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Theranostic carbon dots 'clathrate-like' nanostructures for targeted photo-chemotherapy and bioimaging of cancer

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ABSTRACT

The drug delivery application of carbon-based quantum dots is a new platform in the medical field. We report a unique morphological structure called carbon-dots clathrates (C-dots$_{CL}$) conjugated with folic acid (FA) for targeted delivery of methotrexate (MTX), an anti-cancer drug under physiological conditions followed by photothermal (PTT) and photodynamic (PDT) treatment on cancer cells. Under physiological milieu, the C-dots$_{CL}$–MTX–FA complex are pH-dependent and it can control release the anti-cancer drug *in vitro*. Furthermore, *in vivo* studies were also performed by using genetically-induced pancreatic cancer model to comprehend the *in vivo* pharmacokinetics and tissue distribution pattern of C-dots$_{CL}$–MTX–FA complex. The results demonstrated that the CQD was able to deliver high concentration of MTX in tumour tissues in contrast to the non-tumour tissues. The impact of near-infrared (NIR) laser (1064 nm; Nd-YAG) on the CQDs system was found to be extremely effective for the rapid release of MTX in tumour tissues. The generation of reactive oxygen species (ROS) by C-dots$_{CL}$ upon irradiation of NIR laser was evaluated using flow cytometry. After conjugation with the C-dots$_{CL}$, the half-life of the MTX drug ($t_{1/2}$), elimination constant (K_{el}), area under the curve (AUC) was drastically improved to prove the efficacy of C-dot$_{CL}$ as a powerful drug vehicle. This study has significantly improved the cancer theranostics research using C-dots$_{CL}$ to act as a multi-functional nanomaterial for imaging and as a drug delivery agent.

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Introduction

10–18 Over the past several decades, cancer has been an atrocious disease and need an utmost attention to decrease the tremendous rate of mortality. Unpredictable bio-physiological and molecular biological alterations during the onset of progression of the cancers make their diagnosis and treatments extremely intimidating task [1]. One of the major concerns in cancer chemotherapeutics is the delivery of the active pharmaceuticals within the microenvironment of the tumours [2] principally due to the altered or aberrated surrounding. These aberrations result due to metabolic and structural changes that occur during cancerous transformation making the cancer therapy difficult task [3,4].

19–35 With the advancement of medical nanotechnology, novel nanoparticle became crowned materials for diagnosis and treatment of a variety of cancers [5]. Carbon dots (C-dots) [6] has been a powerful material for delivery of drugs to the solid tumour [7]. Particles in nano-regimes can evade cardinal issues such as poor mobilisation of the drugs to body fluids, killing non-specific innocent cells (mainly due to lack of targeting), tumour resistance to anti-cancer drugs, permeation and retention (PR effect) of drugs in the cancerous milieu [8]. Recently, C-dots are favourite materials for biological applications due to their unique properties such as inherent fluorescence, extremely high biocompatibility and facile protocols for their synthesis [9–16].

These unique properties allowing the C-dots to be emerged as an alternative to the conventional hostile metal-containing

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semiconductor quantum dots such as CdTe, CdS and CdSe QDs [10]. The strong absorption of light in near-infrared (NIR) is advantageous for photothermal therapy (PTT) of solid tumours [17,18]. Also, their intense fluorescence and high quantum yield enables them to be applied for biological imaging [13,19,20].

Synthesis of porous carbon particles can be an alternative to this provided certain issues such as aggregation in the solution and larger size (*c.a.* 200 nm) and considerable high hydrophobicity which makes their administration in the cells almost impossible [21]. Most of the protocols available for synthesis of porous carbon nanomaterials involve a cumbersome template-assisted method in multiple steps [22,23]. Unlike mesoporous silica, a widely-used nanomaterial for drug delivery, porous carbon nanomaterials can have higher surface areas and pore size along with lower inimical effects on biological systems [24,25].

We report a novel fluorescent mesoporous C-dots clathrate (C-dots$_{CL}$) using cetyl-trimethylammonium bromide (CTAB) as a templating agent using hydrothermal pyrolysis of widely used precursor citric acid. In contrast to the porous carbon nanoparticles which often pose two fundamental issues: first, relative hydrophobicity and second, non-uniformity in size and shape, C-dots$_{CL}$ were found to be extremely dispersible in water and their size was also confined to <100 nm. On the surface of these porous C-dots$_{CL}$, a potential anticancer drug methotrexate (MTX) was attached along with folic acid (FA) as a targeting molecule to make C-dots$_{CL}$–MTX–FA complex. Due to the high affinity of FAs towards folic acid receptors (FARs) overexpressed on a variety of cancer cells, the FA–FAR interaction is one of most widely used targeting strategies in cancer drug delivery [3]. MTX is a widely used antineoplastic drug widely used for the treatment of advanced human malignancies related with acute lymphoblastic leukaemia, osteosarcomas, breast cancers and metastatic lymphomas [26]. The MTX is an antagonist of FA (an essential pre-requisite for DNA synthesis) and is considered as a potential therapeutic drug against many types of cancers having an overabundance of FARs on their surfaces. This C-dots$_{CL}$ has an unique feature of high absorbance in NIR regime, which is most penetrating and safe radiation that can be used for photothermal therapy of cancer cells [13,27]. The cell cytotoxicity as performed on breast cancer stem cells (BCSCs), which often remain as a major reason for the recurrences of cancers after chemotherapy or surgery [28]. Furthermore *in vivo* biological distribution of drug and regression of tumour mass was analysed following photothermal and chemotherapy treatment.

Experimental

Materials

All the solvents used in this experiment were of high purity and used as per instruction of materials data safety sheets. The CTAB, MTX, and poly (ethylene glycol)-2-aminoethyl ether were purchased from Sigma–Aldrich (St. Louis, MO, USA). The breast cancer stem cells (HMLER$^{sh\ E\text{-}cadherins}$) were kindly provided by Prof. Robert Weinberg, MIT and American Cancer Society, USA. The Dulbecco's modified Eagle's medium was purchased from Thermo Scientific, USA (DMEM, HyClone® containing 4 mM/l L-glutamine, 4500 mg/l glucose) fetal bovine serum (FBS) were purchased from Gibco, USA. Phosphate buffer saline (PBS), trypsin-EDTA solution and pen-strep solution were purchased from Lonza, Belgium. 2′,7′-Dichlorofluorescin diacetate (DCFDA) was purchased from Sigma–Aldrich, USA.

Characterization

Spectral properties of the C-dots$_{CL}$ were studied by UV–vis–NIR spectrophotometer (Lambda-25, PerkinElmer, USA) and fluorescence spectrophotometer (PerkinElmer, USA). The morphological details of C-dots were using High-resolution transmission electron microscopy (HRTEM; Zeiss Microimaging GmbH, Germany). The X-ray Diffraction was carried out using XRD; Phillips. The Raman spectra were recorded using Jobin–Yvon Labram spectrometer and the Fourier Transformed Infrared (FTIR) Spectroscopy (Brucker) studies were performed within the spectral window 500–4000 cm^{-1}. The dynamic light scattering (DLS) and zeta potential studies (pH 6.5–7) were performed using Malvern Zetasizer.

Template-assisted synthesis of C-dots$_{CL}$

For the synthesis of C-dots$_{CL}$, 2 g of citric acid was dissolved in 10 ml of poly (ethylene glycol) 2-amino ethyl ether and mixed well by ultrasonication (Cole-Parmer 130-W Ultrasonic Processors) for 20 min at ambient temperature (25 ± 3 °C). To this mixture, 0.1 g of CTAB (100 mM) was added followed by 5 ml of NaOH (0.1 N), under continuous stirring conditions for 1 h. The mixture was sealed in a Teflon-coated container in a stainless-steel autoclave container and then heated at 200 °C for 12 h [13]. The resulting (clear) yellow coloured solution was washed or centrifuged (20,000 rpm, 25 min) thrice and the supernatant was discarded. This ensured the removal of almost all the unreacted CTAB. The solution was then passed through ammonium chloride saturated Sephadex G-100 column and eluted with nano-pure water for 8 h to further remove any minute residual CTAB from C-dots$_{CL}$. An intense blue coloured fluorescence was exhibited by C-dots$_{CL}$ upon observation under UV light (λ_{ex} = 365 nm). This was considered as the most fundamental confirmation of C-dots [11–14,18]. The C-dots$_{CL}$ powders were obtained by vacuum heating at 80 °C for 12 h and the appropriate concentration was obtained by dissolving them in nanopore water and the pH value of the final solution was maintained at pH 7.0 and stored at 4 °C.

Attachment of methotrexate (MTX) on C-dots$_{CL}$

The loading of MTX onto CQDs was done by the following procedure. Briefly, 10 ml of the purified C-dots$_{CL}$ solution (200 μg/ml) was mixed with 5 ml of MTX (0.5 M in 1 M NaOH) and stirred for 3 h at ambient temperature (25 ± 3 °C) under continuous nitrogen purging to avoid external interferences. The C-dots$_{CL}$-MTX solution was washed using nanopure water with multiple round of centrifugation (20,000 rpm, 25 min) to remove any unanchored MTX. The final solution was purified by dialysis against pure water (Mili Q, 18.2 MΩ, USA) for 48 h and freeze-dried.

Formation of C-dots$_{CL}$–MTX–FA complex

The FA was added into 20 ml of DMSO (1.25%) and the mixture was subjected to sonication for 1 h. The carboxylate group of FA was activated by addition of *n*-hydroxysuccinamide (NHS, 225 μg) and *N*,*N*-dicyclohexylcarbodiimide (DCC, 125 μg) to the above solution. The reaction was performed in an inert environment by purging N_2 gas at 25 ± 2 °C for 12 h (FA/NHS/DCC molar ratio was kept at 2:2:1). Attachment of FA is facilitated by adding 15 ml of C-dots$_{CL}$–MTX and 5 ml of activated FA (3:0.1 w/v) and the solution was further subjected to purging with N_2 gas (6 h) under constant stirring using magnetic stirrer (150 rpm) and further incubated in dark for 24 h. The resultant solution was centrifuged at 20,000 rpm for 25 min. The pellet was re-dissolved in appropriate volume of nanopure water and stored at −20 °C. Free FA was removed using a 3000 kDa dialysis bag against phosphate buffer saline (PBS, pH 7.2).

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