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Herceptin-Polymer corona around near-infrared fluorescent carbon dots: A model for Immunofluorescence imaging of MCF7 cancer cells

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Abstract

Fluorescence imaging of cancer cells is a recently emerging technology which is becoming a complementary biomedical method for cancer diagnosis. In this work, we report on an enhanced multi-color fluorescence imaging of MCF7 breast cancer cell using near-infrared emitting carbon dots (CDs) embedded in a hydrophilic (polymer-antibody) corona. For this purpose, mono-dispersed low molecular weight poly(aspartic acid) (PASP) was prepared via a simple and cost-effective procedure. The prepared poly(aspartic acid) was characterized by FTIR, 1HNMR, DSC and gel-permeation chromatography. PASP acid was attached to the specific monocolonal antibody, Herceptin (HER). Ultra-small CDs (~ 1 nm) was prepared and their surface was coated with poly(ethylene glycol) diamine (PDA). Finally, (PASP-HER) conjugate was attached to PDA at the surface of CDs via carbodiimide chemistry producing the highly fluorescent (CDs-PDA-PASP-HER) conjugate. The prepared immunofluorescence conjugate was characterized by UV-Vis absorption spectroscopy, fluorescence spectroscopy, transmission electron microscope (TEM), dynamic light scattering (DLS) and zeta-potential measurements. The prepared conjugate exhibited an enhanced binding capacity to the surface of MCF7 cells which express HER2 receptors. The prepared immunofluorescence conjugate demonstrated a multimodal fluorescence behavior at blue, green and red regions of light. It can be concluded that this preliminary study may encourage biochemists and oncologists to conduct further research to translate the prepared fluorescence probe into in-vitro and in-vivo applications.

Keywords: Poly(aspartic acid); poly(ethylene glycol); carbon dots; breast cancer; immunofluorescence.

1. Introduction

Many pathways have been developed for synthesis of poly(aspartic acid) (PASP) [1]. This anionic polypeptide is featured with versatility, biocompatibility, and biodegradability and process many advantages for use in a wide variety of biomedical applications [2]. PASP is a modular polyelectrolyte that shows positive biomedical criteria such as bone-tissue targeting and prolonged blood circulation time [3]. PASP can be further functionalized for production of applicable biomaterials [4]. Several strategies for fabrication of nanostructured PASP-based hydrogels have been developed [5]. A vast number of cross-linking agents such as diamines, dopamine, and amino-functionalized silanes are also known. PASP hydrogel can be produced via miscellaneous methods such as crosslinking reactions or thermal curing of a freeze-dried mixture composed of PASP and poly(ethylene glycol) diepoxide in aqueous medium [6]. PASP has been utilized in production of pH sensitive polymeric smart nanocarriers to deliver hydrophobic drugs specifically to solid tumor [7]. Carbon dots (CDs) are biocompatible fluorescent species that have superior properties such as colorful photoluminescence and multi-colour emission by a single-light source excitation. CDs are considered as a green substitute of the classical metal-based semiconductor quantum dots (QDs) [8]. Recently, polymeric species have become an important materials for synthesis and modification of fluorescent dots to provide polymeric matrix and enhance their photoluminescence [9]. There are many review papers on incorporation of carbon dots into polymeric matrices such as polymeric gel [10], molecularly imprinting polymers [11], and polymer composite films [12]. The high fluorescence quantum yields of CDs make polymers more efficient in many fields such as cancer imaging and drug delivery. HER2-positive breast cancer is a breast cancer that

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abnormally expresses human epidermal growth factor receptor 2 (HER2) [13]. HER2 amplification is the primary pathway of HER2 receptor overexpression and is a major driver of tumor development and progression in a subset of breast cancers. HER2 is amplified in about 15% to 20% of breast cancers. The overexpressed HER2 receptor is a valuable therapeutic target. [14,15]. Anti-HER2 antibody conjugated to (CdSe/CdZnS) quantum dots (HER2Ab-QDs) has been prepared using different coupling agents [16]. The prepared QDS and (HER2Ab-QDs) showed fluorescence quantum yields of 0.23~0.39 in aqueous solution. Trastuzumab, brand name "Herceptin" is a monoclonal antibody that represents the first defense line in immunotherapy of breast cancer [17]. Herceptin targets cancer cells which expresses HER2 receptor positive. Trastuzumab works by binding to the HER2 receptor and slowing down cell replication [18]. In this study, we conducted direct conjugation of Herceptin antibody (Trastuzumab) with the surface of fluorescent carbon dots using carbodiimide chemistry, poly(aspartic acid) and poly(ethylene glycol) diamine for direct detection of HER2 receptors without the need for a secondary antibody, as is required for indirect detection methods [19].

2. Materials and Methods

2.1 Materials

Thionyl chloride (99.7 %) was a product of Thermo Scientific. All other chemicals were purchased from Merck, Germany. Sterile Milli-Q ultrapure water (UPW) of resistivity 18.2 MΩ·cm at 25 °C was used throughout the work. Trastuzumab (Tra, 5 µg/ml) was a product of Roche, Switzerland.

2.2 Methods

Ground state electronic absorption spectra were recorded at room temperature in the range from 200-1200 nm using JASCO V-730 UV-Visible Spectrophotometer. FTIR spectra were recorded on an infrared spectrometer (type JASCO FT/IR-430, Japan) using the KBr disc technique. Transmission electron microscope (TEM) images were recorded on a JEM-2100, Jeol electron microscope. Dynamic light scattering (DLS) instrument (PSS, Santa Barbara, CA, USA), using the 632 nm line of a He-Ne laser as the incident light with angel 90º and Zeta potential with external angel 18.9º. ^{1}HNMR spectra were recorded on Bruker 400 MHz NMR using d6-DMSO as a solvent. Gel-permeation chromatography (GPC) measurements were carried out using Agilent 1100 series, gel permeation chromatography, Germany equipped with refractive index detector using DMF as an eluent and calibrated PL 5 µm, (100, 104, 105 Å) on series of columns against polystyrene standard. Differential scanning calorimetry (DSC) measurements were carried out under nitrogen on SDT Q600 V20.9 Build 20 with heating rate 10 °C.min^{-1}. Fluorescence spectra were recorded on JASCO FB-8350 Spectrofluorometer. Leica Fluorescence Microscope was used for fluorescent imaging of cells.

2.3 Synthesis of low molecular weight poly(aspartic acid)

Firstly, 1g of maleic anhydride was dissolved in 5 mL of dry DMF and the solution was transferred to a Teflon lined hydrothermal autoclave made up of two parts; outer high-quality stainless steel jacket and inner Teflon chamber. Ammonia gas was bubbled for 10 min. After heating the closed system at 95 ºC for 15 min, the system was opened and cooled to room temperature. The solid was precipitated by addition of ethyl alcohol and filtered off. The solid then washed by ethyl alcohol and dried under reduced pressure. The solid was then transferred to a sublimation system and heated at 140 ºC under reduced pressure (0.5 mm Hg) for 20 min. Finally, the temperature was elevated to 200 ºC for 30 min. It is noteworthy to mention maleic anhydride sublimes as a white solid at the sublimation finger. Poly succinimide (PSI) was collected as an orange product in the bottom of the sublimation system. It is noteworthy to mention that maleic anhydride sublimes as a white solid at the sublimation finger. Poly succinimide (PSI) was collected as an orange product in the bottom of the sublimation system. PSI was purified by dissolving in DMF and re-precipitation with THF. Purified PSI was then dried under vacuum 40 ºC for 2 h. Poly(aspartic acid) (PAA) was prepared from PSI by alkali hydrolysis. PSI (2g) was dissolved in double distilled water and the container was placed in an ice bath (0-4 ºC). A solution of sodium hydroxide (1 M) was then drop-wise added till pH of the solution reaches 10. The hydrolysis process was continued at 40 ºC for 1 h. Acetic acid (2%) was then drop-wise added to reduce pH of the solution to 3. The resulting amber colored solution was then dialyzed versus water (dialysis bag, MWCO 2000 Da) for three days to remove sodium acetate and other low molecular weight impurities. The amber colored clear solution was freeze-died to give PAA powder.

2.4 Characterization of PASP:

FTIR (KBr): ν (cm^{-1}) (Fig. 1) = 569, 633, 736, 755, 910, 1061 (C-O stretch), 1156, 1306 (C-N stretch), 1385, 1488, 1690 (C=O amide), 1773(C=O carboxylic), 2350, 2926 (CH aliphatic), 3212 (NH), 3445 broad (carboxylic OH). ^{1}H-NMR (400 MHz,

D_2O, δ in ppm) 2.65, 2.71 (Fig. 2). DSC (Tm) 309.5 ºC (Fig. 3). GPC (DMF) (Mw ~ 17300, PDI = 1.14).

2.6 Preparation of ($H_2NPEGNH_2$) (PDA)

Poly(ethylene glycol) diamine, abbreviated here as PDA, was prepared according to previous literature [20] using thionyl chloride and sodium azide method (Scheme 1).

2.7 Preparation of near-infrared emissive carbon dots

Nitrogen doped carbon dots were prepared from citric acid and purified according to literature [21]. Concentration of carbon in aqueous carbon dots solution was determined by freeze-drying of a certain volume of the solution and accurate weighing of the residual solid content. The whole process of this study was carried out under dark conditions to prevent fluorescence quenching.

2.8 Synthesis of (CDs-PDA-PASP-HER) fluorescent nanoprobe

Herceptin (trastuzamab) antibody was covalently attached to the carboxylic groups in PAA via carbodiimide chemistry. Firstly, the carboxylic groups in PASP were activated as follows: an aqueous solution containing 100 mg of PASP, 20 mg of EDC and 20 mg of SNHS was stirred in dark at 4 ºC for 3 h. Trastuzamab (25 µg in 100 µL ultra-pure water) was added and the mixture was further stirred in dark at 4 ºC for 1 h. Carbon dots (30 µg dispersed in 200 µL) was then added and the mixture was stirred in dark at 4 ºC for 12 h. (CDs-PDA-PASP-HER) conjugate was separated by centrifugation (10,000 rpm, 4 ºC). Excess polymer and reagents were removed by sonication-assisted dispersion in UPW followed by centrifugation. The samples were finally lyophilized and stored in dark at 4 ºC for further use.

2.9 Determination of the bound antibody

The concentration of antibody bound to carbon dots was determined by a spectrophotometric method by analysing the supernatant for the unbound antibody by monitoring the absorbance at 280 nm as previously reported [22].

2.10 Cell culture and sample preparation for fluorescence microscopy

Culturing method of HER2-overexpressed MCF7 cells have been reported [23,24]. In brief, those cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, HYCLONE) and 1% penicillin streptomycin (PS, GIBCO). Labelling of the cells with carbon dots and conjugates was carried out according to standard protocol [25]. Both CDs and the conjugate were tested for a wide range of carbon concentrations and the best results were obtained for a concentration of 10 µg/mL.

3. Results and discussion

There is an increasing demand for building novel diagnostic models that recognize cancer cells only. Poly(aspartic acid) is water soluble and is considered as a 'green' (non-toxic, non-antigenic and biodegradable) polymer substitute for poly(acrylic acid) or poly(vinyl alcohol) which are poorly recovered after use [26]. Polymeric Antibody Conjugated Nanoparticles (ACNPs) are known to improve both diagnosis and Treatments in Breast Cancer [27]. The cancer marker HER2 is overexpressed in the surface of MCF7 breast cancer cells and thus can be efficiently utilized in fluorescence and surface enhanced raman spectra (SERS) imaging of these cancer cells [25]. Lee et al. [28] reported on SERS imaging of HER2 cancer markers abnormally expressed in MCF7 cells using antibody conjugated gold nanoshells.

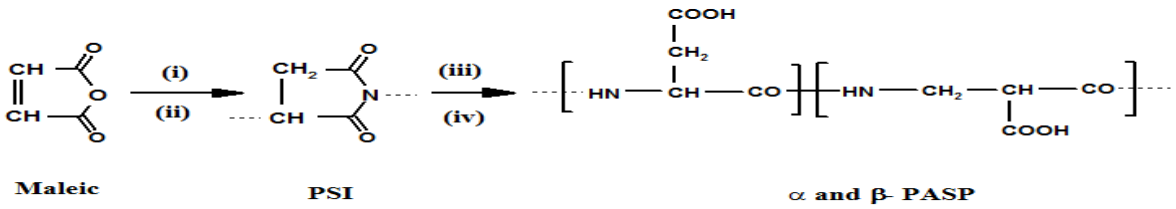
3.1 Preparation of PASP

In this work, a simple and cost-effective procedure was proposed for preparation of mono-dispersed low molecular weight poly(aspartic acid) (Scheme 1). Thermal decomposition of maleic anhydride in DMF previously saturated with ammonia gas resulted in formation of PSI as a brittle orange solid. Controlled alkaline hydrolysis of PSI resulted in formation of sodium salt of Polyaspartic acid. Acetic acid was then used for production of carboxylic ended PASP which is used as a centre for further functionalization with the antibody. Compared to previously published procedures for synthesis of PASP from maleic acid [1], the proposed procedure is characterized with pure product, significantly reduced reaction time and temperature in addition to production of mono-dispersed and low-molecular weight poly(aspartic acid). The prepared PASP was characterized using FTIR, ^{1}NMR, GPC and DSC.

As shown in Fig. 1 and listed in the experimental part, the most characteristic FTIR stretching vibrations of PASP are ($\nu_{C=O}$, amide) at 1690 cm^{-1}, ($\nu_{C=O}$, carboxylic) at 1773 cm^{-1}, (ν_{NH}) at 3212 cm^{-1} and (ν_{OH}) at 3445 cm^{-1}.

Gel permeation chromatography (GPC) of PASP using DMF as mobile phase showed nearly mono-dispersed and low average molecular weight polymer (Mw ~ 17300 Da, PDI = 1.14). ^{1}H-NMR spectrum of PASP (400 MHz, D_2O, δ in ppm) showed two peaks at 2.65 ppm (CH_2) and 2.71 (CH) (Fig. 2)

[6]. A relatively small peak at 2.71 ppm of CH_2 was masked with the strong peak of D_2O at (2.67), however, magnification of this part of the spectrum demonstrated the two peaks (Fig 2. Inset). Differential scanning calorimetry (DSC) of the prepared PASP is shown in Fig. 3. The thermograph exhibited an endothermic peak centred at 309.5 ºC assigned to melting of the polymer [29].



Scheme 1. Synthesis of poly(aspartic acid). (i) DMF, NH3 gas, 95 ºC/ 15 min; (ii) thermal treatment; (iii) NaOH/ 40 ºC, 1h; (iv) AcOH pH=3.

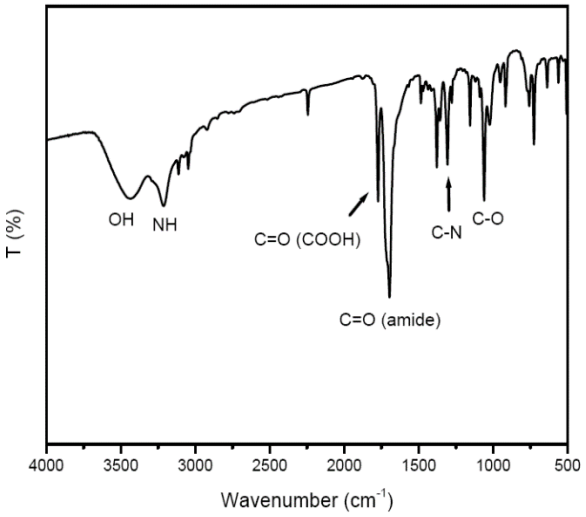


Fig. 1. FTIR spectra of the prepared poly(aspartic acid).

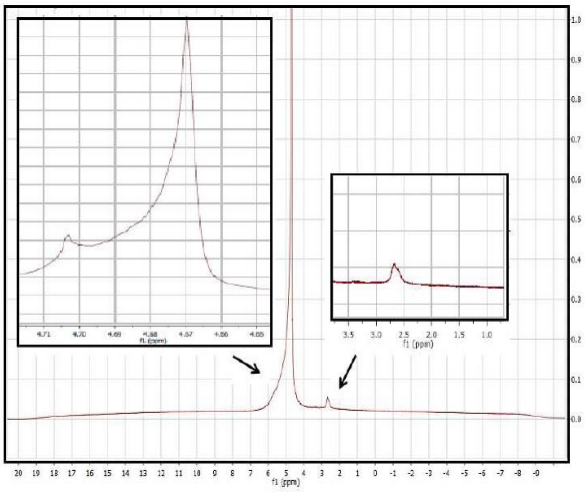


Fig. 2. ^{1}HNMR spectrum of the prepared poly(aspartic acid).

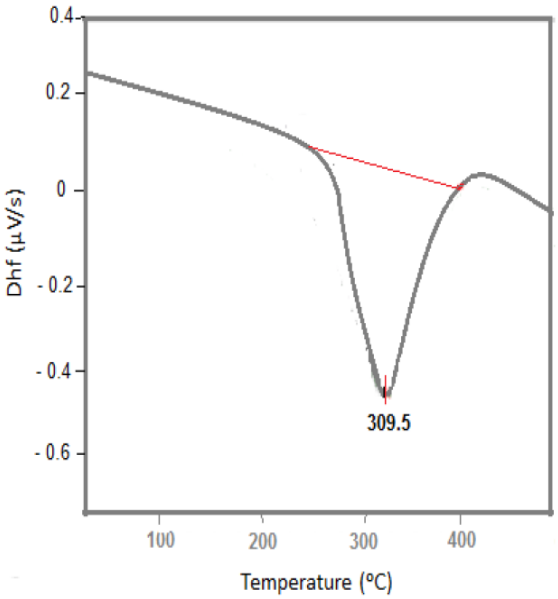


Fig. 3. DSC thermograph of the prepared poly(aspartic acid).

3.2 Carbon dots

A stable colloid of citrate-capped near-infrared emissive carbon dots (Fig. 4) was prepared and characterized according to literature. The prepared CDs showed two absorption bands cantered at 354 nm and 635 nm (Fig. 5). The prepared carbon dots exhibited multimodal fluorescence behaviour at blue, green and red regions of light at different wavelengths (Table 1). Transmission electron microscope image of the prepared CDs demonstrated ultra-small spherical particles of a diameter of about 1 nm (Fig. 6). Dynamic light scattering (DLS) measurements of the prepared CDs revealed a hydrodynamic diameter of ~ 8.6 nm, while zeta potential measurements revealed a negative surface charge of -11 mV (Fig. 10).

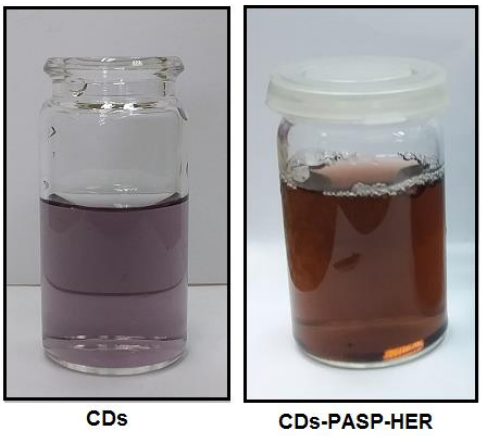


Fig. 4. A photo of the prepared red-emissive CDs (link) and (CDs-PDA-PASP-HER) conjugate (right).

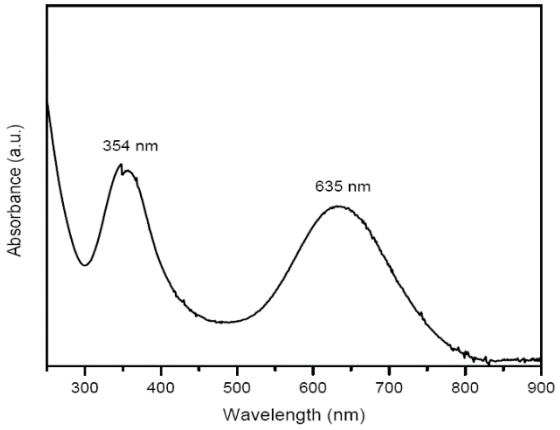


Fig. 5. UV-Vis. absorption spectrum of red-emissive CDs in UPW.

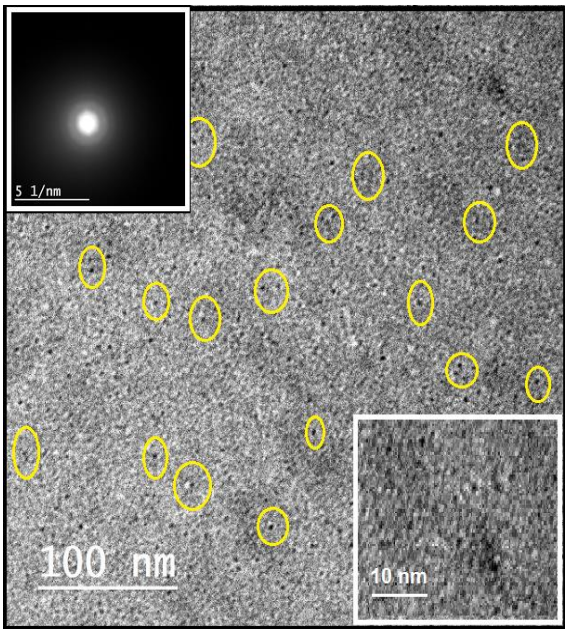


Fig. 6.TEM image of red-emissive CDs; upper inset: electron diffraction pattern, lower inset: high magnification).

3.3 Production of (CDs-PDA-PASP-HER) immunofluorescent conjugate

The carboxylic groups of PASP (pH=3) was activated via carbodiimide chemistry producing NHS ester of PASP. When, pH of the solution is raised to 5.5 in the presence of the antibody, NHS ester is readily hydrolyzed and a covalent bond is formed between the amino group of lysine residue of the antibody and the carboxylic groups of PASP [30]. The non-stoichiometric ratio between PASP and the antibody (~ 10 equivalents) leaves a large number of free carboxylic groups in the polymer backbone. These groups can be attached to the carboxylic groups present at the surface of CDs via crosslinking with PEG diamine (PDA). This led to production of a nanoprobe where carbon dots (~ 1nm) are highly dispersed in the polymer corona and retain their red-emissive property. The presence of antibody molecules dispersed in the matrix led to effective attachment of the nanoprobes to the surface of MCF7 cells, where HER2 are overexpressed.

Polymeric conjugation to the prepared CDs was found to slightly enhance the fluorescence at different excitation wavelengths (Fig. 7). As the CDs do, the conjugate exhibited a multi-color emission behavior at different excitation wavelengths (Fig. 8). TEM image of the conjugate (Fig. 9) showed a polymeric corona formed around the carbon dots. DLS measurements of CDs in the corona revealed a markedly increased hydrodynamic diameter (~ 42 nm) (Fig. 10). Zeta-potential measurements of the conjugate showed a negative surface charge of (-8 mV), probably due to the yet free carboxylate groups at the surface of CDs and those of PASP.

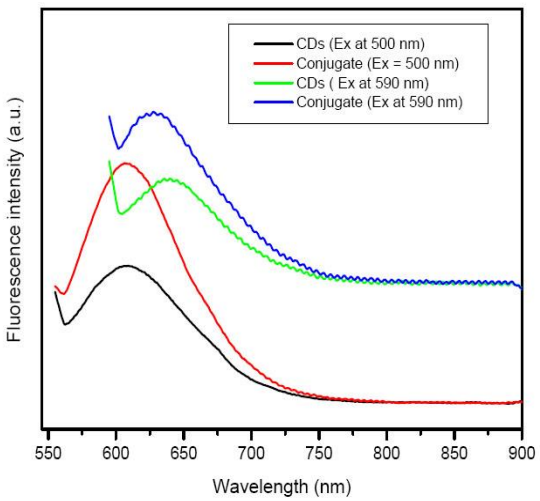


Fig. 7. Enhanced fluorescence of (CDs-PDA-PASP-HER) conjugate compared to spectra of aqueous solution of CDs,

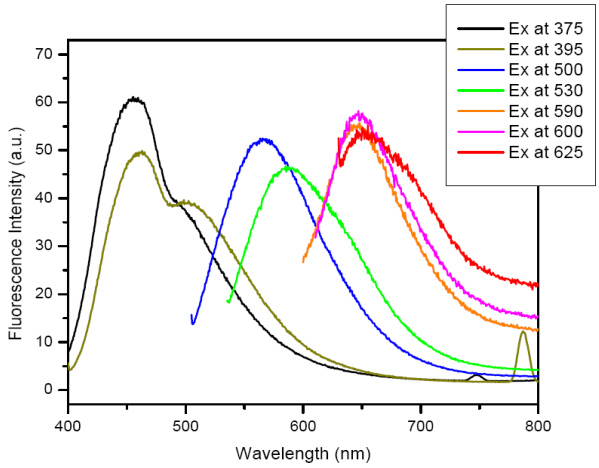


Fig. 8. Multi-colored emission of (CDs-PDA-PASP-HER) conjugate under different excitation wavelengths.

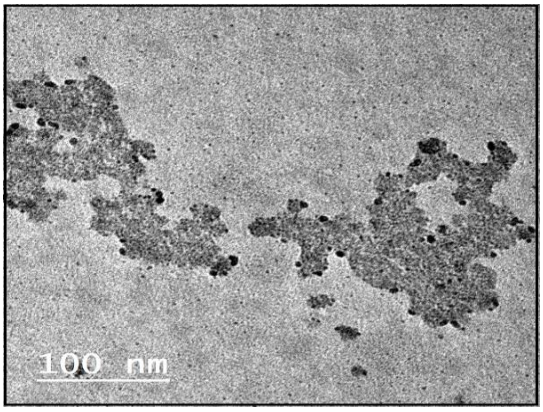


Fig. 9.TEM image of (CDs-PDA-PASP-HER) conjugate.

Table 1 Excitation and emission data of the prepared CDs and (CDS-PDA-PASP-HER) conjugate

	Wavelength (nm)						
Excitation	375	395	500	530	590	600	625
Emission CDs	455	463, 503	565	585	648	647	655
Emission CDs-PDA-PASP-HER	459	463, 506	566	588	649	649	657

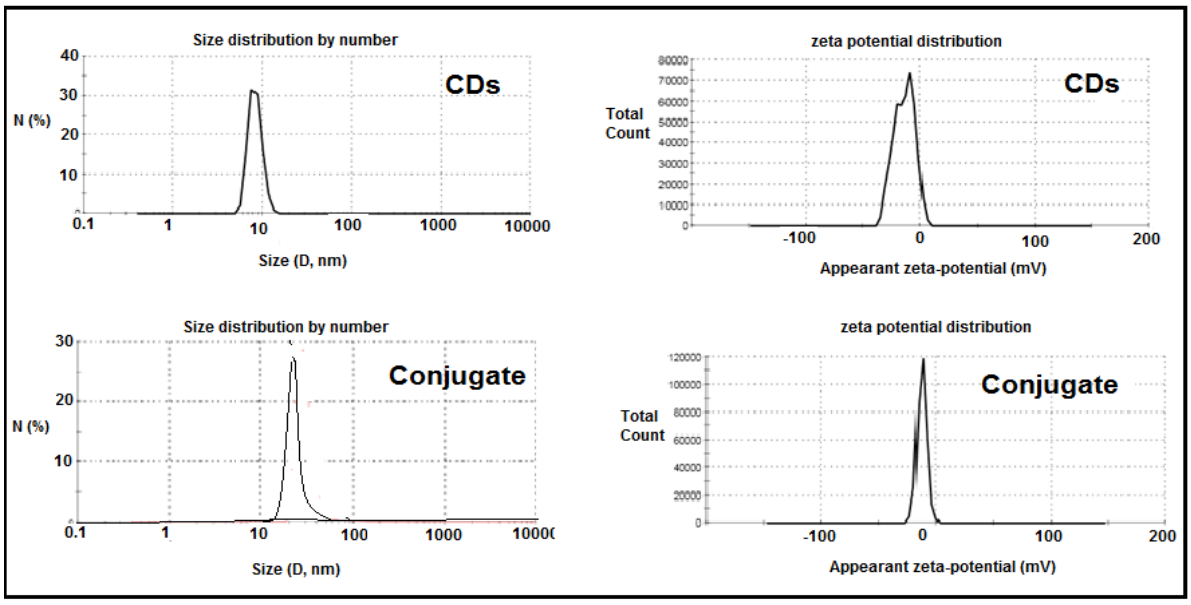


Fig. 10. DLS and zeta-potential of CDs and (CDs-PDA-PASP-HER) conjugate.

3.4 Fluorescence imaging of MCF7 breast cancer cells

HER2 expressed MCF7 breast cancer cells were incubated with the prepared immunofluorescence probe for 3 h followed by rinsing with PBS. The stained cells were imaged with fluorescence microscope in the blue, green and red regions. The conjugate was able to visualize the HER2 expressed MCF7 breast cancer cells as a multi-color fluorescence model (Fig. 11 A-C). The anti-HER2 antibody is responsible for the binding of the fluorescent probe to the surface of the cells. Fluorescence images of stained cells in the blue, green and red regions are shown in Fig. 11 (A,B,C respectively).

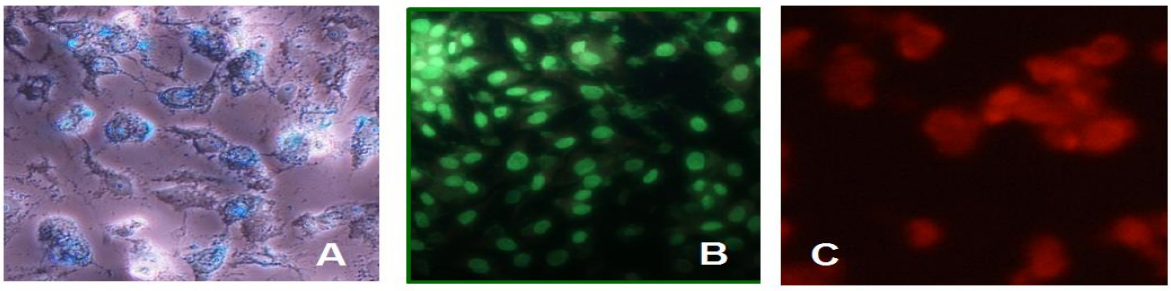


Fig. 11. Fluorescence microscope image of MCF7 cells after incubation with (CDs-PDA-PASP-HER) fluorescent conjugate (A: λex = 500 nm); (B: λex = 530 nm); (C: λex = 625 nm).

4. Conclusions and outlook

In this study, the ultra-bright fluorescent carbon dots were conjugated to anti-HER2 antibody targeting HER2 expressed at the surface of MCF7 breast cancer cells. The attachment was simply achieved via two biocompatible polymers, namely poly(aspartic acid) and poly(ethylene glycol) diamine. This is considered as a cost effective procedure compared to the conventional conjugation methods which uses two types (primary and secondary) antibodies. The prepared conjugate exhibited multi-colour emission behaviour and was used for fluorescence imaging of MCF7 cells in different regions of light. However, extensive studies should be done by the specialists before translation of this probe in biomedical applications such as in-vivo imaging of cancer cells and estimation of expressed genes in cancer cells.

Declaration of interest

The authors report no conflict of interest.

Acknowledgement

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