Journal of Materials Chemistry B

A multifunctional poly(curcumin) nanomedicine for dual-modal targeted delivery, intracellular responsive release, dual-drug treatment and imaging of multidrug resistant cancer cells

Journal:	*Journal of Materials Chemistry B*
Manuscript ID	TB-ART-11-2015-002450.R1
Article Type:	Paper
Date Submitted by the Author:	14-Mar-2016

Complete List of Authors:	Wang, Jining; School of Pharmacy, Shanghai Jiao Tong University Wang, Feihu; School of Pharmacy, Shanghai Jiao Tong University Li, Fangzhou; School of Pharmacy, Shanghai Jiao Tong University Zhang, Wenjun; School of Pharmacy, Shanghai Jiao Tong University Shen, Yuanyuan; School of Pharmacy, Shanghai Jiao Tong University Zhou, Dejian; University of Leeds, School of Chemistry guo, shengrong; university of leeds,

SCHOLARONE™
Manuscripts

Journal of materials chemistry : B

ARTICLE

A multifunctionalpoly(curcumin) nanomedicinefor dual-modal targeted delivery,intracellular responsive release, dual-drug treatment and imaging of multidrug resistant cancer cells†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Jining Wang,‡[a],Feihu Wang,‡[a]Fangzhou Li,[a]Wenjun Zhang,[a]Yuanyuan Shen,*[a] Dejian Zhou,*[b]Shengrong Guo*[a]

Amultifunctionalanti-cancer nanomedicinebased on a biotin-poly(ethylene glycol)-poly(curcumin-dithiodipropionic acid)(Biotin-PEG-PCDA) polymeric nanocarrierloaded with paclitaxel(PTX), magnetic nanoparticle(MNP) and quantum dot(QD) is developed.It combines advantageous properties ofefficient targeted delivery and uptake (via biotin and MNP), intracellular responsive release (via cleavable PCDA polymer), fluorescence imaging (via QD) and combined PTX-curcumin dual-drug treatment, allowing for overcoming drug resistance mechanisms of model multidrug resistant breast cancer cells (MCF-7/ADR). The PTX/MNPs/QDs@Biotin-PEG-PCDA nanoparticles are highly stable underphysiological conditions, but are quickly disassembled torelease theirdrug loadin the presence of 10 mMglutathione (GSH).The nanoparticlesshow high uptakeby tumour cellsfrom a combinedeffect of magnettargeting and biotin receptor-mediated internalization.Moreover, curcumin, anintracellularly cleaved product of PCDA, can effectively downregulate the expression of drug efflux transporters such as P-glycoprotein (P-gp) to increase PTX accumulation within target cancer cells, therebyenhancingPTX induced cytotoxicity and therapeutic efficacy against MCF-7/ADR cells.Taken together, thisnovel tumour-targeting and traceablemultifunctionalnanomedicine is highly effective against model MDR cancer at the cellular level.

1.Introduction

Multi-drug resistance (MDR) is a common but one of the greatest challenges currently facing cancer treatments where cancer cellsare resistance to a variety of structurally and mechanistically unrelated chemotherapeutic agents.[1]The MDRtumour cells can effectively remove drugs from their cellular interior to prevent drug accumulation, reducing the sensitivity of tumor cells to the therapeutic drugs.[2]MDR can be caused by both biochemical and physical obstructionssuch as over-expression of efflux pumps (P-glycoprotein, P-gp), upregulated pathways (NF-κB and PI3K), reduced penetration of drugs into the cells, and so on.[3,4]

An attractive strategyto overcome MDR cancer is to co-administer a specificefflux pump inhibitor along with the cancer chemotherapy drug to increase the drug accumulation and improve treatment efficacy.[5] Curcumin, a natural diphenol compound extracted from the ground rhizomes of curcuma longa, has shown attractive selective cytotoxicity towar cancer cells over healthy cells with broad antitumor properties.[6] More importantly, curcumin is able to independently down-regulate both the PI3K/Aktand NF-kB pathways andsuppress the P-gp expression.[7,8] Therefore, curcumincan be used as an effective sensitizer forMDR cancer cellsto increase their therapeutic response to conventional chemotherapeutic agents.However, the extremely low solubility and poor stability of curcumin under physiological conditions have greatly limited its bioavailability and therapeutic efficacy. To overcome this issue, herein we have directly incorporated curcumin into adisulfide-linked hydrophobic backbone of a PEGylated amphiphilic diblock copolymer(biotin poly(ethylene glycol)-poly(curcumin-dithiodipropionic acid)to improve its stability and water-solubility. Moreover, we have grafted biotin at the hydrophilic end of the copolymer as an active cancer targeting ligand because biotin bindsstrongly to the biotin receptorswhich are widely over-expressed on cancer cell surfaces.[9,10]Importantly, the resultingamphiphilic copolymer (hereafter abbreviated asBiotin-PEG-PCDA, see Scheme 1)canself-assemble into a stable core/shell nanoparticle (NP)in aqueous environment,actingas an efficientnanocarrierforother cancer chemo-therapeutic drug (e.g. paclitaxel, PTX).As a result, both

[a]School of Pharmacy, Shanghai Jiao Tong University, Shanghai, 200240, China
[b]School of Chemistry and Asbury Centre for Structural Molecular Biology, University ofLeeds, Leeds, LS2 9JT, UK. *E-mail: d.zhou@leeds.ac.uk; s.guo@leeds.ac.uk; srguo@sjtu.edu.cn;Tel: +44 (0)113 3436230;
+44 (0)113 3436449

ElectronicSupplementary Information (ESI) available: The synthesis procedure of Biotin-PEG-PCDA and pre-experiment results of MTT.

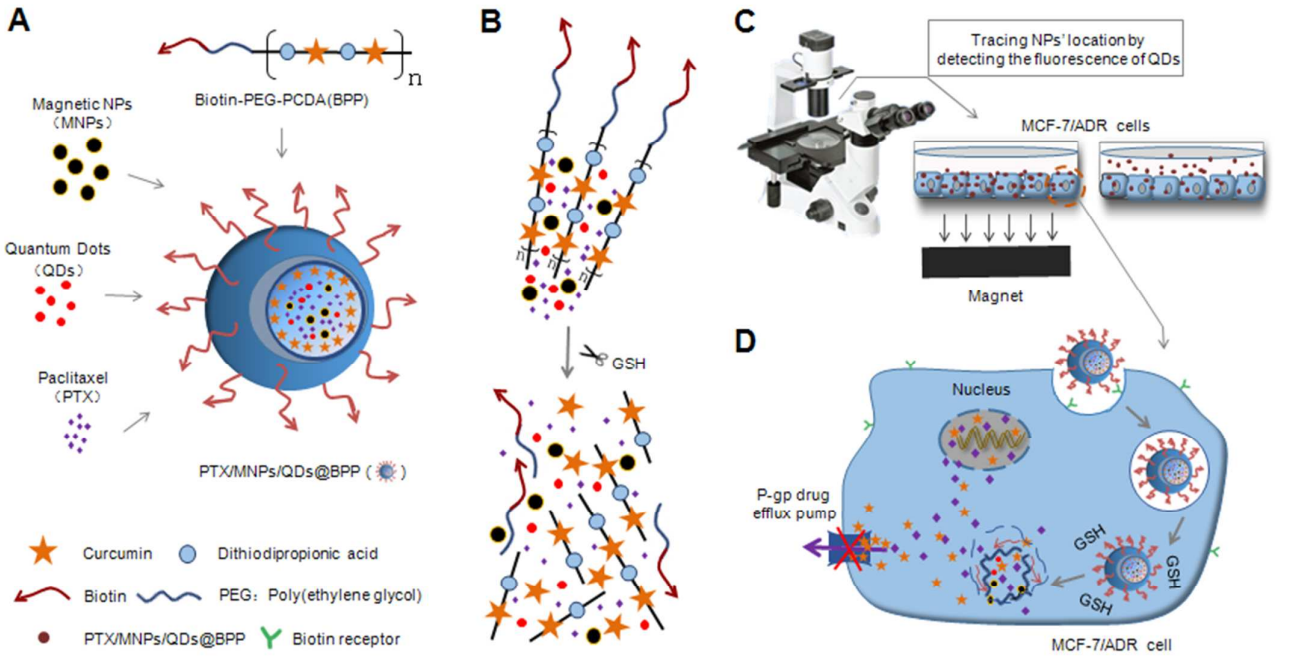
‡ These authors contributed equally to this work.

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

the chemotherapeutic agent (PTX) and chemosensitizer can be efficiently delivered into the target cancer cell at the same time.[11]Importantly, the disulfide linkage at the PCDA hydrophobic sectioncan be readily cleaved by the high intra-cellular glutathione (GSH) content, leading to co-releaseof curcuminand PTX intracellularly. The released curcumin candown-regulate the P-gpexpression, increasingintracellular PTX accumulation and enhancing its cytotoxicity against the model MDR cancer (MCF-7/ADR) cell lines.[12,13]

Meanwhile, the ability of delivering high drug dose to the target tumour cells specifically can greatly reduce the harmfulside effects of most chemotherapeutic reagents.[14,15,16]To achieve this ability, we have further loaded Fe_3O_4magnetic particles into the hydrophobic core of the Biotin-PEG-PCDA nanoparticles (NPs), allowing for magnetic field guided delivery of the multifunctional anticancer nanomedicine to target cancer cells with greatly improved efficiency. To further improve the multi-functionality of the nanomedicine, we have encapsulated a non-toxic, Cd^{2+}-free $CuInS_2$/ZnS core/shell quantum dot (QD) into the hydrophobic core to enable it for traceable delivery.An advantage of using QD over other fluorescent dyes for traceable delivery is its bright, stable and size-tuneable fluorescence, allowing for sensitive, long-term monitoring and tracking.[17,18]



Scheme 1.(A) Construction of the PTX/MNPs/QDs@Biotin-PEG-PCDA hybrid multifunctional nanoparticles.(B)The disulphide linkage of the hydrophobic PCDA core can be quickly cleaved by the high GSH content intracellularly, simultaneously releasingits curcumin and PTX payloads.(C)The incorporated magnetic nanoparticles(MNPs)into the multifunctional NP allows for magnetic field guided concentration of the NPs on target cellsfor increased uptake while the encapsulated QDs allows for sensitive fluorescence detection and tracing.(D) The NP surface biotin groupsallow for efficient uptake via cancer cell surface over-expressed biotin receptors and subsequently leads to GSH-triggered co-release of curcumin and PTX payloads.Curcumin candownregulate the expression ofP-glycoprotein (P-gp)to improvePTXintracellular accumulation and enhancing its cytotoxicity to model MDR cancer (MCF-7/ADR) cells.

2.Experimental

2.1 Materials

Biotin-poly(ethylene glycol)-poly(curcumin-dithiodipropionic acid) copolymer(Biotin-PEG-PCDA, the molecular weights of PCDA and Biotin-PEG-PCDA are 6990 and 10750, respectively) was synthesized as described previously.[19,20]Hydrophobic $CuInS_2$/ZnS quantum dot (QD) capped with dodecanethiol ligands was purchased from PlasmaChem (German). Paclitaxel was obtained from ZiyunBiotechnology Co.,Ltd., (Yunnan, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and dimethyl sulfoxide(DMSO) were purchased from Sigma Co. Ltd.(USA). Penicillin–streptomycin, RPMI-1640 medium (R1145), fetal bovine serum (FBS), and 0.25% (w/v) trypsin–0.03% (w/v) EDTA solution were purchased from Gibco BRL (Gaithersburg, MD, USA). All other reagents were at least analytical gradeand used without further purification.

Breast cancer cell lines MCF-7 and MCF-7/ADR(multidrug resistance) were kindly donated by the Department of Pharmacology, Chinese Academy of Sciences, Shanghai. The cellswere cultured in 75 cm^3

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

flasks in a humidified atmosphere with 5% CO_2 at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine medium (FBS) and 100 U/mL penicillin and 100μg/mL streptomycin. Cells grown to confluence were sub-cultured every other day after trypsinized with 0.25% trypsin–EDTA and diluted (1/3) in fresh growth medium.[21]

2.2 Preparation of magnetic nanoparticles

Fe_3O_4MNPs were prepared by using theco-precipitation method as described previously.[22]Briefly, $FeCl_3$•$6H_2O$ and $FeCl_2$•$4H_2O$(2:1 molar ratio) were dissolved inde-oxygenated pure water, into which NH_3•H_2O was then added dropwise undervigorous stirring and heated to 80℃ to yield Fe_3O_4 MNPs. Then oleic acid (~10% of the mass of MNPs) was added to cap the MNPs and rendering them hydrophobic. The resulting Fe_3O_4@QA were washed repeatedly by ethanoland isolated by using a permanent magnet. The size and morphology of the MNPs was measured by transmission electron microscopy (TEM) on aJEM-2100F TEM (JEOL, Japan). Their IR spectrawere recorded by a Bruker EQUINOX 55 Fourier transformed infrared spectrophotometer (FT-IR, Germany) to detect the surface coating functional groups.

2.3 Preparation of PTX-loadingPTX/MNPs/QDs@BPP NPs.

The PTX-loaded nanoparticles were prepared by the O/W emulsion solvent evaporation method following our previously established procedures. Briefly, a mixture of 10mg MNPs, 5mg QDs, 2mg PTX and 20mg Biotin-PEG-PCDA were co-dissolved in dichloromethane to form a uniform solution, which was thenslowly poured into deionized water under sonication to form an oil-in-water emulsion. Afterstirring at room temperature for six hours, the organic solvent was rotary evaporated at 40°Cunder reduced pressure to obtain a suspension,which was then centrifuged at 3000 rpm for 20 min to remove aggregated particles and unencapsulated free PTX. The resulting clear supernatant was lyophilized to obtain the multi-functional PTX/MNPs/QDs@BPP nanoparticles.

2.4 Evaluation of particle size and zeta potential

The morphology of PTX/MNPs/QDs@BPP NPs was observed by transmission electron microscopy (TEM, JEM-2100F,JEOL, Japan). A drop of the diluted NP solution was placed on a copper grid, stained with 2% phosphotungstic acids and dried before measurement. The average hydrodynamic size and distribution were measured on a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Zeta potentials of nanoparticles were measured onZetasizerNanoZS/ZEN3600 (Malvern Instruments, Herren-berg, Germany) at 25℃. Each sample was tested in triplicate.

2.5 Evaluation of particle rehydration and dilution stability

To investigate the particle rehydration and stability upon dilution which is crucial forefficient drug carriers, the lyophilized powder was redispersed in deionized water by sonication at a concentration of 1mg/mL. The average hydrodynamic size and polydispersity indexof the nanoparticle were tested repeatedly to check whether agglomeration occurred during this process. The resulting solution was then diluted by pure water to investigate whether the NPswere stable upon dilution.

2.6 Investigation of particle magnetic and fluorescent properties

The PTX/MNPs/QDs@BPPNPs weredispersed in an aqueous solution at a concentration of 1 mg/mL, then a permanent magnet was used to determinate their magnetic response.Magnetic NPs were able to be pulled to the side wall byan external magnetic field, but were redispersed uniformly and quickly after the magnet was removed. The fluorescence absorption and emission spectrum of theNPs were measured on a HATACHI F-7000 fluorescence spectrophotometer(Japan) to confirm the loading of the QDs.

2.7 Determination of drug-loading parameters

1mg of the PTX/MNPs/QDs@BPP NPs was dissolved in 2mL of 50% acetonitrile in water being followed by sonicationfor 10 min to completely break of the assembly. The solution was centrifuged at 10,000 rpm for 10 min and the supernatant was filtered with a 0.2μm syringe filter. The resulting PTX concentration was analysed by high-performance liquid chromatography (HPLC) equippedwith a LC 10ADvp pump and a SPD-10Avp UV–vis detector (Waters, USA). The sample solution was injected at least three times at a volume of 20 μL into a Dikma-ODS C18 column (150 mm × 4.60 mm, 5 μm) preceded by a C18 guard column (Dikma, China). The mobile phase was 50% acetonitrile in water with an elution rate 1.0 mL/min.Paclitaxel detectionwavelength was set at 227 nm.The drug concentration of PTX was estimated againsta standard calibration curveestablished under identical conditions. The drug-loading efficiency (DL) and encapsulation efficiency (EE) werecalculated bythe following equations:

$$\mathrm{DL}\% = \frac{\text{weight of the PTX in nanoparticles}}{\text{weight of the nanoparticles dissolved}} \times 100\%$$

$$\mathrm{EE}\% = \frac{\text{weight of the PTX in nanoparticles}}{\text{weight of the feeding PTX}} \times 100\%$$

2.8 In vitro drug release study

The nanoparticle solution was transferred into a dialysis tube (molecular weight cutoff = 3500Da, Snakeskin, Pierce, USA) and suspended in phosphate buffer saline (PBS, 0.15 MNaCl, pH 7.4) containing 1% Tween 80 to imitate physiological environment. PBS solutions containing 10 μM and 10 mM glutathione were used to mimic the blood and intracellular environment. The release experiments were carried out in an incubator (SHA-C,China) under gentle stirring (100 rpm)at 37°C. At predetermined time intervals, the release medium was withdrawn and replaced with an equal volume of fresh release medium. The collected samples wereanalyzed by HPLC asdescribed above to determine the amount of released PTX. Meanwhile PTX release from stock solution was used as control.

2.9 In vitro fluorescence imaging and cellular uptake study

MCF-7/ADR cells in logarithmic growth period were seeded at a density of 2 x 10^5 cells per well in a 6-well plate and incubated overnight.After removing the culture medium, 2 mL of fresh medium containingthe PTX/QDs@PEG-PCDA, PTX/QDs@BPP and PTX/MNPs/QDs@BPP(all containing the same amounts of QDs) were added to each well, respectively. The cells were also scheduled to be treated with or without an external magnet. After

4h incubation, the cells were washed three times with cold PBS to remove unbound NPs, and then imaged on an Olympus IX51 fluorescence microscope (Olympus, Tokyo, Japan) where both fluorescence and bright field photographs were recorded.

For cellular uptake studies, the cells were treated with different NP formulations, PTX/QDs@PEG-PCDA, PTX/QDs@BPP, PTX/MNPs/QDs@BPP, PTX/QDs@BPP plus magnetic field and PTX/MNPs/QDs@BPP plus magnetic field, respectively. At pre-designated time points (2, 4 and 8h), the cells were washed three times with ice-cold PBS, then collected by centrifugation andthen re-suspended in 0.5 mL PBS. The mean fluorescent intensity of the cells was measured on a BD LSRFortessaflow cytometer (Becton Dickinson, America).

2.10 In vitro cytotoxicity assay

The cytotoxicities of the different NPs were determined by the MTT assay. Briefly, MCF-7/ADR cells in their logarithmic growth were seeded in 96-well plates at a seeding density of 6000 cells/well. Following attachment overnight, the culture medium was carefully replaced with 150 µL of medium containing serial dilutions of the different drug/NP formulations: free-PTX solution (PTX), FreePTX+ curcumin physical mixture, PTX@PEG-PCDA, PTX@BPP, PTX/MNPs-/QDs@BPP and PTX/MNPs/QDs@BPPplus magnetic field. The concentrationsof PTX used in the treatment ranged from 0.1 to 100 µg/mL.After incubationfor 48 h,15 µL of the MTT solution (5 mg/mL in PBS) was added to each well. The plateswere incubated for an additional 4 h at 37°C and thenthe medium was removed. Thereafter, 150 µL of DMSOwas added to each well to dissolve the formed formazan crystals.The absorbance of each well was recorded on a Bio-Rad 680 microplatereader (Bio-Rad Laboratories, Hercules, CA)at a set wavelength of 570 nm.

All the cell based experiments were done in triplicate with six parallel samples. Cells treated with culture medium containing 0.1% DMSO were used as controls. The cytotoxicity of the drug-loaded NPs was expressed as IC_{50} valuedefined as the drug concentration required to inhibit cellgrowth by 50% relative to the control.Thesevalues were calculated by nonlinear regression analysis of the response curves.The cell growth inhibition rate and reversingdrug resistance index on MCF-7/ADR cells were calculated as follows:

$$\text{Inhibitory rate} = \frac{(\text{A570 control} - \text{A570 sample})}{\text{A570 control}} \times 100\%$$

$$\text{Resistance reversal index(RRI)} = \frac{\text{IC50 Free PTX}}{\text{IC50 sample}}$$

3.Results and Discussion

Our approach to the multifunctional anticancer nanomedicine is shown schematically in Scheme 1. Anamphiphilic biotin modified poly(ethylene glycol)poly(curcumindithiodipropionic acid)(Biotin-PEG-PCDA)diblock copolymer was prepared. It was subsequently assembled into stable core-shell NPs in the presence of hydrophobic PTX, Fe_3O_4MNPs and QDs, during which the hydrophobic drugs,MNPs and QDs are encapsulated within the hydrophobic core, forming PTX/MNPs/QDs@Biotin-PEG-PCDAhybrid nanoparticles (abbreviated as PTX/MNPs/QDs@BPP).The encapsulation of the hydrophobic entities significantly increases the nanoparticle stability and structural integrityat physiological conditions via enhanced hydrophobic interactions. Moreover, each species brings in a unique function to the multifunctional nano-medicine: QD for sensitive fluorescence tracing, MNP for magnetic targeting, PTX for providing chemotherapy, biotin for active cancer cell targeting, PEG for improving water-solubility, stability and resisting non-specific adsorption, PCDA for curcumin incorporation and GSH-triggered intracellular release. Together, all these functional entities allow us to build a novel, multifunctional nanomedicine platform for traceable, targeted delivery, efficient GSH-triggered intracellular drug release and combinational dual-drug therapy for overcoming MDR cancer at the cellular level.

3.1. Characterization of PTX/MNPs/QDs@BPPnanoparticle

Fig.1A shows the TEM image of oleic acid coated Fe_3O_4 MNPsused in this study. They are mostly appeared in spherical shape with an average MNP core diameter of ~10 nm. Fig.1B shows the FI-IR spectrum of the MNPs. During the MNP preparation by co-precipitation, the MNP surfaces were readily covered with hydroxyl groups in an aqueous environment. The strong absorption bands at 1628 and 3430cm^{-1}were assigned to the O-H stretching and bending vibration modes respectively.[23]Compared with bare Fe_3O_4,

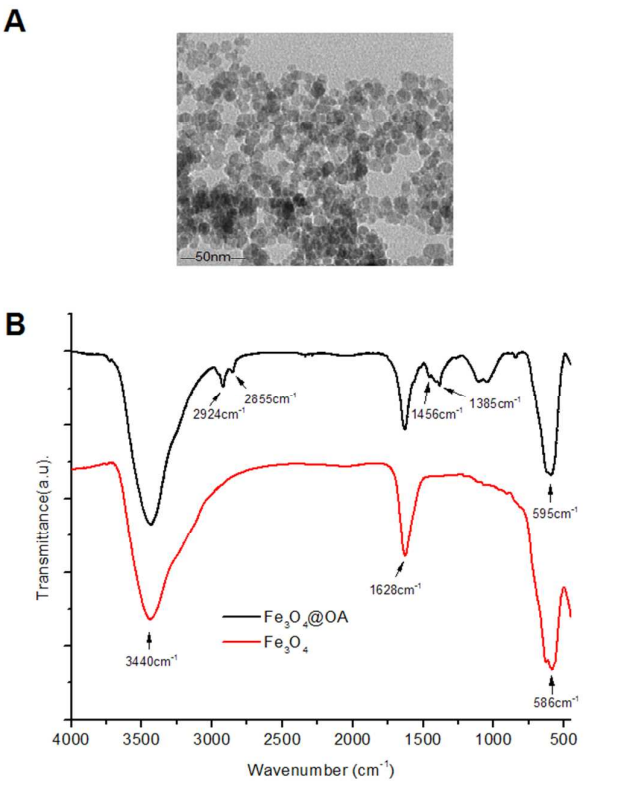


Fig.1(A)Arepresentative TEM image of Fe_3O_4@OA; (B)The FTIR spectra of the bare Fe_3O_4 (red) and Fe_3O_4@OA (black line).

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

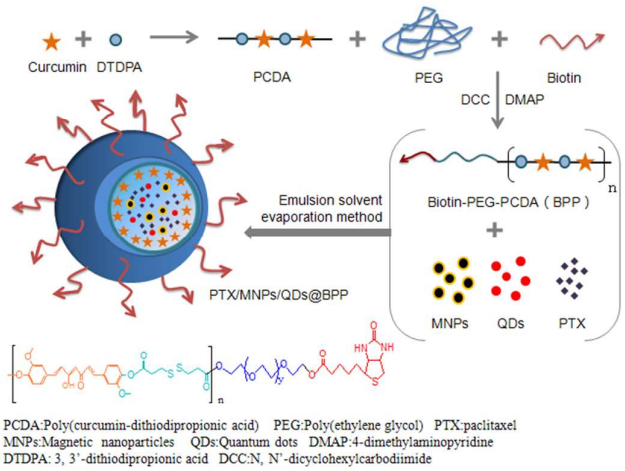


Fig.2. Schematic procedures of synthesising biotin-PEG-PCDA and the assembly of PTX/MNPs/QDs@BPP.

new absorption bands at 2924 and 2855 cm^{-1} were observed for the oleic acid capped MNPs which were attributed to the existing of -CH_2 asymmetric stretching, -CH_2 symmetric stretching of oleic acid, respectively.The band at 1456 cm^{-1} was attributed to COO^- and the band at 586 cm^{-1} was corresponded to Fe-O.[22]These data indicated that the Fe_3O_4NPswere successfullycoated with oleic acid ligands via their COO^- groups, leaving the alkyl chains exposed to render them hydrophobic. This was essential for loading them into the hydrophobic cavity ofthe self-assembled amphiphilicbiotin-PEG-PCDA copolymer NPs.

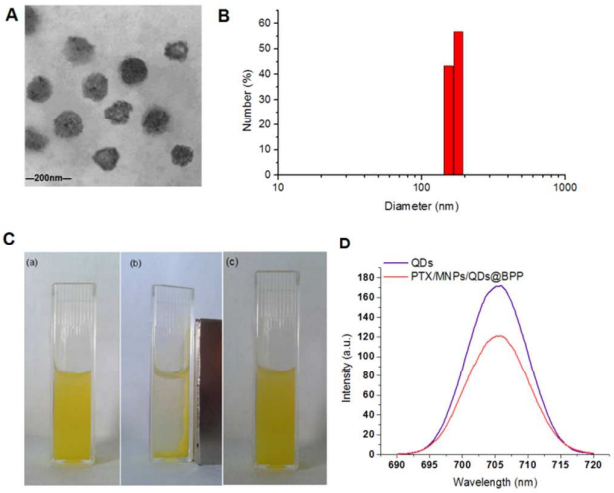


Fig.3 The physicochemical characteristics of PTX/MNPs/QDs@BPP NPs. (A)A typical TEM image of PTX/MNPs/QDs@BPP NPs; (B) Histograms showing the size distribution of PTX/MNPs/QDs@BPP NP; (C)Photograph of an aqueous solution of PTX/MNPs/QDs@BPP NPshowing a uniformyellow colour without any visible aggregation (a), the NPs were rapidly gathered at the side wall of the cuvette by an external magnet (b), the NPswere re-dispersedinto a uniform solution after the magnetwas removed(c);(D)The fluorescence emission spectra of the free QDs and PTX/MNPs/QDs@BPP.

The preparation procedures of PTX/MNPs/QDs@BPPmulti-functional NPsare shown schematically inFig. 2. Biotin-PEG-PCDA was used to load MNPs, QDs and PTX by the simple O/W emulsion and solvent evaporation method. The success of the multifunctional NP assembly was confirmed by TEM imaging (Fig. 3A): smooth spherical NPswith uniform sizes were clearly observed.The sizes ofthe NPs were found to be in the rangeof150-180nm,which was consistent with those found by the dynamic light scattering (DLS) shown inFig. 3B. The zeta potential of the particles was found to be -11.36 mV, consistent with NPs capped with neutral hydrophilicPEGs. Because most of the blood components are negatively charged, the negative zeta potential together with surface PEGs of the NPsshould increase their stability by electrostatic repulsion, reducing the chances ofparticles agglomeration.

The magnetic and optical properties of the NPs were further investigated to examine whether the MNPs and QDs were properly assembled. As shown in Fig.3C, the NP solution exhibited a yellow colour uniformly, suggesting they were fully dissolved without aggregation or precipitation. The NPs wererapidly concentrated toward the side wall of the cuvette upon applying anexternal magnetfield, where the solution colour became much less strong, indicating that the MNPs have indeed been successfully encapsulated into the NPs. Therefore, it is possible to guide the NPs by using an external magnetic field. Upon removal of the magnetic fields, the NPswere rapidly re-dispersed into a uniform solution, indicating no agglomeration of the NPs. Fig. 3D showed that the maximum fluorescence emission wavelength of the free QDand the multifunctional NPs containing the same amount of QDwere almost identical, peaking at 705and 706 nm, respectively. All these indicated that the QDs were successfully loaded into the NPs, allowing them to readily detected via the strong fluorescence of the encapsulated QD.[24]

3.2 Evaluation of particle rehydration and dilution stability

At first, granules existed when the lyophilizedhybrid NP powder was re-dispersed in deionized water.However, the solution became completely clear and uniform aftera brief sonication. The average hydrodynamic diameters ofthe NPs were found to be ~170 nm witha unimodal size distribution (PDI = 0.25), consistent to those measure by the TEM. The re-dispersed NP powder solution thus

Table 1 Particle size and zeta potential of PTX/MNPs/QDs @BPP NPs at different concentrations.

Concentration (μg/mL)	Size (nm)	PDI	Zeta-potential value
100	181.1±3.7	0.15±0.04	-12.2 ± 1.8
10	186.5±2.6	0.21±0.06	-11.1 ± 0.9
1	188.7±2.0	0.22±0.01	-9.6 ± 1.4

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

showed no significant difference to as-prepared NPs, suggesting that the multifunctional NPs can be conveniently lyophilized and then readily rehydrated and redispersed in water. This indicated that the NPs could be readily lyophilized for convenient long term storage without affecting their structural integrity, a very beneficialproperty for nanomedicine.To further investigate theirstabilityagainst dilution, thestocksolution(1mg/ml) was diluted by pure water to give different concentrations, and then the resulting hydrodynamic sizes weremeasured. As shown in Table 1, the NPsat different concentrations exhibited almost identical particle sizes with a narrow size distribution even at a concentration of as low as1 μg/mL. Moreover, theyalso gavevery similar negative zeta potentialsof about -10 mV.These results indicated that the NPs developed herein possessedexcellent stability against dilution and could retain their nanoscale structural integrityeven at very low concentrations.Obviously, these results also demonstrated that there were no leaching of the encapsulated quantum dots andmagnetic nanoparticlesfrom the nanomedicine,otherwise a range of different sized particleswould be observed. Such properties would be extremely beneficial for in vivo applications, allowing for effective minimization of any unwanted premature drug release and degradation and hence reducing side-effects. The outstanding stability of the NPs obtained here is attributed to the strong hydrophobic interactions among the PCDA backbone,hydrophobic PTX molecules, MNPs and QDs at the NP core together with the exposed hydrophilic PEG moieties.

3.3 Paclitaxel-loading parameters

The O/W emulsion solvent evaporation method appeared to be particularly suitable for the incorporation of PTX into self-assembled biotin-PEG-PCDA NPs. The PTX-loading weight efficiency (DL) and encapsulation efficiency (EE) of PTX/MNPs/QDs@Biotin-PEG-PCDAwere determined as 10.3%and 80.7%, respectively. Such high PTX weight loading and encapsulation values were ascribed to the presence of sufficiently large and stable hydrophobic cores of the biotin-PEG-PCDA copolymer NPwhich was further stabilised by hydrophobic interactions with the MNPS and QDs, allowing for efficient encapsulation of highly hydrophobic paclitaxel molecules.

3.4 In vitro drug release studies

In this study, pH 7.4 phosphate buffer solution without and with 10 μM or 10 mM glutathionewere selected to imitate physiological, blood and intracellular environments, respectively. The release medium also contained 1 % w/v Tween 80 as good sink conditions. In addition, prior to conducting release assays, PTX release from stock solution was investigated as a control. It was found that about 80% of non-encapsulated PTXs were released in approximately 5 h, suggesting that free drugs could freely diffuse through the dialysis membrane. Fig. 4represents the cumulated in vitro release profiles of PTX from the multifunctional NPs in different release medium. In contrast to the rapid release observed from PTX stock solution, a pronounced time prolongation of PTX release from the NPs was evident. For example, only about 25% of the PTX load was released from the NPs in pH 7.4 PBS after 80 h. Importantly, the cumulative PTX release profile showed a strong glutathione dependence:PTX was released much more quickly when exposed to PBS containing10 mM glutathione, where ~65% and 90% of drug loads were released at 12 and 48 h, respectively. In contrast,only ~25% and 30% of drug loads were released with 10 μM glutathione at the same time point. This is mainly due to the effective cleavage of disulfidelinkage of hydrophobic PCDA backbone by GSH, allowing for rapid degradation of hydrophobic NP core and the release of loaded drugs.[25]Therefore, the PTX/MNPs/QDs@BPPNPs should be stable under normal physiologicalor blood circulation conditions (with low GSH content), but could readily release its drug load once entered into the target cancer cells/tissues triggered by the high intracellular GSH content (1-10 mM).

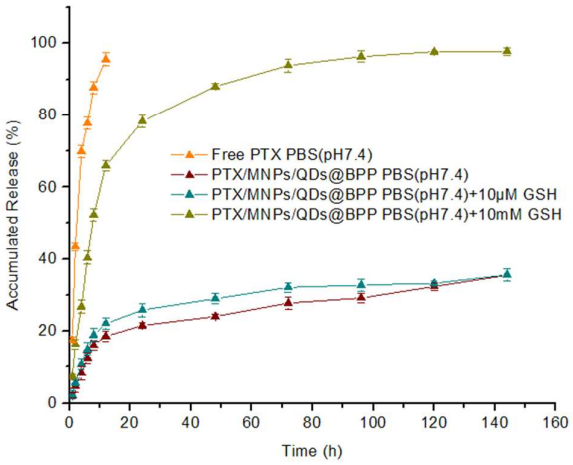


Fig.4. Typical in vitro release profiles of paclitaxel from NPs under different simulated conditions at 37±0.5℃.

3.5 Cell targeting and cellular uptakestudies

The encapsulation of strongly fluorescent Cd^{2+}-free $CuInS_2$/ZnSQDs into the NPs allowed for convenient monitoring of the carrier cell uptake viathe encoded QD fluorescence by fluorescence microscopy. As shown in Fig. 5A, when the PTX/QDs@PEG-PCDA(without biotin) was incubated withthe MCF-7/ADRcells for 4 h, the cells showed minimal QD fluorescence, suggesting minimal cell uptake. The introduction of biotin to the carrier (PTX/QDs@Biotin-PEG-PCDA)was found toimprove the cell uptake significantly as evidenced by a strong QD fluorescence (Fig. 5B). This result confirmed that the biotin could act as a targetingligand to increase cell uptake of the NPs. As shown in Fig.5C, applying amagnetic field to PTX/QDs@Biotin-PEG-PCDA(without MNP encapsulation) incubation system appeared to have no effect on the fluorescence intensity oftreated cells. However, applying amagnetic fieldto help pull down the multifunctional NPs on top of the incubation cells was found to significantly increased the fluorescent intensity of the PTX/MNPs/QDs@BPP(containing MNPs) treated cells, where almost every cell was found to display an strong fluorescence (Fig.5E). The fluorescence intensity was also stronger than that shown in Fig.5D without applying magnet field, suggesting the use of magnetic field for targeted concentration of the NPs are effective in increasing the cell uptake.Taken together, these results revealed that the combination of magneticand biotin targeting could significantly improve the cell uptake efficiency of the PTX/MNPs/QDs@BPP NPs,demonstrating that the amounts of

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

QDs and MNPsencapsulated within the nanomedicine are enough to make it useful for simultaneous fluorescence imaging and magnetic targeting.

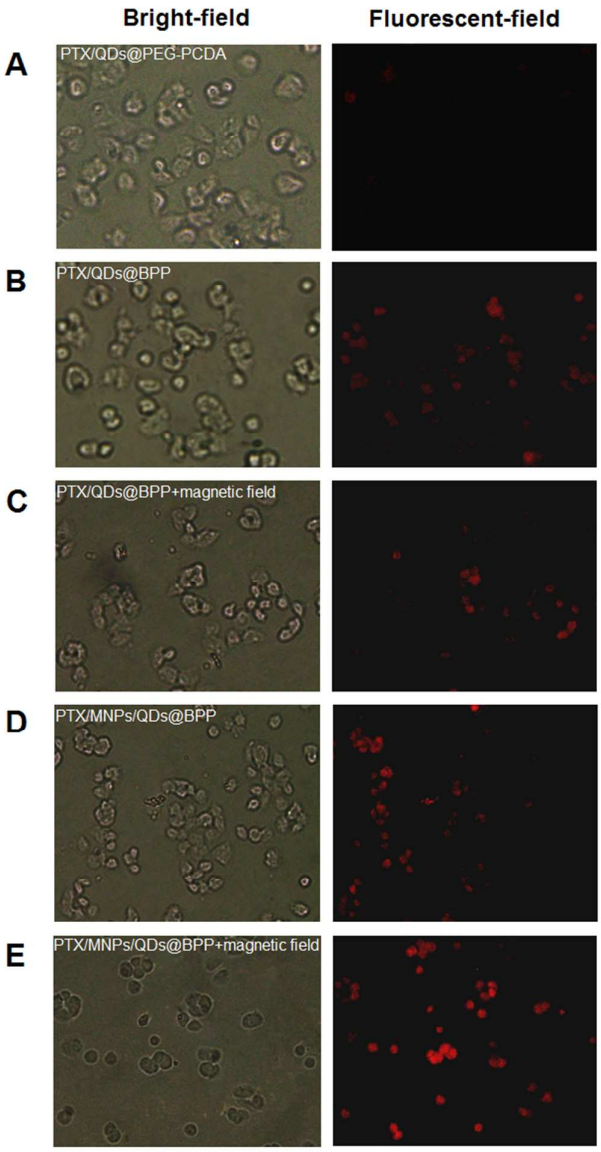


Fig.5. Fluorescent images of MCF-7/ADR cellsafter incubationwith PTX/QDs@PEG-PCDA(A), PTX/QDs@BPP(B), PTX/QDs@BPPplus an external magnetic field (C), PTX/MNPs/QDs@BPP(D), and PTX/QDs@BPPplus an external magnetic field (E).

To further investigate the magnetic field and biotin medicated cell-targeting effects, the mean fluorescent intensity (MFI)ofMCF-7/ADR cells after incubation withdifferent NP formulations for 2, 4, and 8h were quantified by flow cytometry. As shown in Fig. 6, the MFI of cells was found to increase with the increasing incubation time, consistent with more cell uptake of the NPs with time. Moreover, all of the biotinylated NPs (PTX/QDs@BPP; PTX/MNPs/ QDs@BPP) showed higher MFIs than their non-biotinylated NPcounterpart (PTX/QDs@PEG-PCDA). This result was consistent withthat observedinthe fluorescence microscopy imaging experiment described above, confirming biotin modification on the NP surface canimprove cell uptake presumably via efficient binding to over-expressed biotin receptors on MCF-7/ADR cell surfaces. Moreover, cellstreated with the PTX/QDs@BPP(containing no MNPs) showed no measurable difference in thecellular MFIsin the presence or absence of external magnetic field, confirming that the application of external magnetic fieldalone did not impact the cell uptake of the NPs. However,a significantlyhigher MFI (by ~87% at 8 h) was observedfor cells incubated with PTX/MNPs/QDs@BPP (containing MNPs) in the presence ofan external magnetic field than that in the absence of external magnetic field, presumably *via*magnetic field guided concentration of the NPs next to the cells. These results indicated that the MNP containing multi-functional NPs were more efficiently taken up by tumour cells from the combined effects of magnet field and biotin dual-modal targeting.

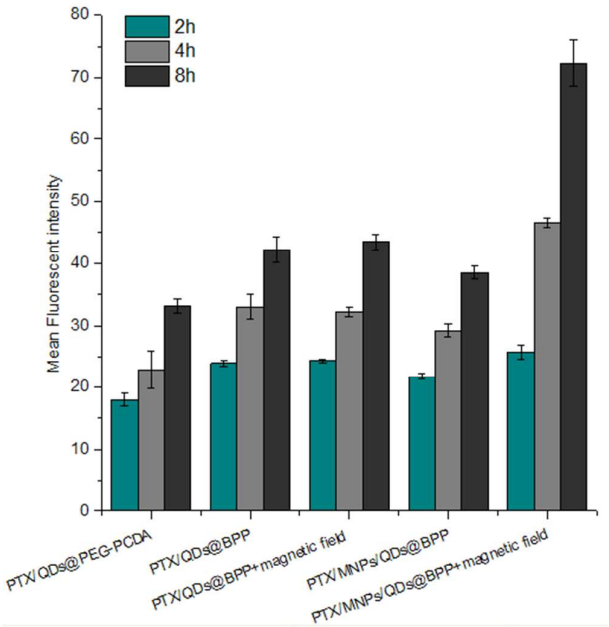


Fig.6. The mean fluorescent intensity of the MCF-7/ADR cells after treatment with different NP formulations with and without applied magnetic field for 2, 4, and 8h. Data are expressed as the mean± S.D. (n=3).

3.6 Cytotoxicity assay in MCF-7/MDR cells

Loading of chemotherapeutic drugsinto polymeric NPscould increasetheircytotoxicityto target cells over free drug.[24,26]Here the cytotoxicity and drug resistant reversal efficacy of the different formulations of thePTX-loadedmultifunctional NPsagainst the MDRcancer (MCF-7/ADR) cells were evaluated by the standard MTT based cytotoxicity assays. Our previous work showed that the MCF-7/ADR cells werehighly resistant to free PTXtreatment compared to the PTX-sensitive MCF-7 cell line, displaying a high resistance index of 248. The results shown in Fig.8also confirmed the high resistance of MCF-7/ADR cells towardfree PTX treatment, givinga high

IC_{50}value of 14.9μg/mL. The combined application of free PTX and curcuminphysical mixture reduced the $IC_{50}$to 9.4μg/mL.Encapsulation of PTX into the PEG-PCDANP (e.g. PTX@PEG-PCDA) further reduced the $IC_{50}$to 7.1μg/ml,suggesting increasedcytotoxicityof PTX against the MCF-7/ADR cells. The high resistance of the MCF-7/ADRcells towards free PTX treatment is most likely due to their surfaceover-expressed p-glycoproteinefflux pumpsthat can efficiently efflux out ofthe internalised PTX molecules from thecellular interior, reducing the intracellular PTX concentration and compromising the treatment efficacy. When the MDR cells were treated withPTX-containing PTX@PEG-PCDA NPs, the cleavage of the disulphide linkage of the PCDA backbone triggered by the highintracellular GSH content simultaneously released the curcumin (a degradation product of PCDA)and PTX payloads. Curcumincould then effectively downregulate the expression of the efflux transporters such as P-glycoprotein (P-gp), allowing for increasedintracellular PTX accumulation,and thereby enhancingthe PTX based chemotherapeutic treatment efficacy.

Thecombined effectof combinational PTX and curcumin treatment over PTX alone was also evident from the freePTX + curcumin mixture (IC_{50}: 9.4*v.s.* 14.9 ug/mL). The different IC_{50} values between freePTX + curcumin mixture and PTX@PEG-PCDA perhaps originated from the latercan provide thesimultaneous intracellular release ofPTX and curcumin, maximising the combinational therapyefficacy.[27] Moreover,the biotin grafted PTX@BPPNP exhibited superior cytotoxicityas compared to the biotin free PTX@PEG-PCDANPs (IC_{50}: 1.68 v.s. 7.1μg/mL). This revealed thatbiotinplayed a critical role in enhancing cytotoxicity of the NPs, presumably by binding to MCF-7/ADR cell surface over-expressed biotin receptors,[28]leading to increased celluptake via receptor mediated endocytosis.This result was also consistent with the flow cytometry measurement results, where the biotin-grafted NPs showed higher cell uptake than those without biotin.More importantly, the PTX/MNPs/QDs@BPPmultifunctional NPswas the most cytotoxic against the MCF-7/ADR cells in the presence of an external magnetic field among all of treatment groups.Its IC_{50} value was considerably lower (0.89 *v.s.* 1.70 μg/mL) than that in the absence of magnetic field, which again was fully consistent with the much higher cell uptake observed from flow cytometry above. This result clearly demonstrates that the combined magnetic field and biotin dual-targeting strategy was more effective than those replying on biotin targeting alone, a considerable advantage of the multifunctional NPs reported herein over other traditional single-modal targeting NPs.[29]

Table 2summarised the ability of the above NP formulations of reversing the drug resistance on MCF-7/ADR cells. This was given as resistance reversion index (RRI), which was defined as the ratio of IC_{50} of free PTX to that of the PTX nanomedicine formulations. As shown in Table 2,treatment with the PTX/MNPs/QDs@BPPin the presence ofexternal magnetic field had a RRI of 16.7 which was significantly higher than any other treatments toward the PTX-resistantMCF-7/ADR cells. This highly encouraging resultwas benefited bytheability of the multifunctional NPto effectively exploit the magnetic/biotindual-modal targeting to achieve high cell uptake, efficient intracellular GSH-triggered co-release of curcumin and PTX for simultaneous dual-drug therapy, and therebymaximising the treatment synergy to overcome drug resistance in the MDR tumour cells. Therefore, thePTX/MNPs/QDs@BPPNP developed herein appears to be a highly effective, targeted, and traceable multifunctionalnanomedicineforeffective treatment of MDR cancer at the cellular levels.

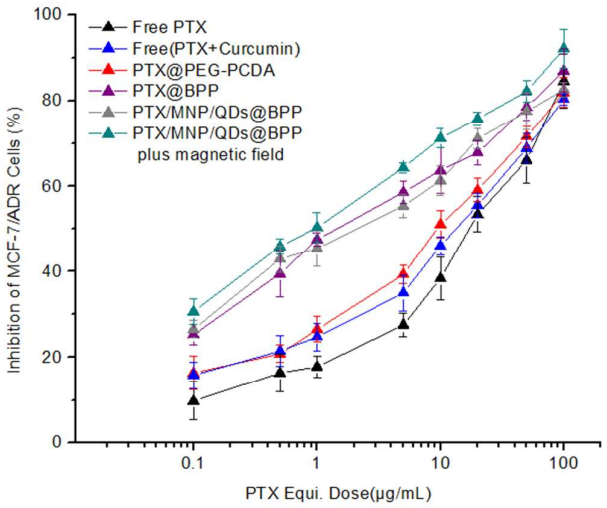


Fig.7.PTX-dose dependent*in vitro* cytotoxicity of the different PTX formulations against the drug resistant MCF-7/ADR cellsafter 48 h incubation.

Table 2 IC_{50} values and drug resistance reversion index (RRI) of various formulations ofPTX against MCF-7/ADR cells.

Formulations	IC_{50} values (μg/mL)	RRI*
Free PTX	14.9	—
Free PTX+Curcumin	9.4	1.6
PTX@PEG-PCDA	7.1	2.1
PTX@BPP	1.68	8.9
PTX/MNPs/QDs@BPP	1.70	8.7
PTX/MNPs/QDs@BPP plus magnetic field	0.89	16.7

*Resistance reversion index(RRI): the ratio of IC_{50} for free paclitaxel to that of paclitaxel withreversal agents against the MCF-7/ADR cells.

4. Conclusion

A novelmagnetic/biotin dual-modal targeting and traceablemultifunctional nanomedicine (PTX/MNPs/QDs@BPP)for efficient treatment of multi-drug resistance breast cancer at the cellular level was developed.Itwas based on co-encapsulation of PTX, MNP and QD into the hydrophobic core of a self-assembled biotin-PEG-PCDA block co-polymer. The NPs werehighly stable under physiological conditions, but were quickly dis-assembled to release itsdrug loads in the presence of 10 mMGSH.The NPs were efficiently up-taken by tumour cellsfrom the combinedeffect of magnet field guided NP concentration and biotin receptor-mediated

This journal is © The Royal Society of Chemistry 20xx

Please do not adjust margins

internalization.It can provide efficient intracellular GSH-triggered release ofcurcumin andPTX to offer simultaneous dual-drug treatment, leading to significantly improved therapeutic efficacy against multidrug resistant MCF-7/ADR cells.Taken together, the PTX/MNPs/QDs@Biotin-PEG-PCDANP whichcombines the ability of fluorescence tracking, MNP/biotin based dual-modal targeting and efficient cell uptake, GSH-triggered intracellular dual-drug release and simultaneous synergistic dual-drug treatment, appear to be a novel and effective multifunctional nanomedicine for overcoming MDR in cancer cells. Further work will be focused on exploiting thismultifunctional NP for in vivo applications.

Acknowledgements

This work is supported by National Natural Science Foundation of China (NSFC, Grant No. 81573352) and the EU FP7 via a Marie Curie International Incoming Fellowship to S.G. (grant No: PIIF-GA-2012-331281)

Notes and references

1 J. I. Fletcher, M. Habere and M. J. Henderson, *Nat. Rev. Cancer,* 2010, 10,147-156.

2 F. H. Wang, D. R. Zhang, Q. Zhang, Y. X. Chen, D. D. Zheng, L. L. Hao, C.X. Duan, L. J. Jia,G. P. Liu and Y. Liu, *Biomaterials*, 2011, 10, 1-13

3 S.H. Jang, M.G. Wientjes and J.L. Au, *J. Pharmacol. Exp. Ther*., 2001, 298, 1236–1242

4 S.J. Fey and K. Wrzesinski, Toxicol. Sci., 2012, 127, 403–411.

5 C. Sarisozen, A. H. Abouzeid, V. P. Torchilin, *Eur. J. Pharm.Biopharm*., 2014, 88, 539-550.

6 J. Sharkh, D. D. Ankola, V. Beniwal, D. Singh, andM.N.V.R. Kumar, *Eur. J. Pharm. Sci*., 2009, 37, 223-230.

7B. H. Choi, C. G. Kim, Y. Lim, S. Y. Shin,andY. H. Lee, *Cancer Lett*., 2008, 259, 111-118.

8 S. Anuchapreeda, P. Leechanachai, M. M. Smith, S. V. Ambudkar, andP. Limtrakul, *Biomaterials Pharmacology*, 2002, 64, 573-582.

9 W. Huang, W. Wang, P. Wang, Q. Zhang, Q. Tian, Y. Zhang, X. H. Wang, R. T. Cha, C. H. Wang and Z. Yuan, *J. Mater. Sci. Mater. Med*., 2011, 22, 853-863.

10 Z. Shen, W. Wei, H. Tanaka, K. Kohama, G. Ma, T. Dobashi, Y. Maki, H. Wang, J. Bi and S. Dai, *Pharmacol. Res*., 2011, 64, 410-419.

11 S. C. Kim, D. W. Kim, Y H. Shim, J.S.Bang, H. S. Oh, S. W. Kim and M. H. Seo, *J. Control. Release*, 2001, 72 191-202.

12 S. V. Bava, V. T. Puliappadamba, A. Deepti, A. Nair, D. Karunagaran and R. J. Anto, *J. Biol. Chem*., 2005, 280, 6301–6308.

13S. Aggarwal, H. Lchikawa, Y. Takada, S. K. Sandur, S. Shishodia and B. B. Aggarwal, *Mol.Pharmacol.*, 2006, 69, 195-196

11 T. Ramasamy, J. H. Kim, J. Y. Choi,T. H. Tran, H. G. Choi and C. S. Yong, *J. Mater. Chem. B*, 2014, 2, 63-74.

12 D. N. Waterhouse, R. Klasa, K. Chi, D. Huntsman, E. Ramsay, E. W ASAN, l. Edwards, C. Tuker, J. Zastre, Y. Z. Zhang, D. Yapp, W. Dragowska, S. Dedha and M. B. Bally, *CurrentCancer Drug Targets*, 2006, 6, 455-489.

13 M. S. Web, S. Johnstone, T. J. Morris, A. Kennedy, T. Gallagher and N. Harasym, *Eur. J. Pharm. Bio*., 2007, 65, 289-299.

14 K. K. Jain, *BMC Med*., 2010, 8, 83..

15 J. W. Park, K. Hong, D. B. Kirpotin, G. Colbern, R. Shalaby, J. Baselga, Y. Shao, U. B. Nielsen, J. D. Marks, D. Moore, D. Papahadjopoulos and C. C. Benz, *Clin. Cancer Res*., 2002, 8, 1172-1181.

16 N. Senzer, J. Nemunaitis, D. Nemnaitis, C. Bedell, G. Edelman, M. Barve, R. Nunan, K. F. Pitollo, A. Rait and E. H. Chang, *Mol. Ther*., 2013, 21, 1096-1103.

17 J. L. Swift, R. Heuff and D.T. Cramb, *Biophys. J*., 2006, 90, 1396–1410.

18 I. L. Medintz, H. T. Uyeda, E. R. Goldman and H. Mattoussi, *Nat Mat*., 2005, 4, 435-446.

19L.Lv, Y. Y. Shen, M. Li, F. H. Wang, M. N. Li,A. J. Guo, Y. Wang, D. J. Zhou, S. R. Guo and S. T. Huang, *J. Biomed Nano*., 2014, 10, 179-193.

20 L.Lv, Y. Y. Shen, M. Li, X. F. Xu, M. N. Li, S. R. Guo,and S. T. Huang, *J. Biomed Nano*., 2014, 10, 324-325.

21 F. H. Wang, Y. Y. Shen , X. F. Xu, L.Lv, Y. G. Li, J. Y. Liu, M. Li, A. J. Guo, S. R. Guo,and F. Jin, *Int. J. Pharm*., 2013, 456, 101-112.

22 K. Yang, K. Yang, H. B. Peng, Y. H. Wen, andN. Li, *Appl. Surf. Sci*., 2010, 256, 3093-3097.

23 G. Zhao, J. J. Feng, Q. L. Zhang, S. P. Li, andH. Y. Chen, *Chem. Mater*. 2005, 17, 3154-3159.

24 A. Colin de Verdiere, C. Dubernet, F. Nemati, M. F. Poupon, F. Puisieux, andP. Couvreur, *Cancer Chemother. Pharmacol*. 1994, 33, 504-508.

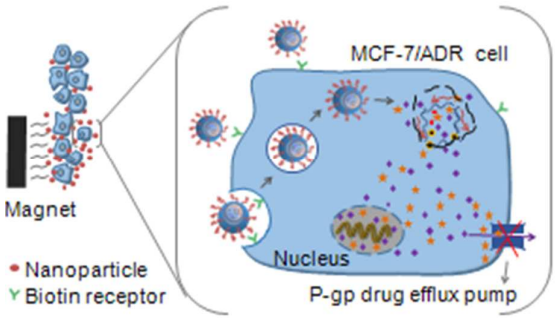
25 L. Lv, Y. Guo, Y.Y. Shen, J.Y. Liu, W.J. Zhang, D.J. Zhou, andS. R. Guo,*Adv. Healthcare Mater*. 2015, 4, 1496-1501.

26 T. Minko, P. Kopeckova, V. Pozharov,and J. Kopecek, *J. Controlled Release*, 1998, 54, 223.

27 V. G. Demillo, M. X. Liao, X. S, Zhu, D. Redelmanc, N. G. Publicover, andK. W. Hunter, *Colloids Surf., A*, 2015, 464, 134-142.

28 J. Kim, J. E. Lee, S. H. Lee, J. H. Yu, J. H. Lee, T. G. Park, andT. Hyeon, *Adv. Mater*., 2008, 20, 478-483.

29. S. Lübbe, C. Bergemann, W. Huhnt, T. Fricke, H. Riess, J. W. Brock, andD. Huhn, *Cancer Res*. 1996, 56, 4694.



A multifunctional anti-cancer nanomedicine PTX/MNPs/QDs@Biotin-PEG-PCDA was developed aiming at overcoming paclitaxel resistance in MCF-7/ADR breast cancer cells with simultaneous imaging.