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ARTICLE

I-motif-coated exosomes as a pH-sensitive carrier for anticancer drugs

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Abstract Nature-derived exosomes have been noted as emerging carriers for anticancer drugs. In this study, as a proof-of-concept, the anticancer drug doxorubicin (Dox) was loaded onto i-motif-modified exosomes (Exo-i-motif) to deliver Dox to cancer cells efficiently. The double-stranded biotin-i-motif/flare (ds-i-motif-bio)s efficiently released Dox in an acidic pH-responsive manner within 1 h. Based on gel electrophoresis, it was clearly confirmed that ds-i-motif-bio successfully interacts with biotin-conjugated exosomes and streptavidin (strep) via the biotin–streptavidin interaction. The particle sizes were below 150 nm without aggregation after strep-mediated modification of ds-i-motif-bio on the surfaces of the exosomes. In addition, released Dox had intact bioactivity for anti-proliferation after immobilization onto the exosomes. This study could serve as a new concept of pH-responsive delivery systems of anticancer drug using nature-derived exosomes with i-motifs.

Keywords Biotin · Doxorubicin · Exosome · I-motif · pH-responsive · Streptavidin

Introduction

It is well known that nanoparticle systems have a wide range of advantages for anticancer drug delivery to cancer cells [1]. In terms of pharmacokinetics, nanoparticles with a diameter over 100 nm provide an enhanced circulatory lifetime of a drug by evading renal clearance [2]. Additionally, nanoparticles whose sizes range from 50 to 200 nm can be delivered by passive targeting via an enhanced permeation and retention effect that is normally found in tumor and inflamed tissue. Considering tumor vasculature is highly dis-organized and angiogenic, nanoparticles can easily go through endothelial cell layers and accumulate inside the target tissue [3]. Accordingly, a wide range of particle systems composed of metal, polymers, and lipids have been developed. Recently, exosomes have been vigorously investigated as drug carriers. Exosomes are nature-derived extracellular vesicles that exist in diverse body fluids, e.g., serum, saliva, and urine [4]. Owing to their homogeneous size distribution, biocompatibility, aqueous stability, and long-term safety compared to synthetic particles, exosomes have been considered as attractive vesicles for drug delivery. In particular, serum-derived exosomes have been considered promising carrier systems in terms of size (below 50 nm), high production yield, and aqueous stability [5]. Several researches reported about surface engineering of exosomes with antigen, targeting ligands, and DNA oligomers to enhance delivery efficiency for delivery of small drugs or biomolecules [6–8].

Tumor microenvironments are acidic compared to normal tissues probably owing to the high rate of glycolysis and increased production of lactate that occurs in them [9]. Accordingly, pH-responsive drug delivery systems have been vigorously studied to target tumors and reduce side effects of drugs [10, 11]. The intercalated motif (i-motif), a

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cytosine-rich DNA strand, is a pH-responsive molecular machinery [12–14]. The cytosine of the i-motif could be easily hemi-protonated to form cytosine–cytosine base pairs in antiparallel fashion only in an acidic condition. Using this pH-responsiveness, i-motif conjugated inorganic nanoparticles, e.g., gold nanoparticles, have been used as nucleic acid sensors and therapeutic carriers [15, 16]. In addition, anticancer drugs could be loaded into i-motif-based nanomaterials to release intercalated drugs in a pH-sensitive manner [17, 18]. However, exosome-conjugated i-motifs have not been reported yet. In addition, development of carriers for anticancer drug to multi-drug-resistant cancer cells is crucial. Due to overexpression of efflux pump, P-glycoprotein, multiple chemotherapeutic drugs have shown negligible effects on multi-drug-resistant cancer cells. Recently, particle-based drug delivery systems have been considered as promising carriers for anticancer drugs to multi-drug-resistant cancer cells by escaping from P-glycoprotein-mediated efflux [19, 20].

In this study, the surfaces of exosomes (Exo) were modified with biotin via NHS coupling chemistry. The hydrodynamic diameters and size distributions of biotin-modified exosomes (Exo-bio) were analyzed by dynamic light scattering (DLS). As an anticancer drug, doxorubicin (Dox) was intercalated within a double-stranded biotin-i-motif/flare duplex (ds-i-motif-bio). The pH-responsive release of Dox from ds-i-motif-bio was examined at different incubation times. Using the biotin–streptavidin interaction, Exo-bio and ds-i-motif-bio were complexed with streptavidin to form Exo-bio-strep-ds-i-motif-bio complexes (Exo-i-motif). Formation of the Exo-i-motif complex was examined by DLS and gel electrophoresis at different Exo-bio/strep/ds-i-motif-bio molar ratios. To examine bioactivity of Dox incorporated Exo-i-motif (Exo-i-motif-Dox), a multi-drug-resistance breast cancer cell line (MCF-7/MDR) was treated with Exo-i-motif-Dox complexes at different Dox concentrations for 48 h, and anti-proliferation activity was examined.

Materials and methods

Materials

Streptavidin from streptomyces avidinii (strep), (+)-biotin-N-hydrosuccinimide ester (biotin-NHS), anhydrous dimethyl sulfoxide (DMSO), diethyl pyrocarbonate (DEPC), fluorescamine, phosphate-buffered saline (PBS, pH7.4), and $MgCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dox was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The 5′-end biotin-modified i-motif (i-motif-bio, biotin-5′-TTTTTTTTTTCCCTAACC CTAACCCTAACCC-3′) and flare (5′-GTTAGTGTT AGGGTTAGTT-3′) were obtained from Bioneer (Daejeon, South Korea) [12, 13, 18]. Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin/streptomycin (P/S), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). An ExoQuick-TC precipitation kit was obtained from SBI system Biosciences, Inc. (Mountain View, CA, USA). A desalting column (molecular weight cutoff [MWCO], 40 kDa), a microbicinchoninic acid (BCA) assay kit, and a Quanti-iT PicoGreen dsDNA assay kit were purchased from Thermo Scientific (Rockford, IL, USA). Nanosep centrifugal devices with omega membranes (Nanosep, MWCO = 300 kDa) were purchased from Pall (Ann Arbor, MI, USA). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of analytical grade.

Preparation and characterization of biotin-modified exosomes (Exo-bio)

Serum-derived exosomes were prepared according to our previous study with a slight modification [5]. Briefly speaking, FBS was centrifuged at $3000\times g$ for 15 min. The supernatant of the centrifuged serum solution (0.95 mL) was mixed with the ExoQuick-TC EXO precipitation solution (190 μL) via inversion. After incubation at 4 °C overnight, the mixture was centrifuged at $1500\times g$ for 30 min. After additional centrifugation at $1500\times g$ for 5 min, the resultant pellet was resuspended in PBS solution and stored at 4 °C. The amount of protein in the exosomes was determined using a BCA protein assay kit according to the manufacturer's protocol.

To prepare biotin-modified exosomes, the amount of amine groups in exosomal protein was quantified using fluorescamine reagent as described previously [21]. Amine groups (250 nmol) on the exosomes (protein amount, 216.1 ± 25.78 μg) in PBS solution were reacted with biotin-NHS (170.7 μg) in anhydrous DMSO at an amine groups/NHS molar ratio of 1:2 for 3 h. Excess and unreacted biotin-NHS was removed via a desalting column. At each step, the hydrodynamic sizes of exosomes or Exo-bio were measured with DLS (Malvern Instruments Ltd., Malvern, UK).

Dox loading and release test

I-motif-bio (29 μg) was annealed with complementary oligonucleotide with one base mismatch named flare (17.8 μg) in DEPC distilled water (DEPC D.W.) using Biometra Thermocycler (Analytic Jena, Yena, Germany). The temperature of the solutions was lowered in decrements of 5 °C from 95 to 25 °C, and each step was maintained for 5 min, except for the final step. The

resulting solution was stored in 4 °C. To determine loading capacity of Dox, double-stranded i-motif-bio and flare (ds-i-motif) was mixed with Dox (0.5 nmole) at ds-i-motif/Dox molar ratio of 0, 0.1, 0.5, 1.0, 1.5 in phosphate buffer (pH 8.0). After incubation for 1 h at 24 and 37 °C, fluorescence intensity of each solution was measured by fluorospectrophotometer (GeminiEM Microplate Reader; Molecular Devices, San Jose, CA, USA) at an excitation and emission wavelength of 480 nm and 590 nm, respectively. Loading capacity of Dox was calculated as following equation; Loading capacity (%) = (initial fluorescent intensity of free Dox—fluorescent intensity of intercalated Dox)/initial fluorescent intensity of free Dox × 100. To determine the time-dependent release profile of doxorubicin from double-stranded biotin-i-motif/flare (ds-i-motif-bio), Dox (2.2 μg, 4 nmole) was intercalated into ds-i-motif-bio (62.4 μg, 4 nmole) at a molar ratio 1:1 in a PBS solution containing 1 mM $MgCl_2$ at pH 7.4. After intercalation for 1 h, the DNA solution was diluted with two types of buffer solutions, MES buffer (pH 5.0) and a PBS solution containing $MgCl_2$. The fluorescence intensity of released Dox was measured after predetermined time intervals (0, 1, 3, and 5 h) using a fluorospectrophotometer. Relative release efficiency of Dox was calculated as following equation: releasing efficiency (%) = released amount of Dox in solution/initial amount of Dox × 100.

Preparation of i-motif-coated exosomes (Exo-i-motif)

I-motif-bio (29 μg) was annealed with flare (17.8 μg) in DEPC D.W. as described above. The resulting ds-i-motif-bio (2.2 μg) was incubated with streptavidin in PBS solution at a biotin/streptavidin molar ratio of 1 for 1 h. Four types of samples including flare (2.2 μg), ds-i-motif-bio (2.2 μg), streptavidin (8 μg), and ds-i-motif-bio conjugated streptavidin (ds-i-motif-bio-strep, 2.2 μg of DNA and 8 μg streptavidin) were loaded onto 20% PAGE gel (acrylamide/bis-acrylamide = 37.5:1). After running at 200 V for 45 min, the gel was stained with EtBr solution (0.5 μg/mL) and observed using UV transilluminator (Spectroline, Westbury, NY, USA).

Exosomes were modified with biotin as mentioned above. Exo-bio (biotin 0.2 or 1 nmole) was allowed to react with streptavidin (0.2 and 0.4 nmole) in PBS solution for 1 h at room temperature. The resulting solution was mixed with ds-i-motif-bio (biotin of 0.2 and 0.4 nmole) in DEPC D.W. and allowed to react for an additional hour at room temperature. Then, incubated samples with different Exo-bio/strep/ds-i-motif-bio molar ratios (1:1:1, 5:1:1, and 1:2:2) were analyzed by gel electrophoresis. For the negative control, the same protein content of free exosomes without biotinylation was reacted with streptavidin and ds-i-motif-bio at the same molar ratios. To visualize double-stranded DNAs, three types of samples (ds-i-motif-bio, ds-i-motif-bio-strep, Exo-i-motif) were stained with 1/10 diluted PicoGreen reagent in DEPC D.W. Each prepared sample (ds-i-motif-bio amount of 1 μg) was loaded onto 20% PAGE gel (acrylamide/bis-acrylamide = 37.5:1). After running at 200 V for 1 h, the fluorescence intensity of intercalated PicoGreen within DNA strands was visualized at excitation and emission wavelengths of 465 nm and 509 nm, respectively, using IVIS instrument (Lumina II; Caliper Life Sciences, Waltham, MA, USA). The hydrodynamic sizes of Exo-i-motif before purification, Exo-i-motif after purification, the Exo-strep at Exo-bio/strep molar ratio of 5:1 were measured by DLS.

Anti-proliferation study

Multi-drug-resistant breast cancer cells of the MCF-7 (MCF-7/MDR) cell line were maintained in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2, according to our previous study [22]. For the anti-proliferation study, cells were seeded at a density of 1×10^4 cells per well in a 96-well plate 24 h before treatment. I-motif-bio (290 μg) was annealed with flare (178 μg) in DEPC D.W. as described above. The Exo-bio in PBS solution was reacted with strep and ds-i-motif-bio in the same order and conditions described above (Exo-bio/strep/ds-i-motif-bio = 1:1:1, molar ratio). The resulting solution was concentrated and purified via nanosep (MWCO = 300 kDa) to remove unreacted streptavidin and ds-i-motif-bio. Dox (16.3 μg) was intercalated into the Exo-i-motif in the PBS solution and incubated for 1 h. Cells were treated with free Dox and doxorubicin-incorporated Exo-i-motif (Exo-i-motif-Dox) at various Dox concentrations (0, 10, 20, 40, and 60 μM) for 48 h. The cell proliferation was measured using the CCK-8 assay according to the manufacturer's protocol.

Results and discussion

Synthesis and characterization of streptavidin-introduced exosomes

Figure 1A shows the preparation of streptavidin-coated exosomes (Exo-strep). The amine groups of the surface proteins on exosomes have been greatly facilitated for covalent conjugation of biomaterials such as polymers and targeting ligands [8, 23]. Accordingly, biotin can be chemically conjugated onto amine groups of exosome surfaces via NHS coupling chemistry. After treating exosomes with biotin-NHS, streptavidin could be easily coated

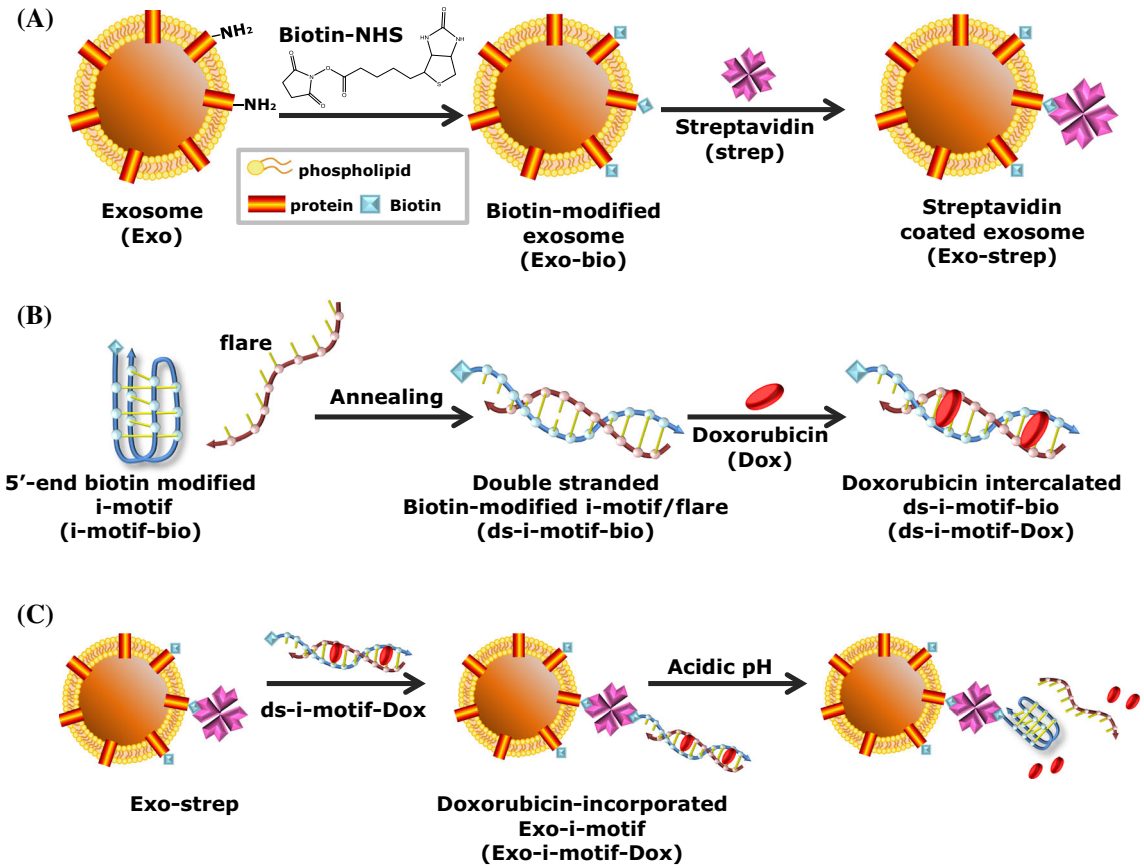

Fig. 1 Schematic illustration of (**A**) preparation of biotin-modified exosomes (Exo-bio) and streptavidin-coated exosomes (Exo-strep). (**B**) Intercalation of Dox within double-stranded DNA composed of the biotin-modified I-motif (i-motif-bio) and flare (ds-i-motif-bio). (**C**) Complexation of Exo-bio-strep-ds-i-motif-bio (Exo-i-motif) and incorporation of Dox for pH-responsive delivery of Dox

onto the exosomal surface via noncovalent biotin–streptavidin interaction. To load Dox onto exosomes, Dox was intercalated to the double-stranded oligomer, double-stranded biotin-modified i-motif (i-motif-bio)/flare (ds-i-motif-bio) (Fig. 1B). The ds-i-motif-bio was easily attached to Exo-strep to form i-motif-coated exosomes (Exo-i-motif) (Fig. 1C). Dox was intercalated into the double-stranded I-motif DNA on the surfaces of the exosomes (Exo-i-motif-Dox). In previous studies, the structure of the i-motif could be easily regulated by the pH of its environment. Accordingly, it was hypothesized that Exo-i-motif-Dox could deliver Dox in a pH-sensitive manner to cancer cells.

After purification of exosomes from FBS via a polymer precipitation method using ExoQuick-TC, the hydrodynamic sizes of the exosomes were 33.2 ± 1.1 nm, which corresponded with the findings of previous studies [5], as shown in Fig. 2A. The amount of amine groups in the proteins of the exosomes quantified using fluorescamine assay was 1.2 ± 0.17 mmol/g of exosomal proteins. For biotinylation, amine groups of exosomal proteins were reacted with biotin-NHS at a amine/biotin-NHS molar ratio of 1:2. After removal of unattached biotin through a desalting column (MWCO 40 kDa), the amount of biotin on the exosomes was 0.8 nmole per μg of exosome proteins. The hydrodynamic size of Exo-bio was 37.5 ± 1.1 nm without any significant aggregation, which was quite similar to that of free Exo. Figure 2B shows size distribution of free Exo and Exo-bio. It is noticeable that there was no significant aggregation in Exo-bio after chemical modification and purification.

Intercalation and pH-dependent release of Dox

As shown in Fig. 3A, fluorescent intensity of Dox was significantly decreased as elevating ds-i-motif/Dox molar ratios, which might be attributed to intercalation of fluorescent Dox within ds-i-motif. Dox fluorescent signal at ds-i-motif/Dox molar ratio of 1:1 was significantly reduced below 10% both at 24 °C and at 37 °C. More than 90% of initial Dox was intercalated within ds-i-motif, which is consistent to previous studies [17, 18, 22, 24]. In this study, we used a one base pair mismatched DNA sequence (flare) as a complementary sequence for the i-motif. Previously, it

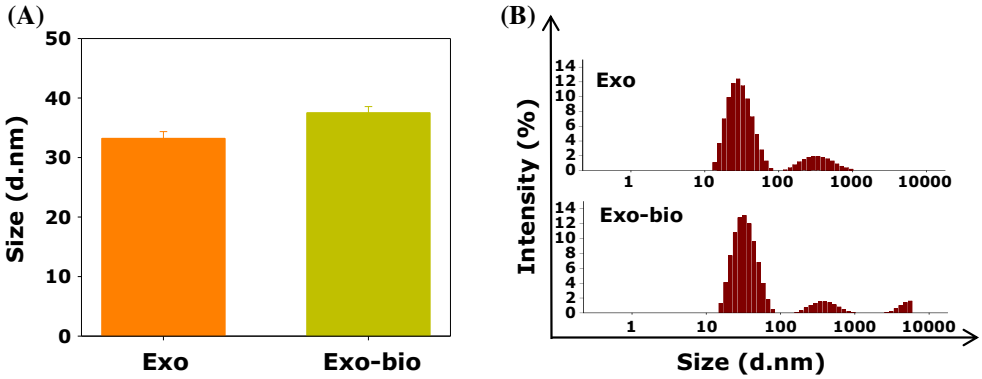

Fig. 2 (**A**) Hydrodynamic sizes and (**B**) size distribution of exosomes and Exo-bio after modification

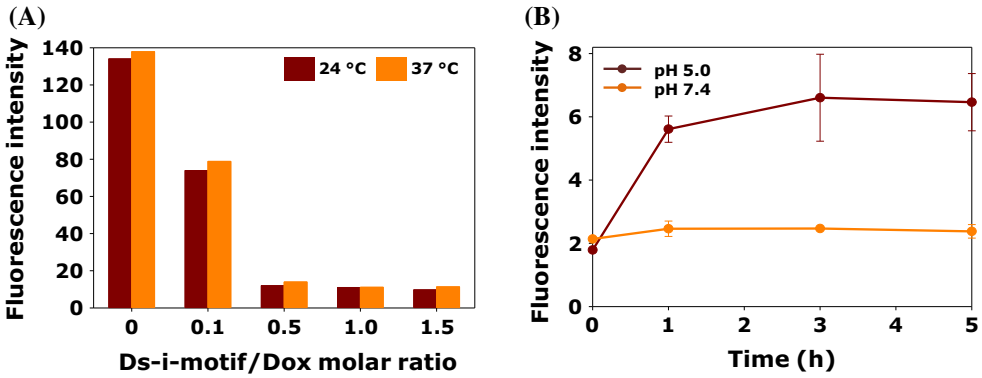

Fig. 3 (**A**) Intercalation of Dox in to ds-i-motif with different ds-i-motif/Dox molar ratios and (**B**) the pH-responsive release of Dox from ds-i-motif-bio at different pH (pH 5.0 and 7.4)

was reported that one base pair mismatch in flare allowed for big differences in pH responses in aqueous solutions at physiological temperature [13]. We confirmed that the one base pair mismatched flare was readily dissociated at an acidic pH at 37 °C compared to completely matched flare (data not shown). Time-dependent release of intercalated Dox from ds-i-motif is examined in Fig. 3B. In our study, relative amounts of released Dox from i-motif double strands were 17.5 ± 1.5 and 21.0 ± 4.9% after 1 and 3 h incubation at acidic pH, respectively. In the previous study, Song et al. [18] showed higher Dox release (∼ 70%) from i-motif double strand on gold nanoparticle within several hours. It is necessary to optimize i-motif sequences to allow prompt release of Dox from ds-i-motif-bio at acidic condition. However, negligible release of Dox was observed up to 5 h of incubation at neutral pH.

Preparation and characterization of i-motif-coated exosomes (Exo-i-motif)

Figure 4A shows the gel electrophoresis of the ds-i-motif-bio after annealing of the i-motif-bio with flare. Annealed ds-i-motif-bio showed strong fluorescence signals due to formation of a double strand. In addition, compared to the free flare, the ds-i-motif-bio exhibited significantly retarded gel migration. To examine if ds-i-motif-bio could bind to streptavidin, prepared ds-i-motif-bio-strep complexes were also loaded onto gels. The ds-i-motif-bio was successfully attached to streptavidin, which resulted in considerable retarded gel migration in lane 5, compared to sole ds-i-motif-bio in lane 3. This result clearly indicates that ds-i-motif-bio can interact with streptavidin and form complexes. This complexation was also confirmed after staining the double-stranded DNA with PicoGreen dye (Fig. 4B). To confirm streptavidin could interact with Exo-bio, streptavidin was incubated with Exo-bio, and reacted with ds-i-motif-bio. As a negative control, free exosomes were also examined. The resulting streptavidin-mediated Exo-i-motif complexes were analyzed by gel electrophoresis and visualized with an IVIS instrument. The ds-i-motif-bio was selectively labeled with PicoGreen dyes, which allowed easy monitoring of complexation with streptavidin. Owing to binding of ds-i-motif-bio with streptavidin, negligible migration was observed in ds-i-motif-bio-strep. After reacting Exo-bio with strep and then ds-i-motif-bio, complexes were observed near the upper wells. Free ds-i-motif-bio was not observed in the presence of sufficient biotin-binding sites of streptavidin after the reaction of Exo-bio and strep at Exo-bio/strep molar ratio of 1:1 and 1:2. It is well known that streptavidin is a homo-tetramer member of the avidin family and has four biotin-binding sites [25]. When streptavidin reacted with Exo-bio at an Exo-bio/strep molar ratio of 1:1 or 1:2, some of biotin-binding sites remained free, which allowed

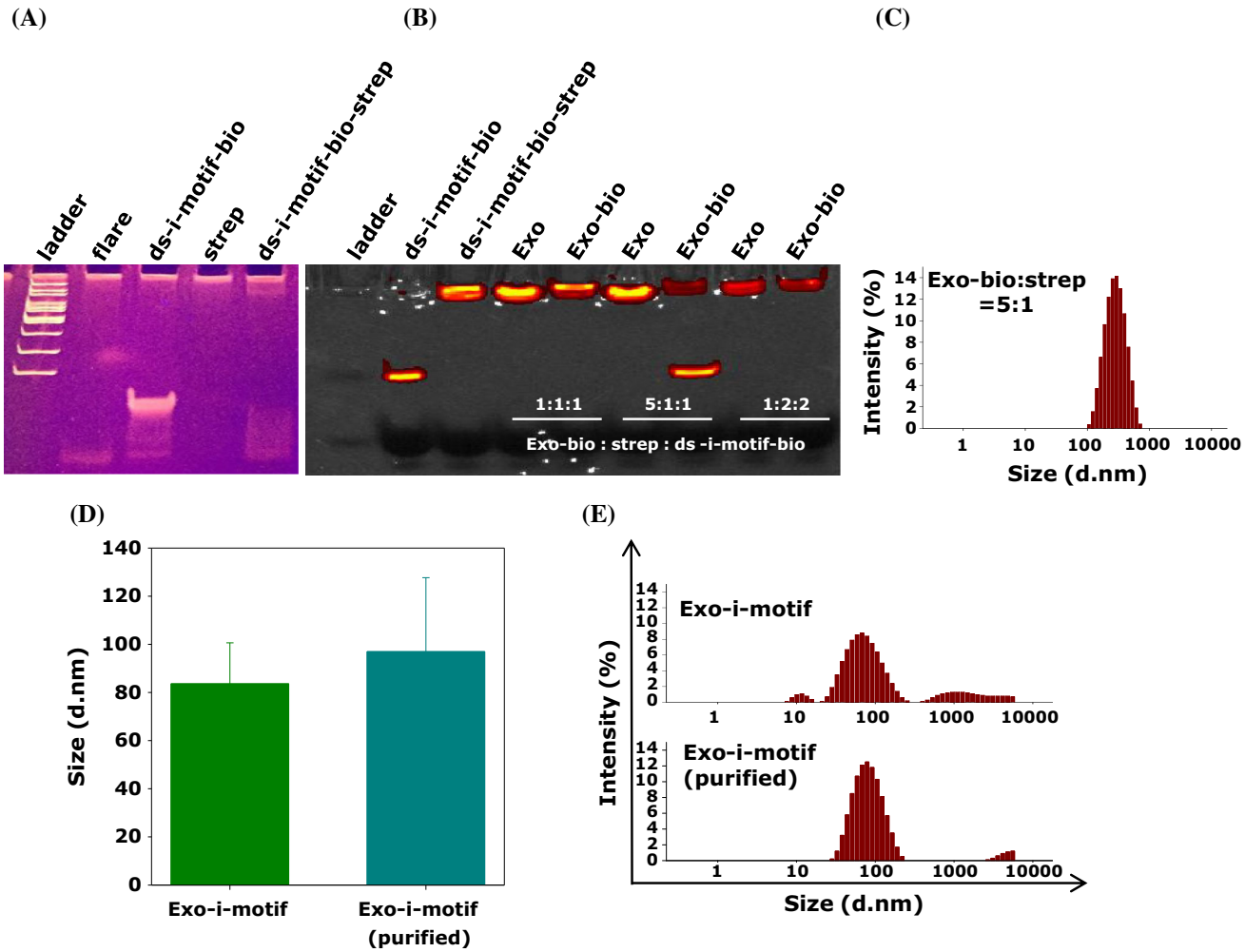

Fig. 4 Characterization of the Exo-i-motif. Poly-acrylamide gel electrophoresis of (**A**) ds-i-motif-bio-strep complexes visualized by EtBr staining and (**B**) Exo-bio-strep-ds-i-motif-bio complexes (Exo-i-motif) visualized by PicoGreen staining at different Exo-bio/strep/ds-i-motif-bio molar ratios. (**C**) Size distribution of Exo-Strep when Exo-bio/strep = 5:1 in molar ratio showing clear aggregation. (**D**) Hydrodynamic sizes and **e** size distribution of Exo-i-motif before and after purification

attachment of the ds-i-motif-bio onto streptavidin. However, when streptavidin reacted with Exo-bio at a Exo-bio/strep molar ratio of 5:1, all of the biotin-binding sites on streptavidin were occupied by Exo-bio, which resulted in aggregated exosomes with hydrodynamic diameter of 239.5 ± 4.1 nm (Fig. 4C). It is conceivable that ds-i-motif-bio could not interact with streptavidin in the presence of excessive Exo-bio. Therefore, unattached ds-i-motif-bio was clearly observed in lane 7, while ds-i-motif-bio added after the reaction of non-biotinylated exosomes and strep was not observed (lane 5). This result clearly indicates that streptavidin interacts both with Exo-bio and ds-i-motif-bio, which could generate i-motif-coated exosomes (Exo-i-motif).

The hydrodynamic size of Exo-i-motif was measured before and after purification. The diameters of Exo-i-motif before and after purification were 83.6 ± 17.0 and 97.0 ± 30.7 nm, respectively (Fig. 4D). The streptavidin-mediated ds-i-motif coating onto exosomes showed significant increase in particle size. However, the average diameter was still below 150 nm, which is appropriate for anticancer drug delivery in vivo [3]. In addition, the purification process using nanosep elicited no noticeable particle aggregation peak in the DLS result (Fig. 4E).

Anti-proliferation activity of i-motif-coated exosomes with DOX

Dox is a well-known anticancer drug that inhibits cell proliferation through inhibition of DNA and RNA polymerase and so induction of apoptosis [26]. To examine whether released Dox from Exo-i-motif was biologically active or not, MCF-7/MDR cells were incubated with Exo-i-motif-Dox. After 48 h of incubation, the amount of cell

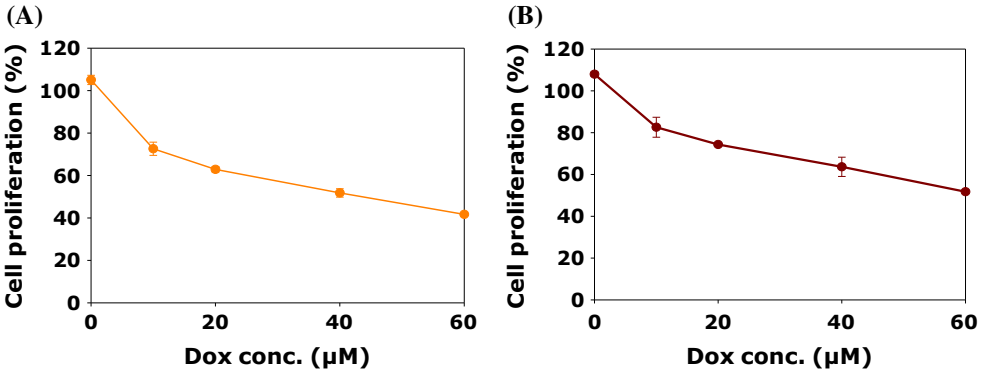

Fig. 5 Anti-proliferation activity of free Dox (**A**) and released Dox from Exo-i-motif complexes (**B**) for MCF-7/MDR cells after 48 h incubation

proliferation was assessed by the CCK8 assay. Figure 5 shows the relative cell proliferation of MCF-7/MDR cells after treatment with free Dox (A) and Exo-i-motif-Dox (B) at different Dox concentrations. Cells treated with Dox at a concentration of 60 μM in free form or incorporated onto Exo-i-motif showed proliferation percentages of 41.7 ± 0.5% and 51.7 ± 0.8%, respectively, compared to no-treated cancer cells. This data clearly indicates that released Dox has evident anti-proliferation activity for MCF-7/MDR cells. It should be also noted that intracellular pathway of particle-loaded Dox is different to that of free Dox. While free Dox could be pumped out of cells via P-glycoprotein after internalization into cells via simple diffusion, Exo-i-motif-Dox might be internalized into cells via endocytosis or phagocytosis [27]. After internalization of Exo-i-motif-Dox, Dox could be released from exosomes at acidic late endosomes [28, 29]. Although Song et al. [18] demonstrated that i-motif-Dox on gold nanoparticles and free Dox showed similar anti-proliferation effect for Hela cells, Exo-i-motif-Dox exhibited slightly reduced anti-proliferation effect compared to free Dox. This result is likely due to incomplete release of Dox from i-motif (Fig. 3B). In addition, previously, several studies reported that the tumor microenvironment is highly acidic compared to normal extracellular matrixes [30]; the acidity of the environment could serve as a signal for targeted delivery of anticancer drugs to tumors. It is conceivable that released Dox from the i-motif in an acidic tumor microenvironment could elicit anti-proliferation activity.

In this study, as a proof-of-concept, the anticancer drug Dox, was loaded onto the Exo-i-motif to be delivered to cancer cells efficiently. After chemical modification of exosomes with biotin and ds-i-motif-bio conjugation via streptavidin on the surface of the exosomes, the particle sizes were below 150 nm without noticeable aggregation. The ds-i-motif-bio efficiently released Dox in an acidic pH-responsive manner within 1 h. Based on gel electrophoresis, it was clearly confirmed that ds-i-motif-bio successfully interacted with Exo-bio through streptavidin via biotin–streptavidin interaction. In addition, the released Dox from the Exo-i-motif had intact bioactivity for the anti-proliferation of MCF-7/MDR cells. This study could serve as a new concept of pH-responsive delivery systems of anticancer drug using the nature-derived Exo and i-motif.

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