Functionalized Nanoparticles Enable Tracking the Rapid Entry and Release of Doxorubicin in Human Pancreatic Cancer Cells

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Abstract

Efficient drug delivery is critical to therapy. Using electron microscopy, X-ray, and light microscopy, we have characterized functionalized superparamagnetic iron oxide (SPIO) nanoparticles, and determined their ability for rapid entry and release of the cancer drug doxorubicin in human pancreatic cancer cells. Dextran-coated SPIO nanoparticle ferrofluid, functionalized with the red-autofluorescing doxorubicin and the green-fluorescent dye fluorescein isothiocyanate as a reporter, enables tracking the intracellular nanoparticle transport and drug release. This engineered nanoparticle enables a >20 fold rapid entry and release of the drug in human pancreatic cancer cells, holding therapeutic potential as an advanced drug delivery platform. The low extracellular pH of most tumors precluding the entry of a number of weakly basic drugs such as doxorubicin, conferring drug resistance, can now be overcome.

1. Introduction

Low extracellular pH of most tumors, preclude the entry of a number of weakly basic drugs, conferring drug resistance. To overcome this resistance, superparamagnetic iron oxide (SPIO) nanoparticles (NPs) that enter cells through endocytosis, possess low toxicity, excellent biodegradability, produced inexpensively and rapidly, capable of magnetic resonance imaging (MRI) and therapeutic hyperthermia, have been engineered in the current study for rapid entry and release of the cancer drug Doxorubicin (DOX) in human pancreatic cancer cells. The novel 0.5-1.0 nm dextran functionalization of SPIO NPs to obtain a ferrofluid, and the chemical linking of a reporter fluorescent dye fluorescein isothiocyanate (FITC) and the red-autofluorescing cancer drug DOX, enables tracking the rapid intracellular entry, transport, and the release and localization of the drug to the target organelle, holding therapeutic potential as an advanced drug

delivery platform. The unique properties of nanoparticles (NPs) have been extensively exploited due to the broad spectrum of their use ranging from applications in electronic materials to medical therapy (Davis and Shin, 2008). In the last decade, gold (Chithrani et al., 2006; Huang et al., 2006), silver (Foldbjerg et al., 2011; Jeyaraj et al., 2013) and magnetic NPs (Pankhurst et al., 2003) especially superparamagnetic iron oxide (SPIO), have emerged as potential candidates for the diagnosis and treatment of various forms of cancer, due in-part to their low toxicity, biodegradability, drug delivery, magnetic resonance imaging (MRI), hyperthermia, and its inexpensive and rapid synthesis (Pankhurst et al., 2003). The interaction of these NPs with cancer cells continues to be studied for understanding their usage in drug delivery and therapy (Dam et al., 2012; Veiseh et al., 2010; Verma and Stellacci, 2010). The SPIO NPs exhibiting unique superparamagnetic properties (no magnetic hysteresis) combined with biocompatibility are ideal for use in nanomedicine (Gupta and Gupta, 2005; Peng et al., 2008; Petri-Fink et al., 2005; Thorek et al., 2006). Surface functionalization of these SPIO NPs with anti-cancer drugs have been conducted in recent years to determine their efficacy in cancer treatment (Berry and Curtis, 2003), however the major issue has been the entry of a number of weakly basic cancer drugs, among them Doxorubicin (DOX), conferring drug resistance (Gerweck and Seetharaman, 1996; Stubbs et al., 2000). Studies report that Polyethylene Glycol (PEG)-functionalized porous silica shell on DOX-conjugated Fe_3O_4 nanoparticle cores (Chen et al., 2010), Poly(amidoamine) or PAMAM-coated Fe_3O_4 NPs-DOX complex (Nigam et al., 2014), and DOX-loaded Fe_3O_4 NPs modified with PLGA-PEG copolymers (Akbarzadeh et al., 2012), hold potential promise in cancer therapy. Pancreatic cancer, among the most aggressive forms of cancer, is a leading cause of mortality (Siegel et al., 2015). Although DOX-conjugated NPs have been used as drug carrier and delivery platform especially in treating human breast cancers (Brigger et al., 2012), their use

in the treatment of human pancreatic cancer cells has been little explored. This necessitated a detailed understanding of the entry, intracellular distribution, and drug release of SPIO-drug functionalized NPs in human pancreatic cancer cells, which is the subject of the current study. In this paper, we report the rapid synthesis of 10 nm SPIO-dextran ferrofluid, with the novel conjugation of drug (DOX) and a fluorescent tracker (FITC) using minor modification of a published EDC/NHS procedure (Young et al., 2009), as well as the demonstration of its rapid entry, subcellular drug release and accumulation in the target organelle in human pancreatic cancer MIA PaCa-2 cells. This unique drug-dye dual conjugate has enabled the tracking of SPIO NPs entry into cells and the subcellular release and distribution of the drug, with drug accumulation in the nucleus (target organelle) and little or no FITC in the organelle. Our study further demonstrates that the rate of intracellular entry of the drug is >20 fold greater when conjugated to the SPIO NPs, as opposed to when free.

2. Materials and Methods

2.1 Synthesis of SPIO NPs

An aqueous solution of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a molar ratio of 1:2 was mixed in a beaker. A 2M NH_4OH solution was added into the mixture at once, under continuous magnetic stirring at 700 rpm. Upon addition of the base, a brown color precipitate was formed which gradually turned black, indicating the formation of iron oxide (Fe_3O_4) NPs. The NPs were then isolated with a magnet and washed several times with deionized double distilled water until a neutral pH was achieved. The synthesized NPs were used for further surface functionalization while a small fraction was dried to obtain a powder for use in structural and magnetic characterizations

2.2 Synthesis of dextran-coated SPIO NPs

Equivalent masses of SPIO NPs and dextran (MW: 15-20 kDa) were dispersed in similar volumes of 0.5 M NaOH separately. The dextran solution was kept under continuous sonication while the suspension containing the NPs was gradually added drop-wise. The resultant mixture was further sonicated for 24 h to obtain a well-dispersed ferrofluid.

2.3 Structural and Magnetic Characterizations of synthesized SPIO NPs

The crystalline nature of the synthesized SPIO powder NPs was confirmed using a Rigaku MiniFlex 600 x-ray diffractometer, generating Cu $K\alpha$ radiation at a wavelength of 1.54 Å. For determine the shape, size, and detailed morphology of both bare and dextran coated SPIO NPs, a JEOL-2010 FasTEM transmission electron microscope (TEM) operated at 200 kV was employed, with the NPs sonicated in alcohol before placing them on a carbon coated copper grid for obtaining high resolution images. The *dc* magnetic properties of these bare superparamagnetic NPs were investigated by a Quantum Design physical property measurement system (PPMS). For conducting the magnetic measurements, approximately 30 mg of the nanoparticle sample was mounted in a gelatin capsule packed with cotton (to restrict the motion of the particles).

2.4 Crosslinking of dextran coated SPIO NPs

The solution containing 1 ml of dextran-coated NPs, 5 ml of 5 M NaOH, 2 ml of deionized water and 2 ml of ECH was prepared. This mixture was then incubated at room temperature under continuous shaking to promote the interaction between the aqueous and the organic phases. Following 24 h of incubation, the black colloidal suspension was dialyzed several times against deionized water to remove any excess ECH and residual ions.

2.5 Carboxymethylation of crosslinked SPIO NPs (CMD-SPIO)

10 ml of the crosslinked SPIO NPs having a concentration of 4mg/ml was adjusted to pH 11 using 0.1 M NaOH under continuous stirring for 1 h at room temperature while purging with N₂ gas. 100 mg of monochloroacetic acid (MCA) was then slowly added to the solution and the mixture was heated at 60°C in a water bath and under N₂ atmosphere. The reaction mixture was then dialyzed against deionized water to obtain the CMD-SPIO.

2.6 Double labeling of Dextran-SPIO NPs with DOX and FITC (SPIO-DOX/FITC)

The DOX and FITC were attached to the CMD-SPIO NPs through EDC/NHS chemistry. 10 mg of EDC and 6 mg of NHS was added to 2.5 ml CMD-SPIO NPs suspension and stirred for 15 minutes at room temperature to activate the carboxyl groups. 1 μmol of DOX was added to this mixture and subjected to stirring for 10 minutes, followed by the addition of 1 ml Ethylene diamine hydrochloride (EDA). The resulting mixture was then dialyzed for 24 h to get rid of unbound drug and EDA. 1 μmol of FITC solution was added to this amino functionalized CMD-SPIO NPs solution. After 2 h, the mixture was subjected to short time interval frequent dialysis. The final concentration of the SPIO NPs in the resulting DOX/FITC conjugated CMD-SPIO NPs (Dex-SPIO-DOX/FITC) was estimated to be 2 mg/ml. All these experiments were carried out in the dark.

2.7 Determination of hydrodynamic size and Zeta potential

The stability and hydrodynamic size of the colloidal dispersion of the bare and functionalized NPs was obtained using magnet-induced aggregation, Zetasizer and Zeta Potential measurements (Nano ZS90, Malvern Instruments), and Dynamic Light Scattering (DLS). Small amounts of Dex-SPIO and Dex-SPIO-DOX/FITC nanoparticle samples were diluted with deionized water (~100 μg/ml) for Zetasizer measurements. All measurements were performed at 25°C under a pH of 7.4.

2.8 Estimation of drug/dye encapsulation efficiencies

The fluorescence (FL) intensity of the fluorescent dyes was estimated using a Hitachi F-2000 Fluorescence spectrophotometer. The calibration plots of fluorescence intensity versus the concentration of the drug/dye were initially obtained as follows. The different concentrations (1, 5, 10, 25, 50 $\mu\text{g/ml}$) of DOX and FITC solutions were prepared from a stock solution of 500 $\mu\text{g/ml}$. A drop (equivalent to 10 μl) of DOX and FITC solutions was then added on a glass slide and the fluorescence images were taken at an optimized exposure time with the red channel and the green channel respectively using a 40X oil immersed objective lenses in a Axiovert 200 Microscope (n=4). For each concentration, the intensity plot profile was determined and the area under the curve was calculated which is a measure of the intensity. Using these intensities, the calibration curves were plotted (Fig. 1A and B). Adopting similar experimental procedure, the fluorescence intensities (red and green) of the conjugated nanoparticle samples were also determined and accordingly the drug/dye concentrations were estimated from the calibration curves.

2.9 Cell culture and treatments

Cell studies involving membrane binding and intracellular distribution of surface functionalized NPs in the human pancreatic carcinoma cell lines MIA PaCa 2, were imaged with an optical microscope (Axiovert 200, Zeiss). Equivalent amounts of MIA PaCa-2 cells were seeded in 35 mm poly L-lysine coated petri dishes with 2 ml of cell growth medium containing Dulbecco's modified essential medium (DMEM), 10% fetal bovine serum (FBS) and 5% penicillin. The cells were then incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere. Following 24h of incubation, half the medium was replaced with fresh medium, containing free DOX, FITC, or 200 μg of Dex-SPIO-DOX/FITC. After 15 min of incubation, cells were washed three times

with 1ml of PBS in each wash, to remove any excess and unbound NPs. Cells were then fixed with 4% glutaraldehyde in PBS at 25°C for 30 min prior to imaging. The same procedure was followed with cells incubated with free DOX and FITC. The concentration of free DOX and FITC were kept approximately identical to the respective drug and dye, present in Dex-SPIO-DOX/FITC. All experiments were carried out in triplicates.

3. Results and Discussion

The hydrophilic SPIO NPs were synthesized using a minor modification of the established chemical co-precipitation technique (Laha et al., 2013; Nemala et al., 2014), and mildly coated with dextran to obtain a stable ferrofluid (Nemala et al., 2014). Our synthesis of dextran coated SPIO NPs, which is schematically illustrated in Fig. 2A, demonstrates success in the formation of a stable dispersed ferrofluid (Fig. 2B), where the magnet-induced aggregation is overcome in the dextran-coated NPs. To determine if the synthesized SPIO NPs are phase-pure, powder x-ray diffraction (XRD) was performed, demonstrating the bare SPIO NPs to contained all the peaks indexed to the crystal structure of phase-pure Fe_3O_4 (JCPDS card number: 85-1436) (Laha et al., 2013) (Fig. 2C). The average crystallite size of NPs was estimated to be around 7.2 ± 0.8 nm applying the Debye-Scherrer's equation (Nemala et al., 2014). The magnetization (M) as a function of magnetic field (H) is depicted in Fig. 2D, and the sigmoidal but anhysteretic nature of the curve demonstrates superparamagnetic behavior of the NPs (Laha et al., 2013; Nemala et al., 2014). The saturation magnetization of these NPs at 300 K was found to be approximately 61 emu/g, which is lower than the reported values of bulk Fe_3O_4 (92 emu/g) (Han et al., 1994). The reduction in saturation magnetization is attributed to the surface disorder or spin canting at the nanoparticle surface (Lin et al., 2006). In further characterization of the

synthesized SPIO NPs, the detailed morphology of both bare and dextran-coated NPs were examined using transmission electron microscopy (TEM). TEM images reveal that the NPs are roughly spherical with a narrow particle size distribution (Fig. 2E and F). Dextran (Dex)-coated NPs demonstrate a 0.5 nm outer coating, resulting in approximately a 1 nm separation between them (Fig. 2G and H). To estimate particle dimensions, particle size from multiple images were measured, and plotted as a histogram to illustrate their size distribution. The histogram data is

fitted to a log-normal distribution function given by
$$f(D) = \frac{1}{\sqrt{2\pi}\sigma D} \exp\left\{-\frac{\left[\ln\left(\frac{D}{D_0}\right)\right]^2}{2\sigma^2}\right\}$$

where D_0 is the most probable particle diameter and σ is the width of the distribution (Nemala et al., 2014). The histogram and the log-normal fits shown in Fig. 2F demonstrate a narrow distribution, with majority of the NPs in the 6-11 nm range. Using the following equations, $D_{TEM} = D_0 \exp(\sigma^2/2)$ and $\sigma_{TEM} = D_{TEM} [\exp(\sigma^2-1)]^{1/2}$, the average particle size and the standard deviation of the distribution were estimated to be 8.4 nm and 2.6 nm respectively. This slight increment in the particle size estimated using TEM over XRD (XRD: 7.2 nm, TEM: 8.4 nm) is in conformation with earlier published studies (Laha et al., 2013; Nemala et al., 2014) and is primarily attributed to the presence of an amorphous layer on the surface of the NPs observed using TEM.

The dextran coated SPIO NPs (Dex-SPIO) were crosslinked with epichlorohydrin (ECH) using an established procedure (Wunderbaldinger et al.) and subsequently functionalized with carboxylic groups (-COOH) using monochloroacetic acid (MCA) to obtain carboxy-methylated SPIO NPs (CMD-SPIO). Both DOX and FITC were conjugated to the CMD-SPIO NPs through EDC/NHS chemistry (Young et al., 2009). EDC and NHS were added to CMD-SPIO NPs

suspension to activate the carboxyl groups, and DOX was added to that mixture followed by Ethylene diamine hydrochloride (EDA), which reacted with the activated carboxyl groups to generate amine groups ($-NH_2$) for further conjugation with FITC (Fig. 3). The encapsulation efficiencies of DOX and FITC in the conjugate were estimated to be 1% and 10% respectively using fluorescence micrograph image analysis (Fig. 1). The surface charge or zeta potential values of Dex-SPIO and Dex-SPIO-DOX/FITC were estimated to be -17 ± 3 mV and -9 ± 1 mV respectively. This change in surface charge of Dex-SPIO upon functionalizing with DOX/FITC, further attests to the successful conjugation of the dye and drug to the Dex-SPIO complex, besides the observed co-association (yellow fluorescence) of the green (FITC) and red fluorescence (DOX) with the SPIO NPs.

To determine the potency and efficacy of DOX entry into human pancreatic cancer cells, the human pancreatic cancer cell line MIA PaCa 2 was used. Cells were exposed to free DOX, free FITC, and Dex-SPIO-DOX/FITC, and time-dependent entry of DOX is summarized in Fig. 4. Results from the study demonstrate that it takes nearly 6h for free DOX to localize to the nucleus, the target subcellular organelle (Fig. 4A), resulting in blebbing and cell death. At the 15 min time point, free DOX and FITC bind to the cell plasma membrane, demonstrating little entry into the cell (Fig. 4B) top control panel. In contrast, exposure of the MIA PaCa 2 cells for 15 min to Dex-SPIO-DOX/FITC, demonstrates rapid (15 min) cellular entry of the functionalized NPs and the release and accumulation of DOX in the nucleus (Fig. 4B, white arrowheads). It has been well established that free DOX is taken up by cells very slowly only through Fickian diffusion (Dalmark et al., 1981; Dash et al., 2010) while the cellular uptake of SPIO is most likely via endocytosis (Arora et al., 2012; Oh and Park, 2014). In this context, the Dex-SPIO-DOX/FITC NPs facilitated cellular uptake of DOX through endocytosis. It is interesting to note that the red

and green fluorescence are separated within the cell since the red autofluorescing DOX accumulate within the nucleus while green fluorescence remain primarily contained in the cytosol, indicating the release of DOX from the NPs following their entry into the pancreatic cancer cell. An efficient drug delivery system requires both the rapid uptake as well as the targeting and subcellular release of the drug for effective therapy, all these attributes being met by our Dex-SPIO-DOX/FITC NPs.

4. Conclusions

In conclusion, we were able to design and develop a drug delivery platform with multimodal tracking (FITC fluorescent and MRI) and treatment (cancer drug and hyperthermia) modality, based on SPIO NPs as the vehicle for an anti-cancer drug DOX therapy, combined with the capability to precisely track the intracellular position of the NPs and of the released drug (Fig. 5). Employing fluorescent microscopy, we were able to demonstrate that even in the absence of any targeting ligand, cellular internalization and localization of DOX in the cell nucleus, is much efficacious when conjugated to SPIO NPs. In addition to the rapid uptake of DOX by live pancreatic cancer cells, our results also demonstrate that upon entering the cell, DOX is efficiently cleaved from the NPs, and accumulates in the target organelle, i.e., the nucleus. Further functionalization of the SPIO-DOX/FITC with peptide antibody, will help in tissue-specific whole-body targeting and therapy, given that SPIO are also capable of detection using magnetic resonance imaging. Additionally, the use of targeted SPIO for hyperthermia-mediated cell death is also possible (Singh et al., 2010).

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FIGURE LEGENDS

Figure 1 Calibration curves of (A) FITC and (B) doxorubicin (DOX) to estimate the drug/dye quantification incorporated with the NP conjugate.

Figure 2 Synthesis and characterization of bare and dextran-coated SPIO nanoparticles. (A) Schematic illustration of the synthesis of bare SPIO NPs using a chemical co-precipitation approach. (B) Left: The red arrow indicates aggregation of bare SPIO NPs dispersed in deionized water in presence of a magnet. Right: Dextran-coated SPIO NPs show no such aggregation in the presence of a magnet, demonstrating a stable dispersed ferrofluid. (C) X-ray diffraction pattern of approximately 8 nm sized bare SPIO NPs. (D) M (H) curve for SPIO NPs at 300 K demonstrating the superparamagnetic behavior with saturation magnetization of 61emu/g. (E) Transmission electron micrograph (TEM) of bare SPIO NPs. The inset shows a high-resolution TEM image, exhibiting the atomic lattice planes. (F) Particle size distribution plot with log normal fitting. (G) TEM image of dextran coated SPIO NPs. (h) TEM image showing the inter-particle separation to be approximately 1 nm in dextran coated SPIO NPs.

Figure 3 Schematic representation of the steps involved in dextran-coated SPIO surface conjugation chemistry of the cancer drug Doxorubicin (DOX) and intracellular tracking system (FITC).

Figure 4 The cancer drug doxorubicin potentiates cell death and is rapidly targeted into cells for release and accumulation in the nucleus when SPIO-conjugated. (A) Phase and fluorescent micrograph of MIA PaCa 2 cells incubated with DOX. Note cellular entry, especially into the nucleus (red fluorescence) of the free drug 6h following exposure. The inset shows blebbing of cells exposed to DOX prior to cell death (Scale = 20 μ m). (B) Phase and fluorescent micrograph of MIA PaCa 2 cells incubated with free DOX and free FITC (control) and DOX-FITC conjugated Fe₃O₄ NPs at 15 min. Green and red fluorescence represent FITC and DOX respectively. Note the accumulation of DOX in the nucleus in cells exposed to the DOX and FITC—conjugated SPIO NPs. White arrowheads indicate DOX entry into the nucleus.

Figure 5 Synthesis and functionalization of superparamagnetic iron oxide (SPIO) nanoparticles for rapid cellular entry and release of the cancer drug Doxorubicin (DOX) in human pancreatic cancer cells. Transmission electron micrographs (TEM) show approximately 8 nm core SPIO nanoparticles with 0.5–1.0 nm dextran coats, as demonstrated from the inter-particle separation. Note the high-resolution TEM image exhibiting the atomic lattice planes and the X-ray diffraction patterns of the synthesized SPIO nanoparticles. Dextran coated Fe₃O₄ core with DOX (red fluorescence) and FITC (green fluorescence) surface conjugation chemistry, and the rapid

entry (15 min) and intracellular release and accumulation of the cancer drug in the nucleus (white arrow head) of human pancreatic cancer Mia Paca-2 cells is shown.

A. **B.** **C.** **D.** **E.** **F.** **G.** **H.**

Step 1

Step 2

Step 3

A.

B.