

**Methotrexate-conjugated quantum dots: synthesis, characterisation and cytotoxicity in
drug resistant cancer cells**

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Determination of the size and concentration of CdSe nano-crystals by UV-Vis spectroscopy

As reported previously in the supporting information of reference (Johari-Ahar et al. 2015), the first excitonic absorption peak value (λ), indicated in the Uv-Vis absorption spectrum, was employed for determining the diameter of QDs using the empirical equation (1). The calculated diameter was used for the determination of molar particle extinction coefficient (equation 2). Finally, the concentration of QD nanocrystals was estimated by Lambert-Beer's law (equation 3) in which A (a.u.), ϵ (L/mol.cm), C (mol/L) and L (cm) are the absorbance at the first excitonic absorption peak, the extinction coefficient per mole of CdSe nanocrystals, the molar concentration of the QD nanocrystals, and the path length (1 cm) of the radiation beam respectively. During the synthesis of CdSe QDs, different-sized nanocrystals with tunable fluorescence emission were obtained through increasing the reflux time from 1 h to 8 h. More detailed explanation for the background of these measurements is available from the paper previously published (Yu et al. 2003).

Equation (1):

$$D = (1.6122 \times 10^{-9})\lambda^4 + (2.6575 \times 10^{-6})\lambda^3 + (1.6242 \times 10^{-3})\lambda^2 - (0.4277)\lambda + (41.57)$$

Equation (2): $\epsilon = 5857(D)^2 + 65$

Equation (3): $A = \epsilon CL$

Coupling efficiency determination

In the case of MTX-conjugated QDs, the concentration of EDC and NHS used for the conjugation reaction showed profound effect on the quantum yield of conjugated QDs; therefore, various amounts of EDC and NHS were tested for attaining the optimised concentration of these coupling reagents.

Table S1 Effect of the coupling reagents (EDC and NHS) on the optical properties of QDs

QD (mg)	EDC (20 mg/mL) (μ L)	NHS (10 mg/mL) (μ L)	Initial fluorescence intensity (%)
10 ± 0.3	0	0	100 ± 4 (no significant change)
10 ± 0.8	30	30	100 ± 6 (no significant change)
10 ± 0.6	40	40	100 ± 11 (no significant change)
10 ± 0.9	50	50	100 ± 7 (no significant change)
10 ± 0.2	60	60	50 ± 4
10 ± 0.4	70	70	25 ± 6
10 ± 0.1	100	100	10 ± 4
10 ± 0.5	150	150	Quenched

In order to determine the maximum coupling efficiency, various amount of MTX was used for the conjugation reaction in the presence of fixed concentration of QD and EDC-NHS. It was found that the amount of conjugated MTX on the surface of QDs gradually increased with increase in the content of free MTX molecules added to the reaction solution until reached its maximum value.

Table S2 MTX loading efficiencies calculated for various amounts of MTX used for the conjugation reaction in the presence of fixed concentrations of QD and EDC-NHS

MTX (mg)	QD (mg)	EDC (10 mg/mL) (μ L)	NHS (10 mg/mL) (μ L)	Loading efficiency (%)
5 ± 0.5	10 ± 0.2	50	50	61.8 ± 0.8
7 ± 0.3	10 ± 0.3	50	50	64.3 ± 0.5
10 ± 0.6	10 ± 0.6	50	50	70.5 ± 0.7
12 ± 0.4	10 ± 0.2	50	50	58.7 ± 0.9
15 ± 0.2	10 ± 0.7	50	50	46.9 ± 1.1

Quantitative analysis of intra-cellular uptake of the prepared nano-systems

To quantitatively analyze intra-cellular uptake of MTX-conjugated QDs in MTX-resistant and MTX-sensitive KB cells, we conducted two set of experiments. In the first experiment, MTX-conjugated QDs with different concentrations were introduced to the cells and intracellular uptake of cadmium ions that are indirectly indicative of intracellular MTX-conjugated QDs content was determined.

Table S3 In vitro cellular uptake of MTX-QD nanoconjugates in MTX-resistant and MTX-sensitive cells

MTX-QDs nanoconjugates ($\mu\text{g/mL}$)	MTX-resistant cells uptake (% of initial MTX)	MTX-sensitive cells uptake (% of initial MTX)
10 ± 0.4	54 ± 3	42 ± 0.2
50 ± 0.6	65 ± 4	54 ± 0.6
100 ± 0.3	71 ± 2	61 ± 0.4

Release profile of MTX molecules from MTX-conjugated QDs

High performance liquid chromatography (HPLC) analysis of MTX

HPLC analyses were carried out based on the method reported previously with minor modifications (Emara et al. 1997). A stock solution of MTX (500 mg/mL) was prepared by dissolving an accurately weighed amount of 50 mg in 100 mL sodium chloride (0.9%) injection. MTX solutions in the concentration range of 5–200 $\mu\text{g/mL}$ were prepared by serial dilution of appropriate volumes of the stock solution with sodium chloride (0.9%) injection. The solutions were stored frozen at $-20\text{ }^{\circ}\text{C}$ and protected against light until required. The liquid chromatograph was an Agilent 1100 HPLC Chemstation system (Santa Clara, CA, USA) consisting of a quaternary pump, a system controller, an auto-injector, a sample cooler kept at 4°C , and a fluorometric detector. The chromatographic separation was performed on a 25 cm \times 4.6 mm (i.d.) Terra MS C18 column (5- μm particle size; Waters; Milford, Massachusetts, USA) protected by a guard column. The excitation and emission wavelength of the fluorescence detector were set at 367 and 463 nm, respectively. The mobile phase was acetonitrile–50 mM ammonium acetate in water (7:93, v/v) with a flow rate of 1 mL/min. Spiked plasma samples were prepared by diluting

the appropriate volume of standard solution of MTX with drug-free human plasma in a ratio of 1:9 to obtain a concentration of 100 ng/mL. An aliquot of 500 μ L absolute ethanol was added to 500 μ L spiked plasma sample for QD-MTX nanoconjugates precipitation and then vortexed for 2 min, quite vigorously. The supernatant obtained after centrifugation (4500 \times g, for 5 min) were subjected to protein precipitation by addition of 5 μ L of perchloric acid, vortexed for 10 s, and centrifuged for 5 min (1000 \times g). The supernatant was introduced to 20 μ L of hydrogen peroxide, then irradiated with ultraviolet irradiation (254 nm) for 10 min for the conversion of methotrexate into highly fluorescent 2,4-diaminopteridine derivatives. A total volume of 20 μ L of the sample was injected directly into the HPLC system and analysed for MTX.

Figure S1 represents the HPLC analysis of the human serum sample evaluated for the release profile of MTX-QD nanoconjugates. The release of MTX molecules from MTX-QD nanoconjugates in the human serum during 72 h of incubation at 37 $^{\circ}$ C was found to be negligible, which was similar to the release profile observed in PBS buffer.

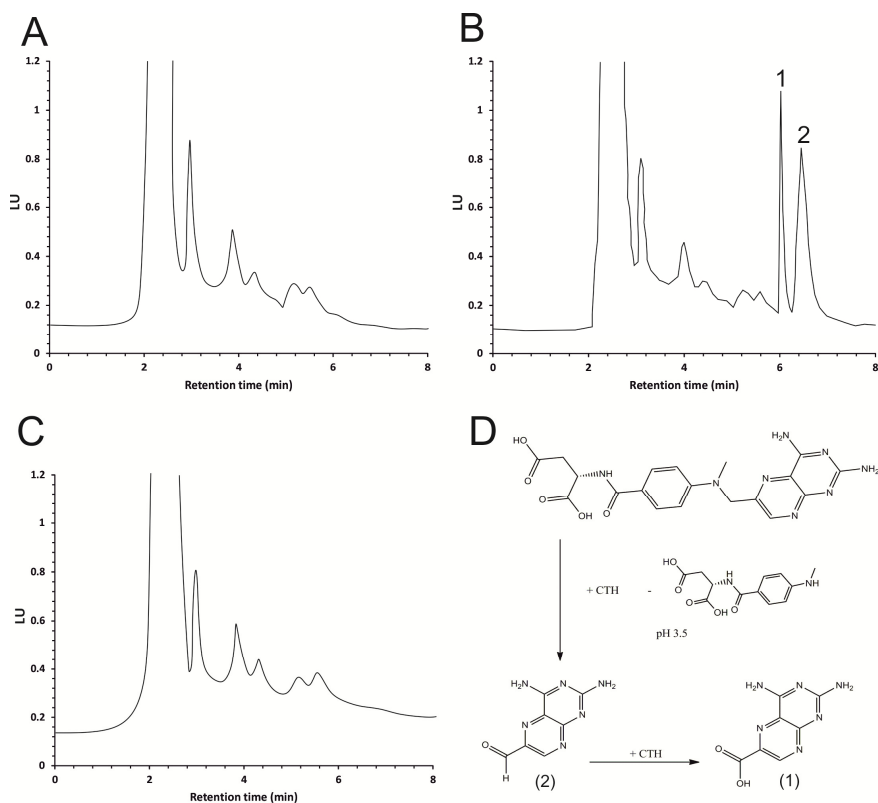


Fig. S1. HPLC chromatograms of (A) the blank human serum sample, (B) the human serum sample spiked with 100 ng/mL of free MTX, and (C) the human serum sample introduced to

MTX-QD nanoconjugates 72 h after incubation at 37 °C. Peaks of 1 and 2 refer to 2, 4-diaminopteridine-6-carboxylic acid and 2, 4-diaminopteridine-6-carboxaldehyde, respectively.

References:

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