme 1, in a typical process the CDs-PEG was prepared as follows: an adequate amount of gelatin was diluted with MiliQ water to form an aqueous solution, then PEG was added under stirring. After stirring for 30 min, the mixture was heated for 10 min under 600 W microwave oven. With time, the colour of the solution gradually changed from light yellow to dark yellow. The colour-changed solution was centrifuged at 12,000 rpm for 15 min to eliminate the less-fluorescent deposit. The purified CDs-PEG solution was maintained at 4°C until needed for further experimentation. The QY of the CDs-PEG was calculated about ~34%, which was higher than the pure CDs at similar excitation wavelengths.

**QY measurements**

The QY of the CDs-PEG has been calculated using the following formula. Quinine sulphate (QS) was chosen as a reference to determine QY. QS (QY is 0.54) was dissolved in 0.1 M H2SO4 and the CDs-PEG were dissolved in distilled water (η = 1.33).

\[
\varphi = \varphi_R \times \frac{I_S}{I_R} \times \frac{A_R}{A_S} \times \frac{\eta_S^2}{\eta_R^2}
\]

where \(\varphi\), \(I\), \(A\) and \(\eta\) are the QY, measured integrated emission intensity, absorbance and refractive index of the solvent,

![Scheme 1. A schematic illustration of the preparation procedure of PEG passivated carbon dots (CDs-PEG) through microwave carbonization.](image-url)
respectively. The subscript S and R refers to the sample and reference fluorophore of known QY, respectively.

**MTX-loading and release**

For MTX/CDs-PEG complex formation, 1 mL of the prepared CDs-PEG solution (10 mg mL\(^{-1}\)) and 1 mL of MTX solution (1 mg mL\(^{-1}\)) were mixed with 2 mL PBS at pH 7.4 under stirring. This solution was then allowed to react for 24 h under dark conditions at ambient temperature. The resultant solution was dialysed using a dialysis membrane (molecular weight cut off 12000 Da) against 20 mL deionized water for 2 h to eliminate any unreacted MTX or CDs. Subsequently, the percentages of loading capacity (LC) and encapsulation efficiency (EE) were calculated using the following formulas, respectively:

\[
LC\% = \frac{\text{Mass of drug encapsulated}}{\text{Mass of nanoparticles}} \times 100
\]

\[
EE\% = \frac{\text{Mass of drug encapsulated}}{\text{Mass of feed drug}} \times 100
\]

The pH-dependent MTX release in vitro experiment was carried out in dark conditions, under stirring at 200 rpm at 24°C. Briefly, 2 mL aliquots of the MTX/CDs-PEG solution were dialysed using a dialysis bag (molecular weight cut off 12,000 Da) against 20 mL of PBS, in both simulated tumour tissue (pH 5.3, 41°C) and normal physiological (pH 7.4, 37°C) conditions. After certain time intervals, adequate amounts of the PBS solutions were removed and the concentration of released MTX was evaluated using UV–vis spectroscopy. The percent of the drug released was calculated by the following formula:

\[
\text{Drug release} = \frac{\text{the amount of released drug}}{\text{the amount of loaded drug}} \times 100
\]

**In vitro cell cytotoxicity**

The cytotoxicity and biocompatibility of the CDs were assessed against the MCF-7 cell line by comparing the CDs-PEG (without MTX), free MTX and MTX/CDs-PEG with MTT assay. Briefly, the cells were seeded into a 96-well plate at a density of \(2 \times 10^4\) cells per well and incubated for 24 h at 37°C under the humidified atmosphere with 5% CO\(_2\). Then, the culture medium was removed and 200 mL of growth medium with various concentrations of the free MTX, MTX/CDs-PEG and CDs-PEG was replaced to each well and incubated for 24 and 48 h. Then, the MTT solution (5 mg.mL\(^{-1}\)) was added to the wells and stored for an additional 4 h. After aspiration of the medium, MTT-formation was dissolved in 200 \(\mu\)L of DMSO, and the optical density (OD) values were measured at 570 nm using a microplate reader, Elx808 (Biotek, Winooski, VT).

**In vitro cell imaging studies**

The fluorescence imaging of cells treated with MTX/CDs-PEG was performed by an inverted fluorescence microscope, Bh2-RFCA (Olympus Corporation, Tokyo, Japan). The cells were seeded onto glass coverslips in 6-well plates and cultured in RPMI 1640 medium. After 24 h, the medium was discarded and the seeded cells were washed with PBS buffer. Then, the cells were treated with 2 mL of MTX/CDs-PEG (50 \(\mu\)g.mL\(^{-1}\)) for 3 h. After that, the coverslips were put onto the glass microscope slides and the fluorescent images were captured from stained cells.

**Results and discussion**

**Characterization of CDs-PEG**

As shown in Figure 1(a), FT-IR spectra of the MTX, CDs-PEG and MTX/CDs-PEG have revealed many common characteristics peaks. The most important peaks in the FTIR spectrum of the CDs-PEG can be listed as the stretching vibrations of C¼H at 2938 and 2864 cm\(^{-1}\), the stretching vibration of C¼O at 1742 cm\(^{-1}\), asymmetric and symmetric stretching vibrations of C¼O¼C at 1244 and 1162 cm\(^{-1}\), respectively, and the stretching vibration of N¼H and O¼H bands at around 3430 cm\(^{-1}\). The successful synthesis of CDs with PEG passivation is confirmed by the appearance of new IR peaks around 1732, 1465, 1353 and 1286 cm\(^{-1}\), respectively \[41,42\]. The FTIR spectrum of the MTX/CDs-PEG, Figure 1. Fourier transform infrared spectroscopy (FTIR) of the PEG passivated carbon dots (CDs-PEG), MTX loaded CDs-PEG (MTX/CDs-PEG) and free methotrexate (MTX) (a) and X-ray diffraction (XRD) pattern of the CDs-PEG (b).
revealed not only the characteristic bands of CDs-PEG but also the clear characteristic bands of MTX at around 1620 cm$^{-1}$ (corresponded to the stretching vibrations of $=\text{CONH}$), around 1476–1694 cm$^{-1}$ (corresponded to the stretching vibrations of aromatic pteridine ring and p-amino-benzoic acid) and at around 3415 cm$^{-1}$ (corresponded to the N–H stretching vibrations of amine groups). These observations validated the presence of MTX in the product structure.

The broad diffraction peak detected in the XRD pattern of CDs-PEG at $2\theta = 21.5^\circ$ representing to amorphous carbon phase in CDs (Figure 1(b)).

The morphology and size of the CDs-PEG were evaluated by TEM and DLS analyses, respectively. The TEM images revealed that the CDs-PEG were well dispersed (without any aggregation) with regular spherical morphology and an average size of 6 nm (Figure 2(a,b)). Additionally, the DLS results of CDs-PEG showed a narrow size distribution around 10.6 nm. This distribution and the rather large size of spheres are representing to aggregation of smaller individual dots. The CDs showed the average ZP value of $-7.54$ mV (Figure 2(c)), which is characteristic of CDs. This observed result can be representing the presence of carboxyl and oxygen functional groups on the surface of CDs.

**Spectral characterization of CDs-PEG**

Figure 3(a) displays the UV–vis absorption and PL spectra of the CDs-PEG. The absorption spectrum showed a characteristic weak absorption peak at 290 nm. When it was excited at 290 nm, the PL spectra exhibited a peak position at 430 nm.

In addition, the PL emission of the CDs-PEG is intensely dependent on the excitation wavelength, regularly decreased with the increasing of excitation wavelength. Such an observation is similar to that of QDs, which may be representing the optical selectivity of variously sized NPs (the quantum effect) or various emissive traps on the CDs surface. Moreover, abundant functional groups such as amines and carboxyl acids, which are presented simultaneously during the microwave pyrolysis, may introduce different defects onto the surface of CDs, acting as excitation energy traps and leading to different PL properties.

The QY of CDs-PEG solution was calculated at around 34% using QS as a reference. In a parallel control experiment, if the PEG passivation was eliminated, a similar colour change and UV–vis absorption spectrum were detected. However, these non-passivated NPs presented a poor luminescence emission spectrum (Figure 3(b)). It should be pointed out that the polymeric passivation agent contains no visible or near-UV chromophores and so no emission should be gained at visible wavelengths. Therefore the observed colourful luminescence emission may be owing to the surface-passivated NPs.

**MTX loading and release studies**

The developed nanocarrier was designed to be responsive to the alterations of the pH and temperature of the environment and MTX as a model anticancer drug was loaded in the nanocarrier. The LC and EE percentages of MTX in nanocarrier were calculated at around 32%. Since the weight of incubated drugs is equal to the nanocarrier weight, so it can be concluded that the LE and EE percentages are the same. As shown in Figure 4(a), the states of MTX/CDs-PEG and MTX in solution are obviously detected from the UV–vis spectrum. The MTX solution strongly absorbs UV photons at the wavelengths of 260, 305 and 380 nm owing to the presence of heteroaromatic pterine chromophore. Compared with free
MTX, the absorbance spectrum of MTX/CDs-PEG solution displays three shoulders at the mentioned wavelengths with a blue-shift (a slight shift to the right). So, these results proposed that CDs-PEG and MTX molecules formed the complex in the solution [44,45]. The above-mentioned presumption supported the FTIR spectrum in Figure 1(a), in which it also exposes the strong loading of MTX onto CDs. The in-vitro pH- and temperature-dependent release behaviours of MTX from MTX/CDs-PEG was determined in PBS under normal physiological environment (pH 7.4, 37°C) and simulated tumour tissue condition (pH 5.3, 41°C) (Figure 4(b)). We detected the highest amount of release for MTX (49%) after 48 h at the cancerous tissue environment condition (pH 5.3) which showed that MTX/CDs-PEG complexes have the capability to selectively release in acidic environments. So, these results illustrated that the developed nanocarrier not only improved the site selectivity of the MTX release but also is favourable to prolonged drug release.

In vitro cytotoxicity study

To evaluate the biocompatibility and pharmaceutical activity of CDs-PEG, MTX, MTX/CDs-PEG and MTT cell assay was performed against the MCF-7 cell line (Figure 5) [46]. As the results were shown, the cell viability of nanocarrier was
assessed and no noticeable cytotoxicity of blank nanocarrier in MCF-7 cell lines after 24 and 48 h, confirming that this nanocarrier presented good biocompatibility. By comparing the cytotoxicity results of the free form of MTX with MTX loaded CDs-PEG, MTX/CDs-PEG revealed higher cell cytotoxicity than free MTX after 24 and 48 h. As the concentration increased, the free MTX showed greater cytotoxicity. The main reason for this result was that the MTX release from MTX/CDs-PEG was more slowly than free MTX. The increased cell viability of MTX/CDs-PEG might be a result of the controlled release of MTX from MTX/CDs-PEG, so it was good news for us. As we know, the NPs have EPR effect, which makes tumour tissue enrich more NPs than other tissues. We supposed that MTX/CDs-PEG may reduce the side effects than free MTX, due to their small particle size and shielding effects.

In vitro bioimaging study

The bioimaging applications of the MTX loaded CDs-PEG was assessed based on the excellent fluorescence properties of the nanocarriers. As shown in Figure 6, after the MTX/CDs-PEG was incubated with MCF-7 cells for 3 h, the fluorescence image of the cells was taken by fluorescence microscope. Upon laser irradiation, the cells treated with MTX/CDs-PEG revealed strong blue fluorescent intensity, representing that the MTX/CDs-PEG have a potential for bioimaging applications. Clearly, an increase internalize drug-loaded nanocarrier into the cells was due to the presence of MTX on the surface of developed nanocarrier and the similar structure of MTX with FA which can act as a potential ligand for folate receptors. The mechanism of cellular uptake and capabilities of MTX/CDs-PEG for active drug delivery to the cancerous tissue are shown in Scheme 2.

Conclusions

This article describes a green, facile and novel process to synthesize the PEG passivated fluorescent carbon dots (CDs-PEG) by the microwave-assisted method to fabricate CDs-PEG without any further chemical modification. The as-synthesized CDs-PEG were found to emit strong blue emission with a maximum QY of 34%. The developed CDs-PEG showed an excellent fluorescence property, water solubility and good stability. These CDs did not impose any significant toxicity to cells and might have potential in more biomedical applications. The in vitro results indicated that MTX-loaded CDs-PEG possessed good biocompatibility, bioimaging function and anticancer effect.

Disclosure statement

No potential conflict of interest was reported by the authors.

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