A Ratiometric Fluorescence Sensor for Phospholipase Based on A Novel Nanohybrid

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Abstract. A facile route to prepare fluorescent carbon dots was developed by hydrothermal treatment of sodium gluconate. We further reported the incorporation of doxorubicin molecules into the prepared carbon dots/ phospholipid hybrids that was synthesized by a self-assembly of a phosphatidylcholine monolayer on the surface of carbon dots. And we demonstrated the fluorescence resonance energy transfer (FRET) process between the fluorescence carbon dots and doxorubicin in the nanohybrid. The phospholipase C can catalyze the hydrolysis of phospholipid, which disrupts the carbon dots-phospholipid-doxorubicin complex. Herein, the developed FRET system was utilized as a ratiometric fluorescence sensor for phospholipase C detection in aqueous and bioimaging in breast cancer cell.

1. Introduction
Recently, fluorescent carbon dots (CDs) have attracted considerable attentions in the area of bioimaging and biosensing owing to their superior properties [1]. So far, a series of techniques were developed to prepare CDs such as fragmentation of bulk carbon sources followed by surface passivation [2] or bottom-up strategies synthesizing from some small precursors such as glycerol, glucose, etc. through oxidation or microwave pyrolysis [3]. Phospholipase C (PLC), an enzyme of the phospholipase superfamily, catalyzes the hydrolysis of the phosphate ester bond in phospholipids that is a major component of biological membranes [4]. This hydrolysis reaction induced by PLC are involved in a range of important biological processes, including metabolism, inflammation response, and etc. Furthermore, PLC has been proved to be highly expressed in several tumors, such as breast carcinomas. Therefore, it is necessary to use PLC as a marker for the malignant phenotype tumor. Acid-base titration, nuclear magnetic resonance (NMR) spectroscopy, and chromatography were used for the detection of PLC in cancer cells [5]. However, the low sensitivity, tedious labeling and modification limited their applications. Recently, fluorescence detection was used extensively as an analytical technique for enzyme analysis owing to its simple operation, high sensitivity and real-time detection [6]. However, the reports on fluorescent CDs-based nanosensor for PLC assay are very rare.

In the present study, we first incorporated anticancer drug doxorubicin (DOX) with reddish-yellow fluorescence into the prepared phospholipid modified CDs. And an obvious fluorescence resonance energy transfer (FRET) process from the CDs emission to the absorbance of DOX molecules occurs in the hybrids. The hydrolysis induced by PLC made the DOX release from hybrids, and the FRET process was disrupted that resulted in a significant change of ratiometric emission at 492 and 596 nm respectively. Therefore, CD-based FRET system could be utilized as a ratiometric fluorescence sensor for PLC detection.
2. Experiment Section

2.1. Synthesis of CDs/phospholipid hybrids
The fluorescent CDs were prepared as following: 5 mL of 0.1 mol/L sodium gluconate solution was added into a 5 mL of deionized water solution with vigorous stirring at room temperature. After 10 minutes, the solution was transferred into a teflon lined stainless steel autoclave with a volume of 25 mL. The autoclave was maintained at 160 °C for 2 h and then cooled down to room temperature by a natural cooling process. The obtained yellow-brown CDs solution was centrifuged with a speed of 12,000 rpm to remove the less deposit. The upper yellow-brown solution was dialyzed in membrane tubing with a molecular weight cut-off of 3 KDa against ultrapure water to remove small molecules and ions and then stored at 4 °C.

The CDs/phospholipid hybrid was synthesized via a simple self-assembly process. Briefly, 2 mL of the prepared CDs solution (1.2 mg/mL) was mixed with 8 mL of aqueous solution containing with 5 mg 1,2-didecanoyl-sn-glycero-3-phosphocholine (PCP) in a erlenmeyer flask (25 mL) and the mixture was sonicated for 30 minutes in a bath sonicator. The mixtures were then left to react for 12 h in the dark at ambient temperature to form the fluorescent CDs/phospholipid hybrid. Excess PCP was removed from CDs/phospholipid hybrid by centrifugation. The freeze-dried CDs/phospholipid hybrid were used for further experiments.

2.2 Synthesis of CDs-phospholipid-DOX complex
The CDs-phospholipid-DOX complex was obtained as follows. Tris-HCl buffer solutions (0.1 mol/L, pH 7.4), CDs/phospholipid hybrid (1.0 mg/mL) and DOX solution (0.2 mmol/L) were respectively added into calibrated centrifuge tube at 2:2:5 in volume. Then the solution was diluted to 2 mL with deionized water for another 6 hours incubation. The purified CDs-phospholipid-DOX solution were freeze-dried into powder, and then dissolved in 1 mL aqueous solution again with 0.52 mg/mL mass concentration.

2.3 The CDs-phospholipid-DOX complex as ratiometric sensor for detection of PLC
The PLC detection process was carried out as follows. 20 µL of 0.1 mol/L Tris-HCl buffer solution (pH 7.4), 100 µL prepared CDs-phospholipid-DOX solution (0.52 mg/mL), and different concentration of PLC was respectively added into 200 µL calibrated centrifuge tube. Then the solution was diluted to 200 µL with deionized water followed by the incubation at 37 °C for 90 minutes. The fluorescence emission spectra were recorded and used for quantitative analysis.

2.4 In vitro fluorescence imaging based on CDs-phospholipid-DOX complex
MCF-7 cells were cultured with 1 mL DMEM media containing as-prepared CDs-phospholipid-DOX complex (0.052 mg/mL) at 37 °C for an incubation of 1.5 h. The medium was removed and the MCF-7 cells were washed thoroughly three times with 0.01 mol/L PBS (1 mL each time) and kept in PBS for the optical imaging. Cellular uptake of CDs-phospholipid-DOX complex by MCF-7 cells tracked via Olympus IX73 inverted fluorescence microscope equipped with a DP72 color CCD under blue light excitation. For control sample (MCF-7 cells without being treated with CDs-phospholipid-DOX complex), MCF-7 cells were only treated with DOX (10 µmol/L) in 1 mL DMEM culture solution. After an incubation of 1.5 h, the medium was removed and the MCF-7 cells were washed thoroughly three times with 0.01 mol/L PBS (1 mL each time) and kept in PBS for the optical imaging. Cellular uptake of DOX by MCF-7 cells tracked via Olympus IX73 inverted fluorescence microscope equipped with a DP72 color CCD under blue light excitation.
3. Results and Discussions

3.1 The preparation and characterizations of CDs
In order to obtain fluorescence CDs with plentiful carboxyl groups, we first developed a green route to synthesize CDs by hydrothermal treatment of sodium gluconate. Sodium gluconate, as a nonpoisonous multihydroxyl molecule, offers excellent water-solubility and biocompatibility. As shown in Fig.1A, when the sodium gluconate solution was heating at 160 ℃ for 2 hours, the yellow brown reaction solution was obtained, which produced bright fluorescence under UV light, correlating to the emission peak around 492 nm in the fluorescence emission spectrum excited at 420 nm. Using quinine sulfate as a reference, the fluorescence quantum yields of the prepared CDs was calculated to be 9.3% [7]. Neither strong acid solvent nor surface passivation reagent is needed in the synthetic process, which occurs in aqueous solution and has the advantage of being very cheap and absolutely “green”. The FITR of prepared CDs was provided to confirm the chemical structure of the synthesized CDs (Fig. 1B). The broad O-H stretching vibration (3410 cm⁻¹), the stretching mode of C=O (1700 cm⁻¹), a pair of asymmetric and symmetric stretching modes of C-O-C (1160 cm⁻¹), and C-OH absorption at around 960 cm⁻¹ were all observed in the FT-IR spectra of the CDs. Besides, the C=C stretching vibration in the CDs was found by the absorption band around 1580 cm⁻¹, and 1390 cm⁻¹ which indicated the surface of CDs was partially carbonized during the hydrothermal process. These results demonstrated that the prepared CDs are functionalized with carboxyl groups, which is benefit for further modification on the surface of CDs. The zeta-potential measurements of prepared CDs was determined to be negative when the pH value was 7.4, also suggesting that the surface of the CDs has the carboxyl groups(Fig. 1BInset). The shape and size of prepared CDs was verified by TEM images. Fig.2 showed that the prepared CDs offered spherical shape and a size distribution in the range of 2.4-5.5 nm, which is in agreement with some previous reports.

3.2 Design and principle of the CDs-phospholipid-DOX complex
Phospholipids, as a major component of cell membranes, have amphiphilic structure and favourable biocompatibility. Due to these intriguing properties, phospholipids have been widely used to modify various inorganic nanoparticles for biomedical application including encapsulating and delivering drugs to numerous cell and disease treatment in the clinic. As indicated in Scheme, CDs-phospholipid-DOX complex were prepared using carboxylated CDs, PCP and DOX. CDs/PCP hybrids were prepared through a simple assembly of phospholipid monolayer PCP driven by electrostatic interactions between positive charge top of PCP and

![Fig.1](image-url) (A) The fluorescence emission (solid line) and UV-Vis absorption spectra (dash line) of prepared CDs solution. Inset: the photograph of prepared CDs solution under visible light A and ultraviolet light B. (B)The FT-IR spectra of as-prepared CDs and zeta potential measurements of CDs solution (Inset).
carboxyl groups on the surface of CDs with negative charge. DOX, as a kind of widely used anticancer drugs, can inhibit the proliferation of cancer cells. In this system, DOX molecules could easily inset into the gap of long alkyl tail of PCP molecules by the hydrophobic and vander Waals interactions between alkyl chain of PCP and anthraquinone ring of DOX. Moreover, Fig.4A indicated that DOX molecules offered an obvious absorption peak around 480 nm and there was a large overlap between the absorption spectra of DOX and fluorescence emission peak of prepared CDs. Therefore, we designed an efficient FRET system between CDs/PCP hybrids and DOX molecules. As shown in Fig.4B, a series of different concentrations of DOX were respectively added into the CDs/PCP hybrids solution for 6 hours incubation, and their fluorescence emission spectra were recorded. As the DOX concentration increasing from 0 to 200 μmol/L, the fluorescence emission intensity attributed to CDs/PCP hybrids at 492 nm (F_{492}) gradually weakened, while a new fluorescence emission intensity belonging to DOX around 596 nm (F_{596}) increased obviously. Therefore, two distinctly different fluorescence emission peak at 492 nm and 596 nm make the CDs-phospholipid-DOX complex be a potential ratiometric fluorescence sensor.

3.3 The CDs-phospholipid-DOX complex system as a ratiometric probe for detection of PLC

As described in Scheme, PLC can catalyze the hydrolysis of the phosphate ester bond in the PCP at the glycerol side to produce the hydrophobic diacylglycerol and hydrophilic phosphocholine[8]. As a result, DOX could be simultaneously released from the CDs-phospholipid-DOX FRET system through PLC-mediated hydrolysis of the phospholipids. Fig.5A displayed the fluorescence emission intensity ratio (F_{492}/F_{596}) changes of CDs-phospholipid-DOX complex system with the addition of increasing PLC concentration (0-25μg/mL) incubated for different time. The fluorescence ratio F_{492}/F_{596} gradually increased until the reaction time reached 90 minutes. Fig.5B showed fluorescence emission peak around 492 nm gradually enhanced, while the fluorescence emission peak belonging to DOX at 596 nm decreased obviously with the increase of PLC concentration that was caused by the decomposition of PCP and exit of DOX from the ratiometric probe system. Fig.5B inset showed the relationship between the fluorescence emission intensity ratio (F_{492}/F_{596}) and PLC concentration in the range from 0 to 10 μg/mL could be well described as following linear equation: F_{492}/F_{596}=0.2423+0.0357[PLC] μg/mL. The corresponding regression coefficient R^2 was 0.993, and the detection limit for PLC was 0.5μg/mL with the dynamic range from 1 to 10 μg/mL. Therefore, the CDs-phospholipid-DOX complex was successfully utilized
Fig. 3 Schematic illustration of the synthetic process of CDs-phospholipid-DOX complex and application as a ratiometric probe system for PLC detection.

Fig. 4 (A) The fluorescence emission spectra of prepared CDs solution excited at 420 nm, UV-Vis absorption spectra and fluorescence emission spectra of DOX excited at 480 nm. (B) The fluorescence emission spectra of CDs/PCP hybrids solution in the presence of different concentration of DOX (curves a to k respectively represent the addition of 0, 1, 2.5, 5, 12.5, 25, 37.5, 50, 100, 150, 200 μmol/L).

Fig. 5 (A) The fluorescence intensity ratio ($F_{492}/F_{596}$) changes of CDs-phospholipid DOX complex system as a function of the PLC hydrolysis time. The PLC concentration is respectively 0, 1.25, 5, 25 μg/mL. (B) Fluorescence intensity ratio of CDs-phospholipid DOX complex incubated with different concentration of PLC (0, 1, 2, 3, 4, 5, 10, 25, 50, 75 μg/mL) for 90 minutes. Inset shows the linear plots of $F_{492}/F_{596}$ versus the PLC concentration in the range of 1-10 μg/mL.
as a ratiometric probe for detection of PLC. Fig. 6A and 6B showed the fluorescence microscope photograph of MCF-7 cell incubated with DOX solution. MCF-7 cells just showed weak reddish-yellow fluorescence upon excitation with blue light (460-490 nm). MCF-7 cell cultured with CDs-phospholipid-DOX showed bright reddish-yellow fluorescence under blue light excitation, which indicated that CDs-phospholipid-DOX complex could effectively increase the DOX intake into MCF-7 cell (Fig. 6C and Fig. 6D). The fluorescence signal belonging to DOX molecules were distributed in the cytoplasm and cell nucleus, while the green fluorescence signal belonging to CDs was not observed, which was consistent with the CDs-phospholipid-DOX FRET system in vitro solution.

4. Conclusions
In this study, we firstly demonstrated fluorescent CDs could form a FRET system with phospholipid and anticancer drug DOX. And the prepared CDs-phospholipid-DOX complex can act as a novel ratiometric sensor for PLC detection in aqueous solution. Moreover, the prepared CDs-phospholipid-DOX complex could effectively increase the DOX intake into MCF-7 cell.

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