

Combined Formulation of Doxorubicin-Arg-Gly-Asp (RGD) and Modified PEGylated PLGA-encapsulated Nanocarrier improves Anti-tumor Activity

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ABSTRACT

In this formulation, Doxorubicin (Dox) was conjugated to Poly (lactic-co-glycolic acid) (PLGA), and formulated via the solvent-diffusion techniques into nanoparticles. The surface of the nanoparticles was subsequently linked with Poly (ethylene glycol) (PEG) and Arg-Gly-Asp (RGD) peptide to achieve both passive and active targeting moieties. The nanoparticles were then tested against several malignant tumor cell lines. The conjugation increased loading efficiency of Dox to PLGA nanoparticles (the encapsulation efficiency was over 85%) and alleviated the drug burst release effect substantially. The drug was released from the polymeric matrix in a sustained release manner over a period of 12 days. The resultant nanoparticles were spherically uniform and well-dispersed. The nanoparticle targeting ability was proven through strong affinity to various *integrin*-expressing cancer cells, and much less affinity to the low *integrin* expression cancer cells. The nanoparticles

also showed high efficacy in inducing apoptosis in specific malignant cancer cells. Taken together, this multifunctional nanoparticles hold potential to treat malignant *integrin*-expressing cancers.

Keywords: RGD; tumor; drug delivery; integrin; nanoparticle

INTRODUCTION

The generation of safe and effective delivery vehicles has resulted in numerous reports on targeting tumor cells without eliciting adverse effects (1, 2). The over-expression of *integrin* receptors on some malignant cancer cells provides a distinct marker for targeted chemotherapeutical or diagnostic contrast agent deliveries. Typically, the Arg-Gly-Asp (RGD) peptide, which has high affinity to $\alpha\beta3$ *integrin*, (3) has been applied in experimental studies to deliver either drugs (4), gene (5) or imaging agents (6) to the desired targeted sites (7).

Site specific targeting nanoparticles with targeting peptides, like RGD, are considered as promising ligands for targeted delivery payloads to specific malignant cancer cells. Nevertheless, active targeting function is hardly successfully accomplished until the passive targeting function is realized (8). Poly (ethylene glycol) (PEG) for passive targeting goal is much favored by many researchers due to its biocompatibility, degradability, and less protein interaction characters in animal body (9).

Whilst Poly (lactic-*co*-glycolic acid) (PLGA) has been noted to be biologically friendly in most biomedical applications, most of the PLGA nanoparticles have been short of either passive or active targeting function, or both. In this report, we utilized PLGA-based nanoparticles with surface modification of PEG and targeting peptide RGD for simultaneous passive and active targeting functions. PLGA was chemically conjugated with Dox, a widely used anticancer drug, before it was fabricated into nanoparticles to avoid the burst effect commonly observed in nanoparticles physically loaded with therapeutic drugs (10). After extensive physicochemical characterization, selective uptake of the Dox-PLGA-PEG-RGD nanoparticles by different cancer cells was investigated via flow cytometry and confocal laser scanning microscope (CLSM). Favorable drug release profile, selective uptake and intracellular localization of the nanoparticles, as well as potent cytotoxicity in malignant cancer cells were demonstrated. Thus, nanoparticles assembling both passive and active targeting functions hold great potential in clinical application.

MATERIALS AND METHODS

Materials

PLGA (RG503H) and Amino-Poly (ethylene glycol) -Carboxylic acid (NH₂-PEG-COOH) with M.W. 2000 were obtained from Boehringer Ingelheim

(Germany). Poly (ethylene-maleic anhydride) (PEMA), *N*-hydroxysuccinimide (NHS), *N*- (3-Dimethylaminopropyl) -*N'*-ethylcarbodiimide hydrochloride (EDC), 4-Nitrophenyl chloroformate (pNC), pyridine, acetone, triethylamine (TEA), *N,N*-Dimethylformamide (DMF), 3- (4, 5-dimethylthiaol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 medium were purchased from Sigma Chemical, Co (St. Louis, MO, USA). Doxorubicin hydrochloride was purchased from Woo-Shin Medics, Co. Korea. B16F10 mouse melanoma cells, DU145 human prostate carcinoma cells, MDA-MB-231 human mammary carcinoma cells, and MCF-7 human mammary carcinoma cells were obtained from American Type Culture Collection (Rockville, MD).

Conjugation of Doxorubicin to PLGA

PLGA (Resome® 503H), with a lactide/glycolide molar ratio of 50/50, and free hydroxyl and carboxylic groups at its terminal ends, was used. Briefly, 0.5 g of PLGA was dissolved in 10 ml of methylene chloride and activated by the addition of 26.3 mg of p-nitrophenyl chloroformate and 18 μ L of pyridine to the solution, kept in an ice bath at 0°C. After isolation, 0.3 g activated PLGA was dissolved in 3 mL of dimethylformamide (DMF) and reacted with 12.9 mg of doxorubicin with 15.5 μ L of triethylamine for 24 h at room temperature under nitrogen atmosphere. The final conjugated product was precipitated by excess cold ether, and dried under vacuum. The efficiency of Doxorubicin conjugated to PLGA was determined by dissolving the conjugated products in DMSO, and the amount of Doxorubicin was quantitated by UV/VIS spectrometry (Perkin-Elmer, Japan) at 480 nm.

Preparation of PLGA-Doxorubicin (PLGA-Dox) Nanoparticles

PLGA nanoparticles were prepared by a solvent diffusion method. Briefly, 50 mg of PLGA-doxorubicin conjugate was dissolved in 3.5 mL of acetone, and the organic solution was added to 0.2% PEMA (35 mL) through a syringe pump (20 mL/h) under stirring at 1, 500 rpm in a fume hood to evaporate acetone overnight. The nanoparticles produced were collected and washed three times with deionized water, and followed by ultrafiltration (Millipore, Amicon®) at 4, 000 rpm for 45 min. Finally, the nanoparticles were dispersed in deionized water to give the desired concentration.

Synthesis of Dox-PLGA-PEG-RGD Nanoparticles

Ten milliliters of the prepared PLGA-Dox nanoparticle solution was diluted with 0.1 M MES (pH 6.0), and then subjected to ultrafiltration, and washed with 0.1 M MES twice. 153 mg EDC, and 230 mg sulfo-NHS were added into the precipitated nanoparticle suspension and incubated for 2 h at room temperature. After washing off the impurities with PBS buffer (pH 7.4), 50 mg NH₂-PEG-COOH with 6.9 μ l TEA was introduced into the activated PLGA-Dox nanoparticle suspension, and incubated for 4 h., further washed with PBS (pH 7.4), and separated by ultrafiltration. Finally, the nanoparticles were dispersed in PBS buffer to the desired concentration. The prepared PLGA-PEG

nanoparticles were once again diluted, washed, and suspended in 0.1 M MES (pH 6.0) for another cycle of EDC/NHS chemistry for conjugation of RGD peptide. The activated PLGA-PEG nanoparticle suspension was mixed with 1.5 mg RGD (Anaspec, USA), and incubated overnight for complete reaction. After that, the synthesized Dox-PLGA-PEG-RGD nanoparticles were dispersed in PBS (pH 7.4) buffer to the desired concentration.

Characterization of Nanoparticles

The mean size, polydispersity and zeta potential of the NPs were determined by size analyzer (Zetasizer 3000 HSA, Malvern Instruments Ltd., England). The encapsulated drug in the NPs and drug encapsulation efficiency were determined via spectrometry under 480 nm with microplate reader. The RGD peptide conjugated on the nanoparticle surface was calculated with the established fluorescamine method (11). The micro-structure of the NPs was visualized by transmission electron microscopy (TEM) (CM10, Microscope Philips) with 2% phosphor-tungstic acid staining.

Cytotoxicity Assay

The cell proliferation assay was conducted based on tetrazolium dye (MTT) method. MDA-MB-231, B16F10 and MCF-7 cells were grown in DMEM medium, and DU-145 cells were grown in RPMI-1640 medium. All the cell culture media were supplemented with 10% FBS, 0.1% nonessential amino acids, and 100 µg/ml penicillin, and 100 unit/ml streptomycin. The cells were separately seeded into 96-well plates at the density of 5×10^3 cells/well and incubated at 37 °C, 5% CO₂ for 24 h before experiment. NPs were diluted with corresponding culture media to different concentrations. Pure drug was used as control. After washing the cells with PBS buffer three times, different concentrations of samples were added to the respective cells at 200 µL/well. After incubation for 48 h, the cells were washed with PBS buffer, and further incubated with 120 µL/well MTT solution (0.5 mg/ml) for another 3 h. At the end of incubation time, the excess MTT solution was removed, and the formed formazan crystals were dissolved by DMSO. The resultant solution was measured at 570 nm with DMSO as blank (n=6).

Cell Uptake and Binding Affinity Assays

Since Dox is intrinsically fluorescent, this facilitated the visualization of the uptake of the Dox formulated NPs into cells under confocal laser scanning microscope (CLSM). To observe the internalization of NPs under CLSM, the desired cells were cultured over night in Lab-Tek® eight well chambers (Nalgene, Nunc Inc., Denmark; seeded at density of 2.0×10^4 cells/well). Then, the cells were washed with 3×0.2 mL/well of PBS and equilibrated with 0.2 mL/well of HBSS/HEPES at 37°C for 30 min. Freshly prepared nanoparticles were diluted with HBSS/HEPES to give the desired concentrations. After balancing the cells with HBSS/HEPES, the medium was then aspirated, and 200 µL/well NPs solution was added into each well. At different time points,

the uptake experiment was terminated by aspirating the test samples and washing the cell monolayers with 0.2 mL/well of ice-cold PBS three times. Then, each cell monolayer was fixed with methanol/acetone (1:1, v/v) followed by nuclear staining with DAPI (20 $\mu\text{mol/mL}$). The cells were observed under CLSM (Zeiss, Heidelberg, Germany) with excitation filter at 488 nm, long band pass emission filter 520 nm for internalized drug; and UV excitation for DAPI staining.

DNA Fragmentation Assay

For the DNA fragmentation assay, cells were grown in DMEM medium before treatment. On the experimental day, cells were treated with 1) cell culture medium; 2) pure drug dissolve in cell culture medium (5 $\mu\text{g/ml}$), and 3) Dox-PLGA-PEG-RGD nanoparticle with equivalent drug concentration in culture medium. After 48 h incubation, cells were washed with ice-cold PBS three times and collected by trypsinization. The cell pellets were re-suspended in 0.5 ml lysis buffer (1% Triton X-100 with 20 mM EDTA and 50 mM Tris-HCl, pH 7.4). The supernatant was subjected to 5 mg/ml RNase A at 56°C for 2 h, followed by incubation with 2.5mg/ml Proteinase K for at least 2 h at 37°C. After addition of 0.5 volume of 10 M ammonium acetate, DNA was precipitated with 2.5 volume of absolute ethanol at -20°C overnight. Then, the DNA was collected via high speed centrifugation, and rinsed with 70% ethanol once, and the DNA pellets were collected after centrifugation for 25 min at 14, 000 rpm. The retrieved pellet was dried in a SpeedVac to remove the residual ethanol. The resultant DNA was dissolved in TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8.0), followed by 1% agarose gel electrophoresis, and stained with ethidium bromide.

Determination of Dox-PLGA-PEG-RGD Nanoparticle Uptake by Flow Cytometry

MDA-MB-231 cells were grown in confluent condition. Dox-PLGA-PEG-RGD nanoparticles were diluted to the desired concentrations with HBSS buffer, and incubated with cells for 4 h. The cells were then washed three times with ice-cold PBS and trypsinized. The cell pellets were further washed with ice-cold PBS twice, and 1 ml 70% ethanol was added to fix the cells at -20°C overnight. The cells were washed once again before flow cytometry (Dako, USA) analysis.

***In vitro* Drug Release**

In vitro drug release profile of Dox-PLGA-PEG-RGD nanoparticles was determined by loading 1 ml of original nanoparticle solution into dialysis tubing (M.W. 12, 000), which was submerged into 10 ml PBS (pH 7.4), and shaken at 37°C, 100 rpm in a water bath. The released drug was retrieved at pre-determined time points, and analyzed by fluorescence spectrometry ($\lambda_{\text{ex}} = 480 \text{ nm}$; $\lambda_{\text{em}} = 590 \text{ nm}$).

RESULTS

Conjugation of Doxorubicin to PLGA

Fig. 1 shows the schematic route of Dox-PLGA conjugation by carbamate linkage between the hydroxyl group on PLGA and amine group on doxorubicin. The available carboxyl groups were ready for the subsequent linkage with the primary amine groups on PEG chain after formation of nanoparticles. The extent of conjugation of Dox to the PLGA was determined to be around 2.93% in weight ratio.

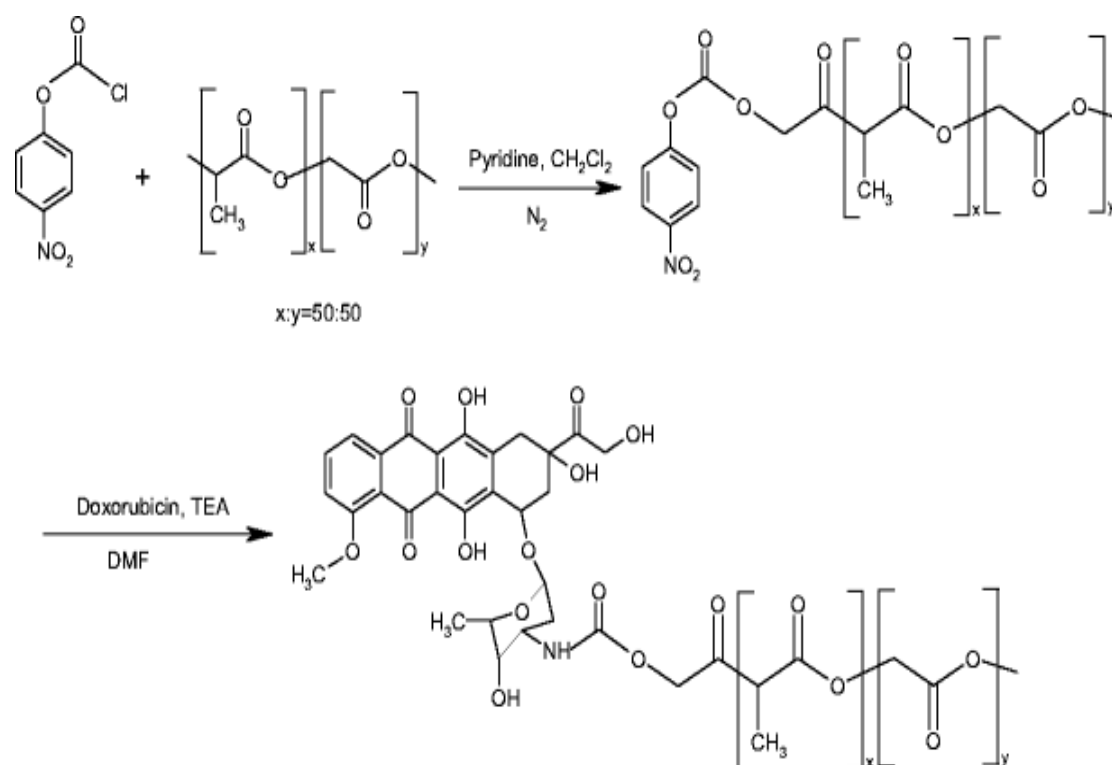


FIGURE 1: Scheme of conjugating doxorubicin to PLGA

Synthesis and Characterization of Dox-PLGA-PEG-RGD Nanoparticle

The schematic procedure of synthesis of Dox-PLGA-PEG-RGD nanoparticles is presented in Fig. 2. In the preparation of initial PLGA NPs, PEMA was used as surfactant instead of the conventionally used poly (vinyl alcohol) (PVA) to increase the surface carboxyl groups. As has been reported previously (12). The surface modification with PEG and RGD makes the PLGA nanoparticles more functional than the conventional unmodified PLGA nanoparticles.

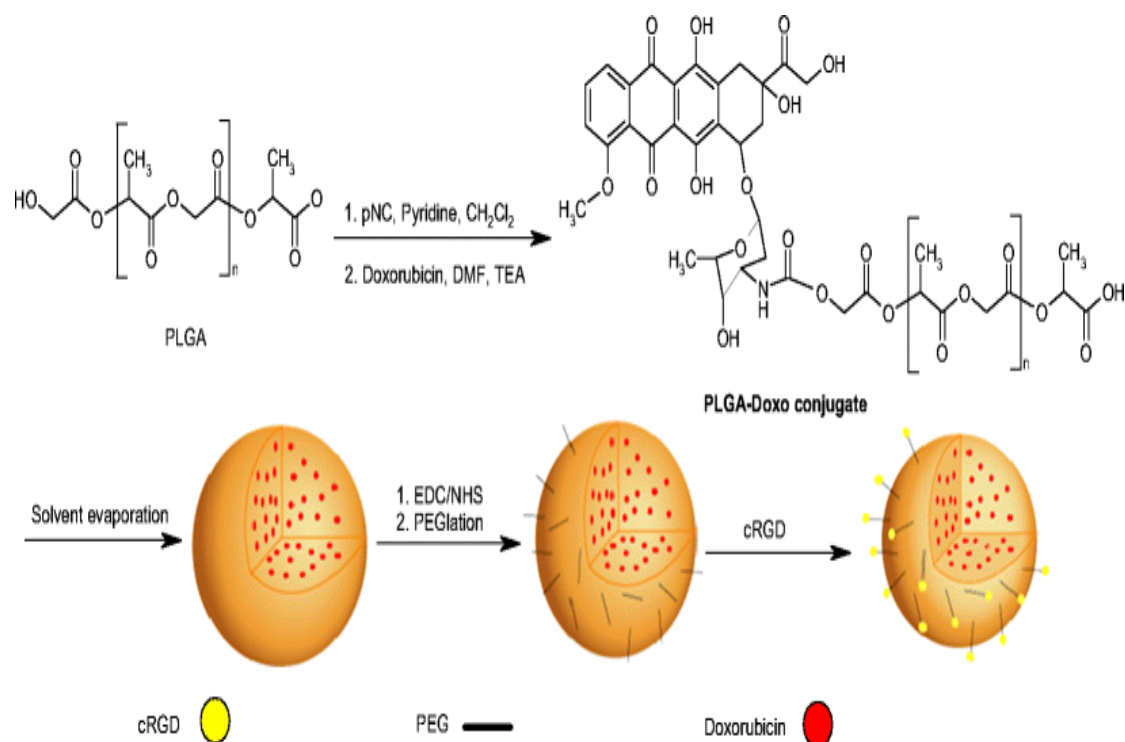


FIGURE 2: Schematic route of preparation of Dox-PLGA-PEG-RGD nanoparticles

The characteristics of PLGA nanoparticle (including conjugated Dox) (PLGA-Dox) and Dox-PLGA-PEG-RGD nanoparticle are summarized in Table I. It is obvious that after two steps of conjugation to the PLGA-Dox nanoparticle's surface with PEG and RGD, the mean size of NPs increased, and also the polydistribution became slightly wider. The surface charge of NPs dramatically increases from -51.7 ± 3.1 to -18.9 ± 2.4 mV, which was attributed to the surface modification with PEG and RGD, both of them consuming the carboxyl groups on the nanoparticle's surface. However, the surface modification did not significantly change the drug encapsulation efficiency (E.E.) and encapsulation capacity (E.C.), indicating that the formulation and series of modification procedures did not lead to dramatic drug loss, whilst the minor decrease of both E.E. and E.C. may be owing to the loss of unencapsulated Dox conjugates available on particle surface, or the minor amount of Dox cleaved from PLGA during the preparation period, due to the slow hydrolysis of liable glycol block on PLGA backbone in aqueous environment.

The morphology of as-prepared PLGA-Dox and Dox-PLGA-PEG-RGD nanoparticles were characterized by TEM, respectively. It can be seen from Fig. 3 that both types of NP were quite uniform and well-dispersed with low size distribution, similar to the results characterized by dynamic light scattering method (zetasizer measurement). Also, both NPs were solid, spherical nanoparticles with smooth surface.

Table I: Physical Characteristics of PLGA Based Nanoparticles (Mean \pm SD, $n=3$)^aEncapsulation efficiency: drug encapsulated in the NP to initially added drug into the formulation^bEncapsulation capacity: the amount of drug encapsulated into milligram of NP

	Size (nm)	Poly dispersity	Zeta potential (mV)	E. E. ^a (%)	E. C. ^b (μ g drug/mg NP)	RGD Quantity (nmol peptide/mg NPs)
PLGA-Dox	366.6 ± 3.1	0.158	-51.7 ± 3.1	88.3	15.32 ± 1.2	N/A
Dox-PLGA-PEG-RGD	423.0 ± 16.6	0.189	-18.9 ± 2.4	87.2	14.99 ± 2.4	0.15 ± 0.3

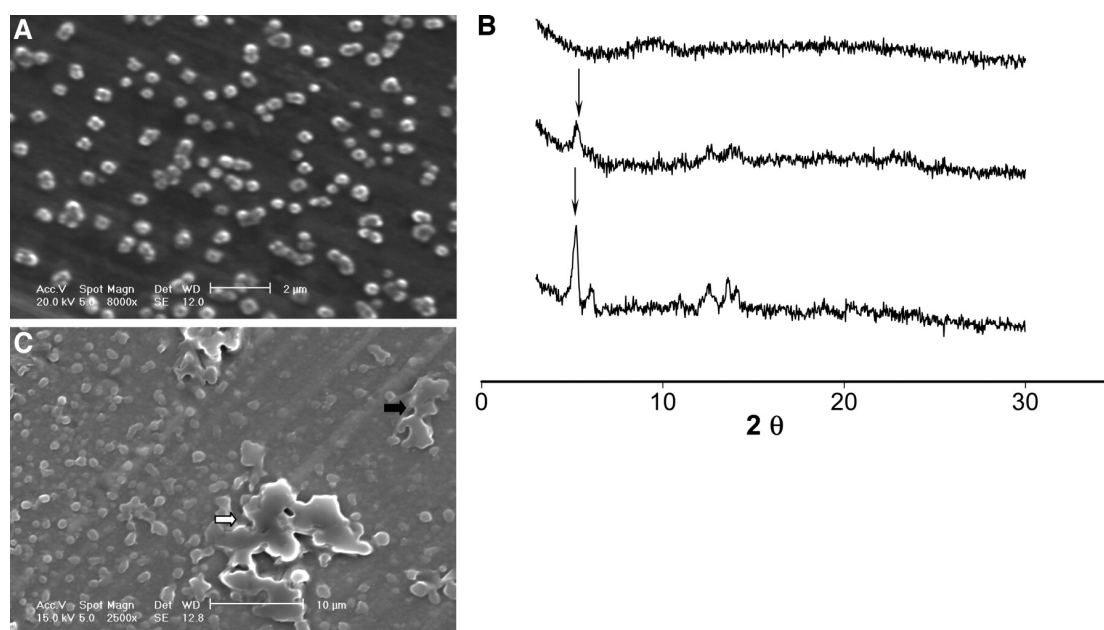


FIGURE 3: Characterization of Dox-PLGA-RGD nanoparticles. A, transmission electron micrographs of nanoparticles. Drug loading was 1.62%. Particle size was 423 ± 16.6 nm (mean \pm SD). B, wide angle X-ray diffraction spectra. Top, Dox alone. Middle, mixture of Dox (2% in weight) and blank nanoparticles. Bottom, Dox-loaded nanoparticles described in A. Arrows indicate the major diffraction peak of crystalline Dox. C, scanning electron micrograph of Dox-loaded nanoparticles. Large aggregates, white arrow; small aggregates, black arrow

Cytotoxicity of NPs to Various Tumor Cells

The cytotoxicity effects of both pure Dox and drug containing nanoparticles were conducted by an established MTT assay (Fig. 4). The drug concentration range was from 0.005 $\mu\text{g/ml}$ to 50 $\mu\text{g/ml}$. Equivalent amounts of drug encapsulated in drug containing nanoparticles were adjusted to the equivalent concentrations by the corresponding cell culture medium. Cytotoxicity of the drug free NPs was also investigated at the highest NPs concentration used in the MTT assay.

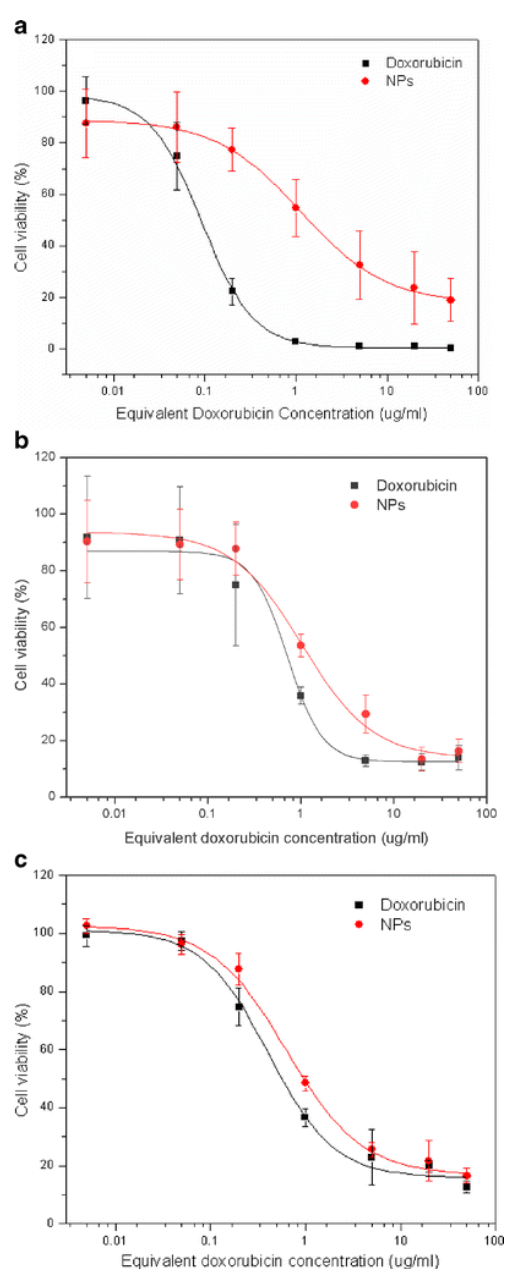


FIGURE 4: Cytotoxicity results of Doxo-PLGA-PEG-cRGD nanoparticles or doxorubicin to a B16F10 cells, b DU145 cells, and c MDA-MB-231 cells.

In terms of IC_{50} values, the three *integrin* expressing cancer cells retained different sensitivity to pure doxorubicin and Dox-PLGA-PEG-RGD NPs, respectively. With pure Dox, B16F10 cells showed the most sensitivity ($IC_{50}=0.09 \mu\text{g/ml}$), whilst DU145 ($IC_{50}=0.71 \mu\text{g/ml}$) and MDA-MB-231 ($IC_{50}=0.41 \mu\text{g/ml}$) cells presented similar, but lower sensitivity. However, this cytotoxicity trend dramatically changed when cells were treated with Dox-PLGA-PEG-RGD nanoparticles. After incubation with NPs, the NPs showed the most cytotoxic to MDA-MB-231 ($IC_{50}=0.63 \mu\text{g/ml}$), median with B16F10 ($IC_{50}=1.13 \mu\text{g/ml}$), and the lowest with DU145 ($IC_{50}=2.29 \mu\text{g/ml}$). It can be seen that for all the three *integrin* expressing cells, the cytotoxic efficacy of the Dox-PLGA-PEG-RGD nanoparticles was lower than that of the pure Dox. The cytotoxicity of PLGA-Dox NPs to these tumor cells was also tested (data not shown), which presented similar but slightly lower efficacy to all the above tumor cells, when compared to the Dox-PLGA-PEG-RGD NPs.

Cell Affinity Assay

Affinity of different malignant cells to pure drug, modified and non-modified NPs was investigated by CLSM. Fig. 5a–i shows the uptake of Dox-PLGA-PEG-RGD NPs at various time points by different tumor cells. Since B16F10 (13), DU145 (14) and MDA-MB-231 (15) cells all express $\alpha\beta_3$ *integrin* on cell membranes, Dox-PLGA-PEG-RGD NPs should be selectively recognized by these *integrin* expressing cancer cells upon exposure, and underwent endocytotic pathway into the cytoplasm. As shown in Fig. 5a–i, the uptake of NPs into cytoplasm followed a time-dependent manner. It was noted that the uptake of NPs was not significant after 2-h incubation, compared to that at 0.5 h. The most significant uptake of NPs was observed at 4 h incubation in B16F10, DU145 and MDA-MB-23 cells. Because of the lack of *integrin* on MCF-7 cell membrane (16), the uptake of RGD modified NPs into these cells was few over the 4-h incubation period, except for few non-specific association to cells after 4 h. The resultant 1-h uptake effect of Dox-PLGA-PEG-RGD NPs, PLGA-PEG NPs, and doxorubicin alone were also compared. It was found that all of the *integrin* expressing cells showed significant uptake of Dox-PLGA-PEG-RGD NPs, whilst the uptake of PLGA-PEG NPs and doxorubicin alone remained low (Figures not shown).

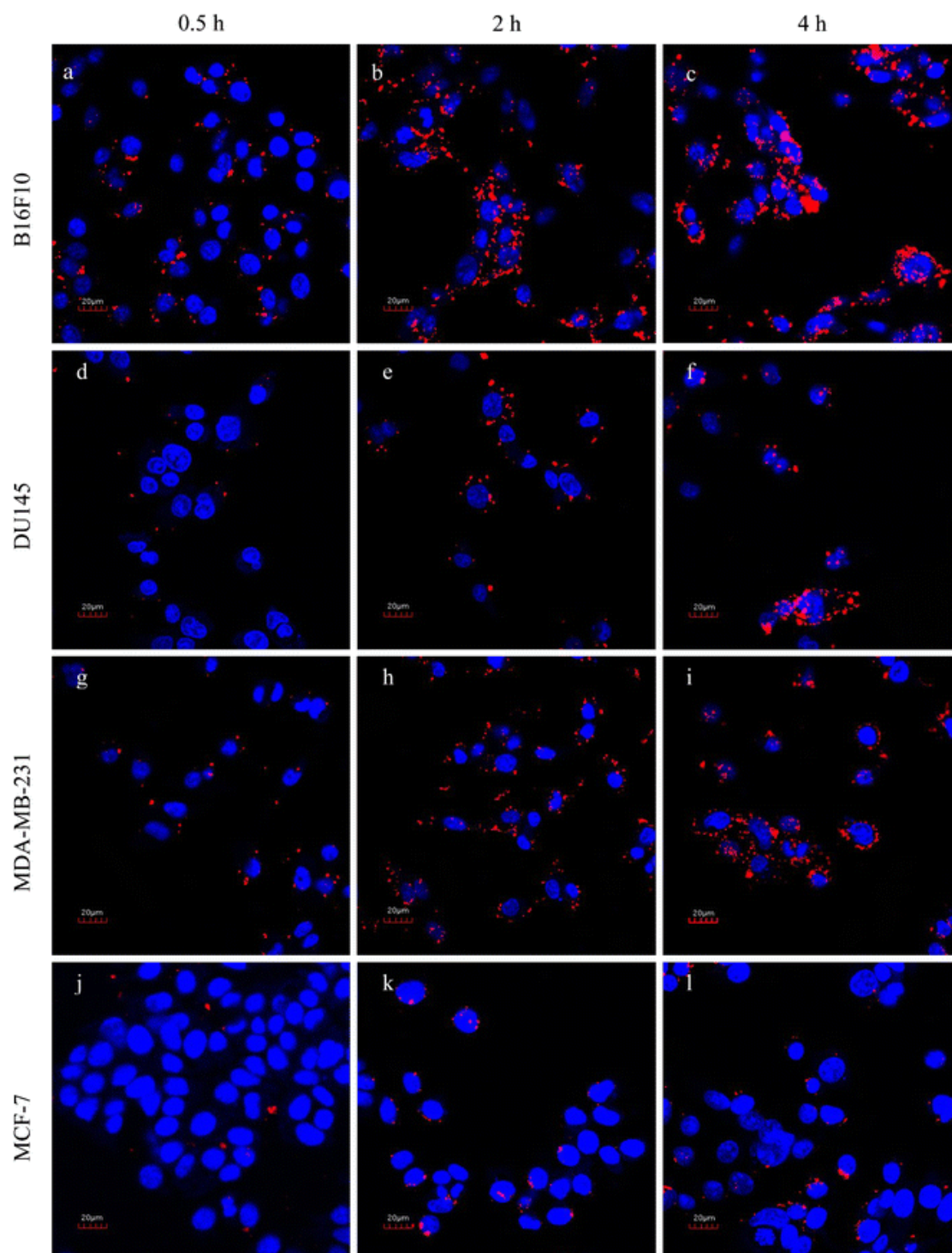


FIGURE 5: Cell Affinity Assay. CLSM results of cellular uptake of Dox-PLGA-PEG-RGD nanoparticles in 0.5, 2 and 4 h incubation of a-c B16F10 cells, d-f DU145 cells, g-i MDA-MB-231 cells, and j-l MCF-7 cells.

***In vitro* Drug Release**

The *in vitro* drug release profile of Dox-PLGA-PEG-RGD NPs is shown in Fig. 6. The drug released from the polymer matrix was in a sustained release manner. The “burst release effect” commonly associated with micro- or nanoparticle delivery system was, to some extent, avoided during the first several hours’ release period.

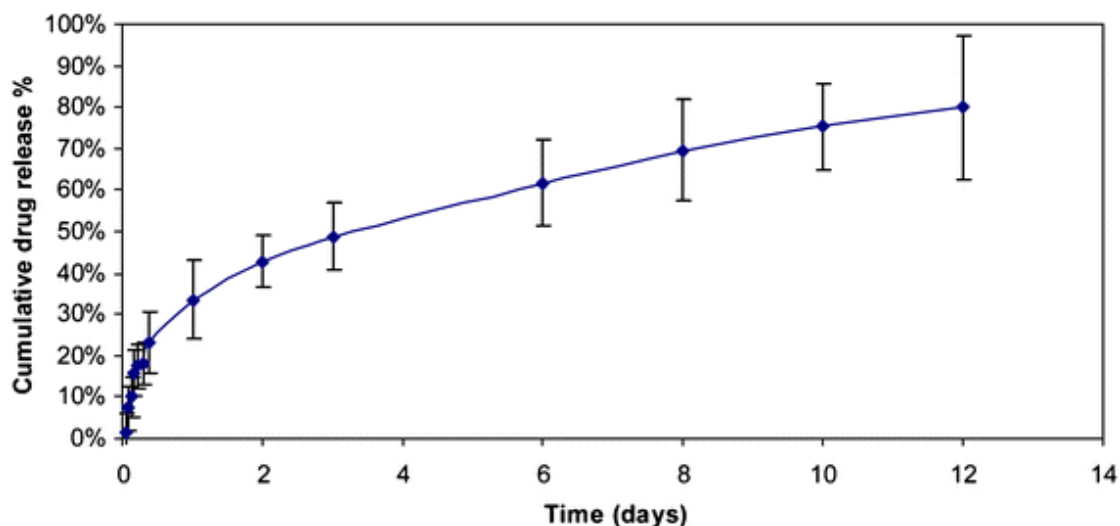


FIGURE 6: *In vitro* drug release profile of Dox-PLGA-PEG-RGD nanoparticles over 12 days at 37°C, 100 rpm in shaking water bath. Results are represented in triplicate

Cell Apoptosis Induced by Dox-PLGA-PEG-RGD Nanoparticles

In this study, agarose gel electrophoresis was employed to detect Dox-PLGA-PEG-RGD NPs induced apoptosis of the cancer cells. DNA fragmentation effect of Dox-PLGA-PEG-RGD NPs was determined on MDA-MB-231 cell line. The respective pure doxorubicin and NPs were adjusted to contain 5 µg/ml of Dox; and the cells were treated with the test solutions for 4 h followed by 44 h incubation in culture media. In Fig. 7, the cells treated with NPs were shown to have more lower-molecular weight (LMW) DNA fragments that were resolved at the bottom of the agarose gel. In contrast, the cells treated with doxorubicin alone had more higher-molecular weight (HMW) DNA fragments found close to the loading well on the gel.

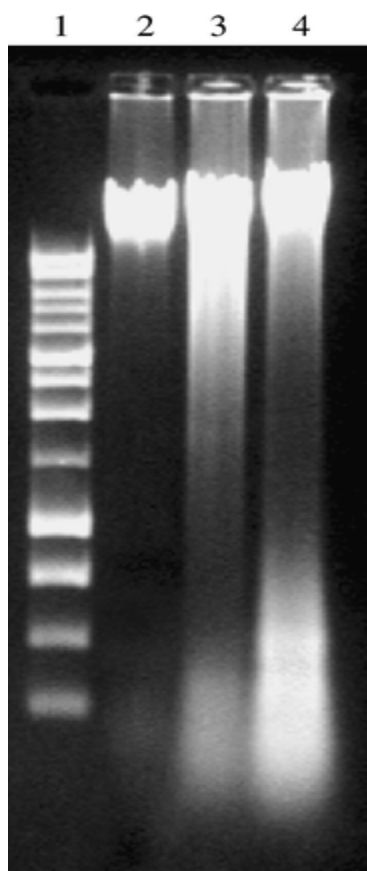


FIGURE 7: Cell Apoptosis Induced by Dox-PLGA-PEG-RGD NPs. DNA fragment results from agarose gel electrophoresis. Lane 1 DNA ladder, Lane 2 MDA-MB-231 cells treated with cell culture medium, Lane 3 MDA-MB-231 cells treated with doxorubicin, Lane 4 MDA-MB-231 cells treated with Dox-PLGA-PEG-RGD nanoparticles.

DISCUSSION

The design of intelligent systems to ensure the selectivity of chemotherapeutic drugs, in order to avoid the notorious side effects cannot be over-emphasized. *Integrin*, like $\alpha\beta_3$, has been extensively investigated to target many malignant cancer cells or endothelial cells for delivering cytotoxic drugs (5–7). As one of the conventional chemotherapeutic drugs, doxorubicin (Dox), is widely used clinically as a result of its efficacy against various cancers (17). It has already been reported that Dox-conjugated novel targeting peptide had significantly improved the therapeutic efficacy (18, 19). RGD peptide, one of the small bioactive molecules, is typically favored by many researchers as targeting ligand to deliver pay-loads to targeted sites (20).

Nowadays, various PLGA based drug/gene delivery systems with a variety of ligands have been investigated, whereas few reports have focused on fabrication of PLGA NPs with both active and passive targeting moieties

simultaneously for treating malignant cancers. In this study, we fabricated the PLGA based Dox delivery system with both passive and active targeting functions for malignant *integrin* expressing cancer cells. PEG (2000 M.W.) was chosen to realize the passive purpose, as PEG was reported to have the least protein interaction in animal body, was able to prolong the circulation period of pharmaceuticals, and was still functional at the distal end (21). The molecular weight of 2000 Dalton was chosen, as PEG of higher molecular weight might hinder the RGD active targeting function. The dynamic light scattering and TEM analysis showed that the as-synthesized Dox-PLGA-PEG-RGD NPs were uniform and well-dispersed; both properties are prerequisites for drug delivery systems suitable for clinical applications. Doxorubicin has moderate water solubility, whereas PLGA is rather hydrophobic. Thus, it is difficult to incorporate Dox into PLGA NPs in high efficiency. Conjugation of Dox to one of the distal ends of PLGA significantly enhanced the loading efficiency from less than 1% (22) to 87.2% as found in this study.

The different cytotoxic effects of Dox-PLGA-PEG-RGD NPs to various malignant *integrin* expressing cancer cells were probably caused by the different *integrin* receptor expression levels on the cell membranes, with MDA-MB-231 the highest and DU145 the lowest, leading to different uptake efficiency of NPs. What's more, with the RGD modification on NPs surface, the threshold for cytotoxic onset may be changed, as the RGD is also an *integrin* inhibitor at appropriate concentrations in tumor microenvironment (23). The inherent different cellular sensitivity to pure drug could also influence the resultant IC_{50} values of the drug loaded NPs. When a cell is already very sensitive to a drug, the advantage of formulating the drug into NP delivery system may not be very evident *in vitro*. As reported by other researchers (23), the conjugation of doxorubicin to PLGA polyester led to compromised cytotoxic effect to cancer cells (Fig. 4). In this study, -PLGA-PEG-RGD showed the least compromised efficacy to MDA-MB-231 cells, which may result from the higher *integrin* expression level in that cell line, facilitating the uptake of NPs into cytoplasm, and/or the less sensitivity of this cell line to pure doxorubicin. When a cell is less sensitive to a drug, the advantage of adding a targeting moiety to the delivery system will become more evident *in vitro*, and recovers some of the compromised effects due to the NP fabrication.

Results from CLSM (Fig. 5) revealed that Dox-PLGA-PEG-RGD NPs were preferred to be up-taken by MDA-MB-231 and B16F10 cells that had overexpressed *integrin* on their cell membranes. However, the uptake efficiency became static for both cells at a longer incubation time (no apparent difference in uptake after 4-h incubation with NPs) ; this might be due to saturation of the cellular surface receptor for NPs. Results from flow cytometry also showed the increasing uptake of Dox-PLGA-PEG-RGD NPs with ascending concentration of NPs (data not shown).

The DNA fragmentation phenomenon is involved in most apoptotic conditions. Cells undergoing apoptosis process often produce short DNA fragments as indicated in our result (Fig. 7). Dox-PLGA-PEG-RGD NPs

showed the most potency to induce dramatic DNA fragmentation compared with doxorubicin alone. Therefore, the results from DNA fragmentation assay confirmed that Dox-PLGA-PEG-RGD NPs were able to deliver chemotherapeutic drugs selectively to the *integrin* expressing cancer cells and induce apoptosis in these cells. Although the cytotoxic efficacy of doxorubicin was compromised after conjugation to PLGA, the selective targeting property and the sustained drug release profile of the doxorubicin NPs could improve the efficacy of the drug *in vivo*. The release kinetics of doxorubicin from the formulation was in zero-order ($R^2=0.97$), in the range from 12 h to 12 days (Fig. 6).

In conclusion, two independent approaches i.e. conjugating doxorubicin to PLGA, and mounting active and passive targeting moieties with PEG and RGD onto to the NPs were adopted to fabricate the Dox-PLGA-PEG-RGD NPs. The synthesized NPs effectively targeted doxorubicin to malignant *integrin*-expressing cancer cells, and showed profound apoptotic induction effect to those cells. Although the drug efficacy to cancer cells was compromised after conjugation with PLGA, the resultant sustained release behavior and the property of selective uptake of the NPs by the cancer cells could still make Dox-PLGA-PEG-RGD NPs a good candidate for anticancer therapy.

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