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Anti-Tumor Activity of Docetaxel PLGA-PEG Nanoparticles with a Novel Anti-HER2 scFv

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Abstract

Background: In this study, we developed pegylated (poly($_{D,L}$-lactide-co-glycolide) (PLGA-PEG) nanoparticles for loading docetaxel and improving active target in cancer cells because it's advantages over other nanocarriers such as excellent biocompatibility, biodegradability and mechanical strength and these nanoparticles were conjugated with molecules of a novel anti-HER2 single chain fragment (scFv) by a simple carbodiimide modified method. ScFvs have potential advantages over whole antibodies such as more rapid tumor penetration and clearance. In addition, to investigate cellular uptake of targeted nanocarriers, many studies had performed by linking with fluorescent factors but in this study 6-histidine-tag fused with novel anti-HER2 scFv antibodies can be used to purify protein and study binding activity and cellular uptake of targeting nanoparticles that it was not changed their characterization *in vitro*. Furthermore, cytotocixity of these nanoparticles was also investigated in BT474 (HER2 overexpress) and MDA-MB-231 (HER2 underexpress) cells.

Results: Docetaxel loaded nanoparticles (Doc-NPs) with a mean size of 105 nm and zeta potential of -25 mV were prepared by nanoprecipitation method. Conjugation of a novel single chain fragment of antibody against epidermal growth factor receptor 2 to Doc-NPs by covalent coupling via cross-linker EDC and NHS resulted in an increase of mean size and zeta potential of targeted nanoparticles (scFv-Doc-NPs) with 135 nm and -32 mV respectively. The scFv-Doc-NPs bound specifically to BT474 cells but no MDA-MB-231 cells was investigated by flow cytometry. Especially, confocal fluorescence scanning microscopy revealed internalization of the scFv-Doc-NPs by the targeted cancer cells through anti-Histag antibodies with Alexa Fluor® 546. Moreover, the scFv-Doc-NPs showed stronger cytotoxicity on BT474 cells than MDA-MB-231 cells with IC_{50} values of 0.234 and 0.535 μM, respectively.

Conclusion: Here we report anti-HER2 scFv labeled and docetaxel loaded PLGA-PEG nanoparticles in order to broaden the applications of this new targeted drug delivery system in the therapy of HER2 overexpressed cancers. Also, this drug delivery system represents a promising approach to improve the efficacy of nanoparticles in active targeting for HER2-overexpressed cancer therapy.

Keywords: Active targeting; Docetaxel; scFv; Polymeric nanoparticles; anti-HER2

Background

Chemotherapy has much side effects because of nonspecific biodistribution of chemotherapeutic agents. Many nanoparticle drug delivery systems have been designed to improve the efficacy of anticancer agents, minimize side effects and to enhance biocompatibility, serum stability [1]. Moreover, to improve more effect of nanoparticle drug delivery, the surface modification of nanoparticles with peptides, nucleic acids, antibodies, aptamers, or small molecules that bind to antigens on the surface of cancer cells or cancer tissues may be considered as an efficiently targeted delivery of cancer drugs [2]. Human epidermal growth factor receptor 2 (HER-2/neu) is one of the major targets for the design of targeted anticancer drugs and monoclonal antibodies specific to certain antigens on the surface of cancer cells have been used most often for the targeting of nanoparticles to tumor sites [3]. This HER-2/neu receptor is a 185- kDa transmembrane glycoprotein encoded by HER2 or the c-erbB-2 proto-oncogene on chromosome 17q21 belonging to the EGFR family. It has three domains: an extracellular domain (EDC), a hydrophobic transmembrane region, and an intracellular tyrosine kinase domain. The ligand-binding portion of the receptor is EDC [4,5]. Overexpression of HER-2/neu protein is detected in many tumors including invasive breast, colorectal, ovarian, pancreatic, stomach and prostate cancers… [6-9].

For scFv, it has advantages over whole antibodies such as greater tumor penetration, lower retention in non-target tissues, more rabid blood clearance, and less immunogenic response in vivo [10]. These antibody fragments have been widely used as targeting moieties of nanocarriers both in vitro [11] and in vivo [12] and are particularly attractive because their binding properties can readily be engineered using directed evolution [13].

Moreover, conjugation of different polymers with chemotherapeutic drugs in order to formulate various nanocarriers have been studied throughout the last decates and have improved safety and efficacy of drugs [14,15]. Especially, PLGA has generated tremendous interest due to its excellent biocompatibility, biodegradability, and mechanical strength [16]. Also, PLGA molecules have hydrophobic nature, therefore hydrophobic drugs, including most anticancer agents, can

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be easily loaded into PLGA nanoparticles [17]. It has also been used by many researchers for passive and active targeting of anticancer agents [18]. Moreover, water-soluble shell around the particle was be able to prevent attachment of plasma proteins [19]. Therefore, PLGA nanoparticles coated PEG results in highly stable drug delivery system and PEG has been used in FDA-approved biotherapeuticals [20]. PEG on the surface of nanoparticles reduced recognition and clearance and prolonged blood circulation time of nanoparticles [21]. Recent studies have used PLGA-PEG to load anti-cancer drugs [22,23] and to formulate targeting drug delivery systems [24-26]. However, no study has investigated PLGA-PEG nanoparticles with anti-HER2 scFv antibodies. Moreover, to produce targeting drug delivery systems conjugated with antibodies, it need to go through multiple steps [27-29]. This may affect the targeted drug delivery and the activity of antibodies. In this report, we use carbodiimide method and a modification with simple steps, therefore targeted docetaxel delivery conjugated with anti-HER2 scFv have good activity for targeted cancer cells.

Docetaxel is a leading chemotherapeutic drug for breast carcinoma, ovarian, head and neck, and lung cancer [30-32]. In this study, we report on a conjugation of polymer and novel anti-HER2 monoclonal antibodies with their most advantages in the preparation and characterization of pegylated PGLA nanoparticles as a targeted delivery system for docetaxel. In addition, to investigate cellular uptake of targeted nanocarriers, many studies had performed by linking with fluorescent factors [33-35] but in this report 6-histidine-tag fused with novel anti-HER2 scFv antibodies can be used to study binding activity and cellular uptake *in vitro*. Their ability to target and enter Her2-overexpressed BT-474 cells was studied and compared with MDA-MB-231 cells as a negative control.

Materials and Methods

Materials

Docetaxel anhydrous (Doc) of purity was purchased from Shanghai Bioman Pharma Co. Ltd, Shanghai, China. Poly($_{D,L}$-lactide-co-glycolide) with terminal carboxylate groups (PLGA, lactide:glycolide, 50:50, Mw ~ 7000 - 1700), bifunction poly(ethylene glycol 2-aminoethyl ether acetic acid (NH_2-PEG-COOH, Mw ~ 3400), 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide (EDAC) and N-hydroxysuccimide (NHS) were obtained from Sigma-Aldrich (St Louis, MO). Molecular and cellular biology buffers were purchased from Sigma. 4-Aminobenzoic acid p-aminobenzoic acid and 3-(4,5-dimethylthiazol-2-yl)-(2,5-diphenyl tetrazolium bromide) (MTT) was purchase from Promega (US). Monoclonal mouse anti-hexahistidine antibody was purchased from Abcam (Cambridge, MA), anti-mouse secondary antibodies conjugated with Alexa 546 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). All other organic solvents were analytical grade from Fisher Scientific.

Cell culture

Human breast cancer cell lines BT474 (HER2-overexpress) and MDA-MB-231 (HER2-underexpress) (American Type Culture Collection) were obtained from Institute of Biotechnology (VAST). The cell lines were cultivated in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO_2. The cells were maintained in an exponential growth phase by periodic subcultivation.

Synthesis of PLGA-PEG

Copolymer PLGA-PEG was synthesized by conjugation of NH_2-PEG-COOH to PLGA-COOH. Briefly, 150 mg of PLGA-COOH were dissolved in 1 ml DCM. PLGA-carboxylate was converted into PLGA-NHS by adding 1 mg of NHS and 2 mg of EDAC in 1 ml DCM with gentle stirring. PLGA-NHS obtained was precipitated with 10 ml ethyl ether/methanol washing solvent by centrifugation at 3000 rpm for 10 min and the washing process was repeated two times to remove residual EDC/NHS. The PLGA-NHS pellet was dried under vacuum for 30 min to remove residual ether and methanol. After drying under vacuum, 100 mg of PLGA-NHS was dissolved in 2 ml DCM followed by addition 10 mg of NH_2-PEG-COOH and 7.5 mg of N,N-diisopropylethylamine. The mixture solution was incubated for 24 h at room temperature under gentle stirring. Then, this mixture washed by ether/methanol washing solvent and centrifuged to remove unreacted PEG. The resulting PLGA-PEG copolymer was dried under vacuum and used for nanoparticle preparation without further treatment. The following are the main nuclear magnetic resonance (NMR) peaks of the sample:

^{1}H-NMR ($CDCl_3$ at 500 Hz) d 5.2 (m, (OCH(CH_3) C(O)OCH_2C(O) n-(CH_2CH_2O)m), 4.8 (m, (OCH(CH_3)C(O) OCH_2C(O)n-(CH_2CH_2O) m), 3.7 (s, (OCH(CH_3)C(O)OCH_2C(O) n-(CH_2CH_2O)m), 1.6 (d, (OCH(CH_3)C(O)OCH_2C(O)) n-(CH_2CH_2O)m.

Preparation of docetaxel-loaded PLGA-PEG nanoparticles

The Doc encapsulated PLGA-PEG copolymer nanoparticles were prepared using nanoprecipitation method. Briefly, 10 mg of PLGA-PEG copolymer and 1mg of Doc were dissolved in acetone. The mixtures were poured into Millipore water solution with the solvent:water = 1:5. Nanoparticles were formed immediately, and gently stirred at room temperature (RT) for 4-5 hours to evaporate the organic solvent. The resulting nanoparticle suspension was then collected by ultrafiltration. The nanoparticles were then freeze- dried using the Thermo Electron's Modulyo freeze dryer (USA) for 8 h.

Characterization of docetaxel-loaded PLGA-PEG nanoparticles

Size and zeta potential measurements: The average size and polydispersity of nanoparticle derivetives were analyzed by dynamic light scattering (DLS). Zeta potential of NPs was evaluated in deionized water (~1mg/ml) using the electrophoretic mode of Zetasizer 3000 HS (Malvern instruments Ltd., United Kingdom) at 25°C. Each sample was measured in triplicate.

Surface morphology: Transmission electron microscopy (TEM) system (Jeol JEM-1010, USA) were used to determine the shape and surface morphology of nanoparticles produced.

Drug encapsulation efficiency: The docetaxel entrapped in the Doc-NPs and measured by NanoDrop® 1000 spectrophotometer (Thermo Fisher Scientific) and quantified by UV-Vis spectrophotometer at 275 nm for Doc was previously described [45]. Briefly, 3 mg nanoparticles were dissolved in 2 ml of DCM (dichloromethane). DCM was evaporated in nitrogen atmosphere and residue was resuspended in dimethyl sulfoxide (DMSO) for analysis by UV-Vis spectrophotometer. The standard curve of Doc was linear in the range of 50-1000µg/ml in DMSO. The encapsulation efficiency of Doc was measured by the amount of Doc encapsulated comparing with the total amount of drug used in formulation, multiplied by 100.

Production and purification of soluble scFv

For the production and purification, the anti-HER2 scFv protein was previously expressed in *E. coli* strain BL21 [42]. Briefly, the fusion gene of anti-Her2 scFv containing His_6-tag was cloned into a T7

promoter-based *E. coli* expression vector, pET-22b(+). *E.coli* bacterial cultures were grown at 37°C in lysogeny broth (LB) growth medium. The anti-Her2 scFv expression was induced by addition of 0.5 mM isopropyl-$_L$-thio-β-$_D$-galactopyranoside (IPTG) and grown to log phase ($A_{600\ nm}$ = 0.8). The cells were harvested, centrifuged, and the pellet was resuspended in lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, 8 M Urea) and sonicated on ice when the solution became translucent. The lysate was then centrifuged at 12,000 g for 30 min at room temperature. The supernatant was used for purification of His_6-tagged protein on HisPrep FF 16/10 column (GE Healthcare Life Sciences) under denaturing conditions using FPLC equipment. The protein was refolded on the column by washing with a linear gradient from 8 to 0 M urea and eluted with 250 mM imidazole. For final purification of anti-Her2 scFv, elution fractions were collected by Centrifugal Devices (Pall Life Science, USA). SDS/PAGE analysis of the proteins was performed according to standard protocols using 12.5% polyacrylamide gels.

Conjugation of scFv to nanoparticles

The conjugation of anti-HER2 scFv to Doc-NPs was accomplished via crosslinking of –COOH and $–NH_2$ using carbodiimide method. Briefly, 1 ml of Doc-NPs solution (2 mg/ml) was incubated with 100 µl of 4 mM EDC and 100 µl of 10 mM NHS for 15 minutes at room temperature with gentle stirring. Then the activated particles were covalently linked to 100 µl of scFv (1 mg/ml) for 2 hours at room temperature and gently vortex. The reaction mixture was quenched by adding hydroxylamine (to give a final concentration of 5 to 10 mM). The Doc-NPs conjugated with anti-Her2 scFv was purified from unconjugated protein and by-products by ultrafiltration. The NPs suspensions were kept at 4°C until use.

Flow Cytometry

Flow cytometry (FCM) was used to evaluate binding of scFv-Doc-NPs to target (HER2-overexpress BT474) and nontarget (HER2-underexpress MDA-MB-231) cells. Cells were collected from culture and centrifuged for 5 min at 4°C and 1500 rpm. Cells were then incubated with 100 µg/ml of Doc-NPs or scFv-Doc-NPs solution for 30 min at RT. Samples were then washed 3 times with PBS 1× and were incubated with monoclonal mouse anti-hexahistidine antibodies for 1 h at RT. After that, samples were washed 2 times by PBS 1×. Samples were incubated with Alexa Fluor® 546 goat anti-mouse antibodies for 1 h at RT and were washed 2 times by PBS 1× and supernatant was discarded. Then, 500 µl of PBS 1× was added to each sample, and flow cytometric analysis was performed on FACSCanto II Cytometer (BD Biosciences) with BD FACSDiva™ software.

Cellular uptake studies

The cellular uptake of Doc-NPs and scFv-Doc-NPs by cells was observed by fluorescence microscopy. BT474 and MDA-MB-231 cells were allowed to adhere to glass coverslips in 12-well plate for 24 h before experiments. The cells were then incubated with 100 µg/ml of Doc-NPs or scFv-Doc-NPs for 1 h at 37°C. After washing twice with PBS, samples were incubated with monoclonal mouse anti-hexahistidine primary antibody for 1 h at 4°C and then samples were washed three times by PBS 1×. Samples were incubated with Alexa Fluor® 546 goat anti-mouse secondary antibody for 1 h at RT and washed by PBS 1× three times. Nucleus was counterstained with Hoechst, the cells were fixed with 4% formaldehyde for 10 min and analyzed by confocal fluorescence scanning microscopy (Zeiss LMS 510 confocal microscopy).

In vitro cytotoxicity assays

The in vitro cytotoxicity of the following docetaxel formulations was tested on BT474 and MDA-MB-231 cells using the MTT test: Doc-NPs, scFv-Doc-NPs, and free docetaxel. Unloaded nanoparticles were used as a control. BT474 and MDA-MB-231 cells were first grown in 96-well plates at the density of 5 x 10^3 viable cells/well and incubated for 24 hours to allow cell attachment. The medium was replaced by 100 µl of the formulation at different concentrations of 0.05-5 µM followed by incubation for 72 h at 37°C. For free docetaxel, a stock solution was prepared in DMSO (1M Docetaxel). The DMSO concentration in the medium was lower than 0.5%, at which level it has no effect on cell proliferation. The cell viability was detected by MTT assay (Promega, US) according to manufacturer's instruction. Each assay was repeated three times. Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = (Int_s/Int_{control}) \times 100 \quad (1)$$

Where Int_s is the colorimetric intensity of cells treated with the samples, and $Int_{control}$ is the colorimetric intensity of cells treated with the DMEM medium only. All experiments were repeated thrice

Results and Discussion

Synthesis of PLGA-PEG polymer

Carboxyl-functionalized PLGA-PEG copolymer was synthesized by direct conjugation of PLGA-COOH with NH_2-PEG-COOH, both having fixed block length, to generate PLGA-PEG. The basic chemical structure of PLGA-PEG copolymer was confirmed by ^{1}H-NMR (Figure1). One of the prominent features is a peak at 3.4 ppm, matching the methylene groups of PEG. Overlapping doublets at 1.6 ppm are attributed to the methyl groups of the D- and L-lactic acid repeat units. The multiples at 5.2 ppm and 4.8 ppm correspond to the lactic acid –CH and the glycolic acid –CH, respectively, with the high complexity of the peaks resulting from different D-lactic, L-lactic glycolic acid sequences in the polymer backbone. The carboxyl group located at the end terminal of the hydrophilic PEG block is available for surface chemistry on the nanoparticle surface.

Characterization of nanoparticles

Docetaxel was encapsulated in the pegylated PLGA nanoparticles with carboxyl end groups by the nanoprecipitation method. The physicochemical characteristics of the nanoparticles are summarized in table 1.

One of the most important characteristic of nanoparticle systems is their size and size distribution. The biodistribution, toxicity, and targeting ability of these systems is determined by their size. Pore sizes

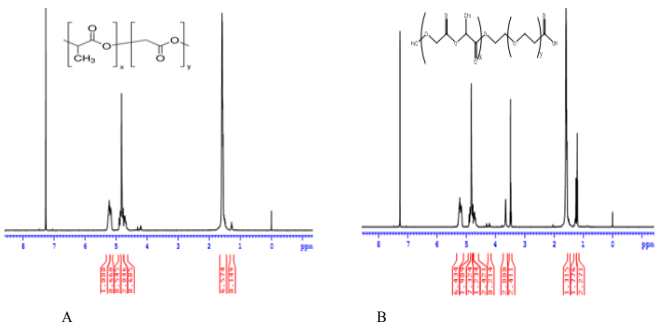


Figure 1: ^{1}HNMR characterization of PLGA-COOH (A) and PLGA-PEG (B). PLGA: δ 5.2 (m, ((OC<u>H</u>(CH_3)C(O)OCH_2 C(O))$_n$)); δ 4.8 (m, ((OCH(CH_3) C(O)OCH_2C(O))$_n$)); δ 1.6 (d, ((OCH($C\underline{H}_3$)C(O)OCH_2C(O))$_n$). PEG: δ 3.7 (s, ($C\underline{H}_2C\underline{H}_2$O)m).

in tumor microvasculature vary between 100 nm and 780 nm [36]. Many studies have recognized the suitable size of these carriers for extravasation and accumulation in solid tumors below 400 nm [37]. As shown in table 1 and figure 2, the nontargeted nanoparticles had a size of 105 ± 4 nm (Figure 2B), whereas the monoclonal antibody-targeted nanoparticles were larger at 135 ± 9 nm (Figure 2C) and the drug-unloaded and nontargeted nanoparticles had the smallest mean size of 87 ± 8 (Figure 2A). Conjugation may be a reason for the larger size of the targeted nanoparticles [38]. Transmission electron microscopy showed that the nanoparticles and targeted nanoparticles were spherical and rather homogeneous in size (Figure 2).

For nanoparticles and monoclonal antibody-targeted nanoparticles, the zeta potential was -24 ± 0.3 mV and – 32 ± 1 mV, respectively. This result shows that the negative value of mean zeta potential of the targeted nanoparticles is increased due to coupling of anti-HER2 scFv to the nanoparticles, containing several ion groups and suggesting that conjugation of monoclonal antibodies to the nanoparticles leads to an increase in the negative surface charge of targeted nanoparticles.

Previous studies have shown that in order to release taxane drugs at a sustainable rate from PLGA NPs, the drug loading concentrations should be limited [39], especially in the case of pegylated NPs [40]. Therefore, NPs containing variable amounts of docetaxel were synthesized by adjusting docetaxel drug loading at 10% by weight of the added polymer and it was satisfactory rate for goog drug encapsulation

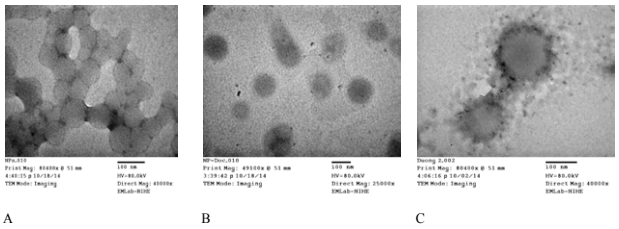


Figure 2: TEM images of NPs (A), Doc-NPs (B) and scFv-Doc-NPs derivetives (C).

Samples	Mean size ± SD (nm)	PDI ± SD	Zeta potential ± SD (mV)	EE
PLGA-PEG	87 ± 8	0.08 ± 0.02	- 24 ± 0.3	
Doc-PLGA-PEG	105 ± 4	0.1 ± 0.05	- 25 ± 0.5	43%
ScFv-Doc-PLGA-PEG	135 ± 9	0.1 ± 0.07	- 32 ± 1	

Table 1: Physicochemical characteristics of PLGA-PEG, Doc-PLGA-PEG and scFv-Doc-PLGA-PEG nanoparticles (n=3).

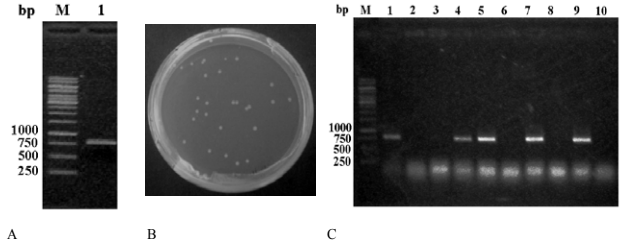


Figure 3: The cloning and expression of pET22b(+) vector containing anti-HER2 scFv gene in *E. coli* cells. 3A. Lane M: DNA marker 1kb (Fermentas), lane 1: anti-HER2 scFv gene; 3B. *E. coli* clones; 3C. Lane M: DNA marker 1kb, lanes 1-10: anti-HER2 scFv gene analyzed by restriction enzymes from *E. coli* clones.

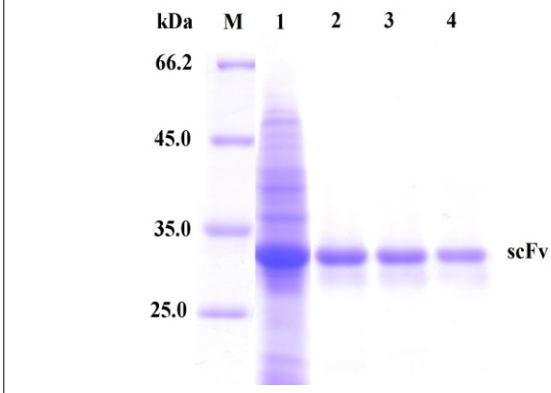


Figure 4: Purification of anti-HER2 scFv. 12.5% SDS-PAGE confiming the purification of anti-HER2 scFv (31 kDa), CoomassieBrillian Blue R-25 stained gel; Lane M: Standard protein marker (Thermo Scientific); Lane 1: total protein of culture extract; Lane 2, 3 and 4: elution fractions ofscFv protein.

efficiency [41]. Table 1 also shows the drug loading efficiency and the PLGA-PEG nanoparticles prepared with nanoprecipitation method in this study resulted in a high drug encapsulation efficiency of 43%.

Results of expression and purification of anti-Her2 scFv

The gene encoding anti-HER2 EDC scFv derived from a recombinant phage-display library (Griffin.1 library) have been screened, selected and registered on GeneBank (NCBI) with code AM402973.1 [42]. This gene was designed to fuse with His_6-tag on *C*-terminus and cloned by using forward 5'-GAGATATACAGAATTCCTGCCCTTAC-3' and reverse 5'-ATACAGCTCCCTCGAGTGAAGATACG-3' primers containing two *EcoR*I and *Xho*I restriction enzyme sites, respectively, a fragment of 800 bp was produced by PCR (Figure 3A).

The PCR fragment was purified using the QIAGEN gel extraction kit and was ligated into pET22b(+) vector. pET22b(+) plasmids containing anti-HER2 EDC gene (pET22b(+)-EDC) were transfected in the *Escherichia coli* DH5α (*E. coli* DH5α) cells (Figure 3B). The recombinant clones were analyzed by restriction enzyme analysis (Figure 3C) and DNA sequencing.

Expression and purification of anti-HER2 scFv in *E. coli* were described in Materials and Methods. These anti-HER2 scFv antibodies were used as targeting molecules for directed delivery of Doc-loaded PLGA-PEG NPs to tumor cells. The proteins obtained were of the expected molecular weight and homogeneity according to SDS-PAGE (Figure 4).

Flow cytometric analysis

To test whether scFv-Doc-NPs would differentially bind to HER2-overexpress and HER2-underexpress cells, the binding of scFv-Doc-NPs to BT474 and MDA-MB-231 cells evaluated by FCM analysis. Previous studies have well established that BT474 cells are a human breast cancer cell line that overexpresses HER2 protein on its surface (3.7×10^6 receptors/cell) and MDA-MB-231 cells are a HER2-underexpress human breast cancer cell line (7×10^4 receptors/cell) [43]. Consistent with the original report, scFv-Doc-NPs demonstrated a higher binding to the BT474 cells than the MDA-MB-231 cells (Figure 5). The mean fluorescent intensity of scFv-Doc-NPs binding with BT474 cells is about 3 times higher than that with MDA-MB-231 cells. Since scFv may facilitate binding with the target.

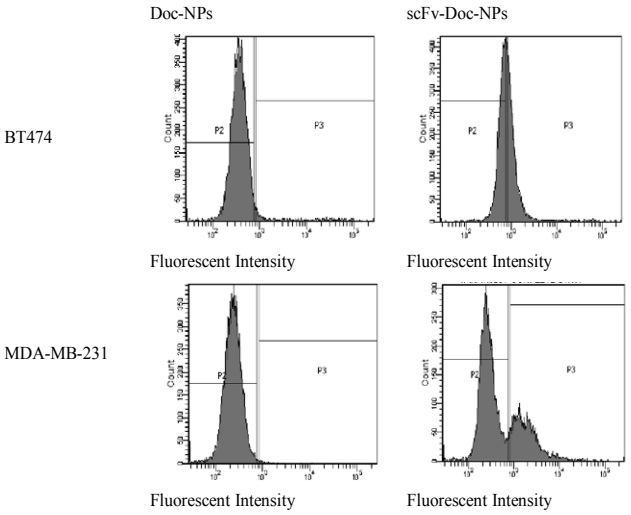


Figure 5: FMC analysis of binding activity of HER2-overexpress BT474 and HER2-underexpress MDA-MB-231 cells treated with Doc-NPs solution as control and scFv-Doc-NPs derivative at RT for 30 min in PBS.

Cellular uptake experiment

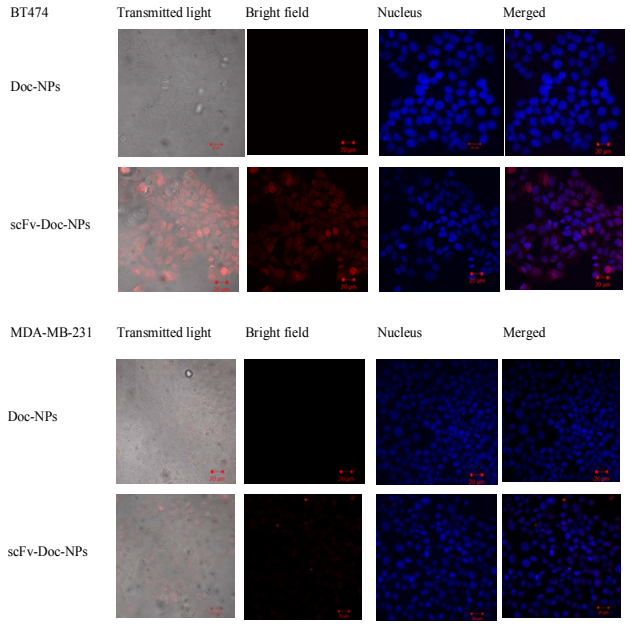
To further explore the *in vitro* cancer targeting of scFv-Doc-NPs against the HER2-overexpressing cells, we perfomed *in vitro* cellular uptake studies with both BT474 and MDA-MB-231 cells using confocal fluorescence microscopy. The cells were incubated with antibody-conjugated or antibody-unconjugated nanoparticles as described and then fixed and detected the bound nanoparticles by indirect immunofluorescence by using mouse anti-histag primary antibody and Alexa 488-conjugated anti-mouse secondary antibody. Nuclei were counterstained with Hoechst. The results showed that scFv-Doc-NPs generated enhanced fluorescence intensity in BT474 cells, comparing with MDA-MB-231 cells (Figure 6). Moreover, the images clearly indicated that the scFv-Doc-NPs were mainly accumulated in the cytoplasm of BT474 cells. Accordingly, scFv-Doc-NPs could be internalized into the BT474 cells and carry the anticancer drugs into the cytoplasm. Compared to BT474 cells, fluorescent signal of scFv-Doc-NPs entered MDA-MB-231 cells was much less; suggesting again that the scFv facilitated the uptake of nanoparticles into the cells.

Figure 6: Confocal microscopy images of HER2-overexpress BT474 and HER2-underexpress MDA-MB-231 cells treated with Doc-NPs or scFv-Doc-NPs. The nuclei were stained with Hoechst. The merged images of Alexa 546 and the Hoechst channels. BT474 cells exposed to scFv-Doc-NPs at 100 μg/ml for 1 h.

In vitro cellular cytotoxicity assay

A series of in vitro cytotoxicity assays was performed to evaluate the anticancer potential of free Doc, Doc-NPs, scFv-Doc-NPs using BT-474 and MDA-MB-321 cells after an incubation of 72 h at 37°C (Figure 7). Anti-HER2 scFv-unconjugated nanoparticles with no drug loading were also tested as negative control. Statistical analysis showed that the drug-unloaded and scFv-uncojugated nanoparticles did not influence on cell viability. Doc-NPs was highly cytotoxic for both BT-474 and MDA-MB-231 cells and they were also more toxic than free docetaxel. The IC_{50} values of free Doc and Doc-NPs were 1,55 and 0.69 μM for BT474 cells and 1.61 and 0.80 μM for MDA-MB-231 cells, respectively. However, the most cytotoxicity of scFv-Doc-NPs was in BT474 cells, while the cell toxicity of MDA-MB-231 cells was lower with IC_{50} values of 0.234 and 0.535 μM, respectively.

Moreover, the higher cytotoxicity of drug-entrapped NPs and targeted NPs can be due to different mechanisms between free drug molecules and various drug-loaded nanoparticle derivetives. Free drug molecules were transported into the cytoplasm by a passive diffusion and transported out by P-glycoprotein pumps. However, drug-loaded NPs penetrate into cells through the endocytosis and they can be escaped from the influence of P-glycoprotein pumps, resulting in a higher cellular uptake compared to free drug molecules [44]. Targeted delivery of nanoparticles can potentially enhance the selective killing of tumor cells. In this study, we utilized a anti-HER2 recombinant single chain Fv antibody fragment (scFv) that bound specifically to extracellular domain of HER2 receptor and explored the therapeutic potential of anti-HER2 scFv-decorated pegylated PLGA nanoparticles. These targeted nanoparticles exhibited much improved cytotoxic activity compared to non-targeted nanoparticles *in vitro*. Thus, synergistic efficiency of the scFv-Doc-NP derivative can be explained



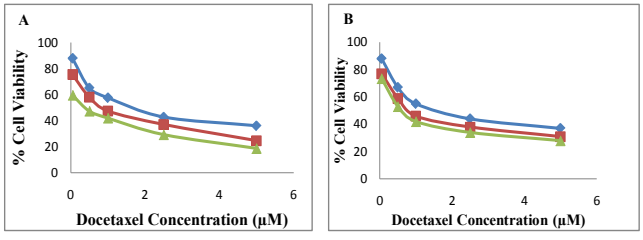


Figure 7: Cytotoxic effects of free Doc and Doc-NPs with or without anti-HER2 scFv on target BT474 (A) and non-target MDA-MB-231 (B) cells. Cell viability was analyzed after incubation with different NPs and free Doc (equivalent docetaxelconcentrations (0 μM, 0.05 μM, 0.5 μM, 1 μM, 2.5 μM, and 5 μM) of free Doc (—◆—), Doc-NPs (—■—) and scFv-Doc-NPs (—▲—) using cytotoxicity assay Kit (MTT).

by their specific interaction with HER2 antigens on the surface of BT-474 cells, indicating the potential of these nanoparticles to treat human HER2-overexpression cancer cells. **Conclusion**

In this study, pegylated PLGA nanoparticles decorated with the novel anti-HER2 scFv suitable characterization were successfully prepared for targeted delivery of docetaxel *in vitro*. We have investigated binding and internalizing activity of anti-HER2 scFv-decorated pegylated PLGA nanoparticles using His_6-tag fused with novel anti-HER2 scFv protein. The cytotoxic results of this targeting drug delivery system showed stronger cytotoxicity with BT474 cells than MDA-MB-231 cells. The results suggest that great synergetic potential of scFv-Doc-NPs may have potential applications in targeted therapy against HER2-positive caner cells for further in vivo applications.

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