International Journal of Pharmaceutics

A simple, fast, and orientation-controllable technology for preparing antibody-modified liposomes

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Abstract

Modification with antibodies is a useful strategy for the delivery of nanoparticles to target cells. However, the complexity of the required chemical modifications makes them time-consuming and low efficiency, and the orientation of the antibody is challenging to control. To develop a simple, fast, effective, and orientation-controllable technology, we employed staphylococcal protein A, which can bind to the Fc region of antibodies, as a tool for conjugating antibodies to nanoparticles. Specifically, we modified the C-domain dimer of protein A to contain a lysine cluster to create a molecule, DPACK, that would electrostatically bind to anionic liposomes. Using this protein, antibody-modified liposomes can be prepared in 35 minutes with two steps: (1) interaction of DPACK with liposomes and (2) interaction of an antibody with DPACK-modified liposomes. Binding efficiencies of DPACK with liposomes and IgG with DPACK-modified liposomes were 75% and 72-84%, respectively. Uptake of liposomes modified with anti-epidermal growth factor receptor (EGFR) antibodies via DPACK by EGFR-expressing cancer cells was significantly higher than that of unmodified liposomes, and the liposomes accumulated in tumors and colocalized with EGFR. This simple, fast, effective and orientation-controllable technology for preparing antibody-modified liposomes will be useful for active targeting drug delivery.

Key words: Antibody-modified liposomes; modified protein A; active targeting

1. Introduction

Targeting delivery of chemotherapeutic agents using nanoparticle carriers, such as liposomes, is an effective strategy for anti-cancer treatments (Allen and Cullis, 2013; Nishiyama et al., 2016). While the enhanced permeability and retention (EPR) effect has been well established as a passive nanoparticle-dependent tumor targeting strategy, active targeting by nanoparticles modified with antibodies that recognize molecules specific to cancer cells potentially offers delivery of drugs with higher specificity (Torchilin, 2008; Manjappa et al., 2011; Bertrand et al., 2014). Various nanoparticles modified with antibodies for anti-cancer therapy have been developed (Torchilin, 2008; Bazak et al., 2015; Muhamad, et al., 2018). For example, effective anti-cancer effects of liposomes modified by antibodies against epidermal growth factor receptors (EGFR) have been reported (Kim et al., 2009; Santos et al., 2021).

Binding of the antibody is a key step in the preparation of antibody-modified nanoparticles. Typically, antibodies are bound covalently to polyethylene glycol (PEG) moieties on PEG-modified nanoparticles or are associated with the surfaces of nanoparticles via N-hydroxysuccinimide (NHS) ester or maleimide (Santos et al., 2021). For example, in one published method, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(succinimidyl-PEG) was incubated with antibody at room temperature for 4 hr to associate antibodies with the tip of the PEG lipid, and the reaction was stopped with excess glycine (Molavi et al., 2013). Column chromatography was required for removal of the excess glycine. In another case, the antigen binding fragment (Fab') of antibodies was conjugated chemically to PEG-maleimide on the surface of liposomes (Nishikawa et al., 2012; Shimizu et al., 2020). To obtain Fab', IgG was treated with pepsin, and then the resultant dimerized fragment (F(ab')2) was reduced with

cysteamine. Fab' was conjugated to maleimide on the surface of the liposomes during a 20 hr incubation at 4ºC. In total, this latter procedure required at least 26 hr to complete the chemical modification of PEG-liposomes with Fab' (Nishikawa et al., 2012).

Currently-available technologies for the chemical modification of nanoparticles with antibodies are complex and time-consuming, and these techniques frequently result in low yields (Okamoto et al., 2018). In addition, it is difficult to control the orientation of the antibodies on the surfaces of nanoparticles, because the chemical modification sites are random, especially in the case of NHS-mediated modifications. Therefore, a simple, fast, effective, and orientation-controllable technology for improved antibody modification of nanoparticles is required. To develop such an ideal technology, we focused on staphylococcal protein A, which can specifically bind to antibodies (Amritkar et al., 2020), as a tool for conjugating antibodies to the nanoparticle surface. Protein A is a 42.0 kDa protein that exists on the surface of *Staphylococcus aureus,* and it can bind strongly to the Fc region of immunoglobulins, especially IgG. Staphylococcal protein A consists of domain X, which does not bind antibody, and five highly similar antibody-binding domains: A, B, C, D and E. Each antibody-binding domain has three helical structures and two loops. The antibody-binding domains bind with the Fc region via hydrophobic interactions on the surface of helices 1 and 2.

In the present study, we used a genetically modified dimer of the C domain of protein A (DPACK), which has a molecular weight of approximately 13 kDa. DPACK is modified to contain a lysine cluster in a region of helix 3 that is opposite the Fc binding domain on helices 1 and 2 (Fig. 1a-c). Since the positively charged lysine cluster of DPACK can associate electrostatically with negatively charged materials, DPACK is expected to bind tightly to the surfaces of negatively charged liposomes upon physical

mixing; upon liposomal interaction, the Fc-binding domain would be presented outward. Thus, we hypothesized that antibody-modified liposomes could be prepared in a short period of time by a two-step binding process. In step one, DPACK binds to the liposome surface. In step two, antibodies are bound to the DPACK-decorated liposomes (Fig. 1d). In the present study, this two-step binding process was evaluated by gel filtration followed by SDS-PAGE analysis and by kinetic analysis of binding among antibody, DPACK and liposomes. Furthermore, functionality of the antibody-modified liposomes prepared by this new technology was examined *in vitro* by quantifying uptake of the liposomes by a cultured cancer cell line and *in vivo* by quantifying accumulation of liposomes in tumors within tumor-bearing mice. Based on the resulting data, we propose that this new method using DPACK is a simple, fast, effective, and orientation-controllable technology for the preparation of antibody-modified liposomes.

2. Materials and methods

2.1. Materials

The sequence of the C-domain of protein A was modified via conventional site-directed mutagenesis techniques to replace amino acids 40, 43, 46, and 53 with lysine to form a lysine cluster in helix 3 (Fig. 1a). This modified protein, DPACK, was produced in *E. coli* and purified via ion-exchange chromatography.

Egg phosphatidylcholine (EPC), dipalmitoyl phosphatidylglycerol (DPPG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(succinimidyl-PEG2000) (DSPE-PEG2000-NHS) were obtained from NOF CORPORATION (Tokyo, Japan). 1, 1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ($DiIC_{18}$) was obtained from Thermo Fisher Scientific (Waltham, MA). Human IgG was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO). Cetuximab, an anti-EGFR antibody, was obtained from Merck Biopharma Co., Ltd (Tokyo, Japan). Other reagents were of the highest grade commercially available.

The human lung adenocarcinoma cell line A549 was obtained from Dainippon Sumitomo Pharma Biomedical Co., Ltd. (Osaka, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2.

2.2. Preparation of antibody-modified liposomes by two-step binding

A chloroform solution containing EPC and DPPG (molar ratio 1:1) in a glass test tube was evaporated to dryness under a nitrogen gas stream. The dried lipid film was hydrated with 1 ml PBS. After a 20 min incubation at 65 °C, freezing and thawing of the hydrated lipid suspension was repeated three times. The glass test tube was sonicated in an

ultrasonic bath for 5 min for sizing of liposomes before filtration. The liposomal suspension was treated using an extruder equipped with a 100 nm polycarbonate membrane filter to control the size of the liposomes. The size and surface charge of liposomes were measured via dynamic light scattering (Zetasizer Nano, Malvern Panalytical Ltd., UK). Liposome diameters and zeta potentials are summarized in Table 1.

Antibody-modified liposomes were prepared by a two-step binding process: (1) DPACK was associated with liposomes and (2) antibodies were bound to DPACK on the modified liposomes (Fig. 1). In step 1, a solution containing DPACK (0.167 mol% of lipid) was added to an EPC/DPPG liposomal suspension (molar ratio 1 : 600), and the mixture was incubated at 37 ºC for 30 min to allow electrostatic association of DPACK with liposomes (DPACK/Lipo). In step 2, an antibody IgG solution was added to DPACK/Lipo suspension to achieve a molar ratio of DPACK to antibody of 50:1, and the mixture was incubated at 37 ºC for 5 min to allow capture of the Fc region of antibodies by DPACK presented on the liposomal surface (Ab/DPACK/Lipo). The diameter and zeta potential of the Ab/DPACK/Lipo were measured as described above.

To evaluate modification efficiency, the sample containing Ab/DPACK/Lipo was processed by gel filtration chromatography using Sepharose 4 Fast Flow resin (GE Healthcare, Chicago, IL), and the absorbance at 750 nm of each fraction was measured. Then, each fraction was subjected to SDS-PAGE using a 15% polyacrylamide gel after incubation with sample buffer containing β-mercaptomethanol. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (CBB), and band intensities were quantified with the image analyzing software ImageJ (https://imagej.net/Citing).

2.3. Preparation of antibody-modified liposomes by a conventional method

EPC/DPPG liposomes prepared as described in section 2.2 were incubated with DSPE-PEG2000-NHS (0.17 mol%) at 60 ºC for 15 min to PEGylate the liposomal surface, and the liposomal suspension was cooled on ice. Then, IgG (0.003 mol% relative to lipid concentration) dissolved in 0.1 M borate buffer, pH 8.4, was added to the liposome suspension, and the mixture was incubated at 25 ºC for 16 hr to chemically conjugate the antibody to the DSPE-PEG2000-NHS. The diameter and zeta potential of the antibody-modified liposomes were measured, and the modification efficiency was evaluated as described above.

2.4. Analysis of interaction among antibody IgG, DPACK and liposomes

The rate constants of association (k_a) and dissociation (k_d) were measured with bio-layer interferometry (BLItz System, Sartorius AG, Germany). In this analysis, biotinylated-IgG was used. Human polyclonal IgG (Kaketsuken, Japan) was incubated with Sulfo-NHS (N-hydroxysulfosuccinimide)-Biotin (Thermo Fisher Scientific, Waltham, MA) at 25 ºC for 30 min. Then, the mixture was dialyzed at 4 ºC overnight to remove unreacted Sufo-NHS-Biotin. We evaluated the dissociation constant (KD) of biotinylated-IgG/DPACK was 4 x 10-8 M. This value was similar to the previously reported value (KD = 9 x 10^{-8} M) of IgG/Protein A (Gülich, et al., 2000). Therefore, it was suggested that the binding ability of the Fc region of IgG to DPACK was not affected by biotinylation, although biotinylated site of IgG was random. First, a DPACK solution was added to biotinylated-IgG fixed on a streptavidin chip after equilibration with PBS containing 0.1% BSA for 150 s. At 300 s, various amounts of liposome in suspension (0-160 μM) were added. Then, dissociation was initiated by incubation with PBS containing 0.1% BSA for 150 s. The

rate constants k_a and k_d were calculated from the resulting sensorgrams, and the dissociation constant K_D was calculated as the ratio of k_d to k_a.

2.5. Quantification of uptake of liposomes by cultured human lung cancer A549 cells expressing EGFR

Ab/DPACK/Lipo was prepared with anti-EGFR antibodies conjugated to liposomes fluorescently labeled with 1% $DiIC_{18}$ (Ab(EGFR)/DPACK/Lipo), and modification efficiency was evaluated as described in section 2.3. In this study, we used anti-EGFR antibody (abcam, ab52894), which can be applied to western blotting, immunoprecipitation, flow cytometry, immunohistochemistry (Formalin/PFA-fixed paraffin-embedded sections), immunocytochemistry/immunofluorescence and ELISA, suggesting that the recognition site of the antibody was extracellular domain of EGFR.

Fluorescently-labeled Ab(EGFR)/DPACK/Lipo (final concentration 0.3 mM) was added to A549 cells cultured on 35mm glass bottom dishes (7.5×10^4 cells/dish), and the cells were incubated at 37 ^oC. After a 1 hr incubation, the cells were washed with PBS and fixed by treatment with 4% paraformaldehyde. The fixed cells were washed three times with PBS and stained with the nuclear staining dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Thermo Fisher Scientific, Waltham, MA) at 37 ^oC for 15 min. Stained cells were washed with PBS and observed by confocal laser scanning microscopy (LSM700, Carl ZEISS, Germany). The fluorescence intensity of the $DiIC_{18}$ liposomes was quantified with the image analyzing software ImageJ (https://imagej.net/Citing).

2.6. Preparation of tumor-bearing mice

Five-week-old balb/c nu/nu male mice were purchased from Japan SLC, Inc.

(Shizuoka, Japan). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of Tokushima University. To prepare tumor-bearing mice, a suspension of A549 cells (5.0×10^6 cells) was injected subcutaneously into the lower left posterior flank. After inoculation of cancer cells, tumor size and body weight were measured daily.

2.7. Microscopic observation of fluorescently labeled liposomes in tumors of tumor bearing mice

To examine the *in vivo* tumor targeting of Ab(EGFR)/DPACK/Lipo, liposomes labeled with $DiIC_{18}$ (100 μmol/kg) were administered via intravenous (i.v.) injection into A549 tumor-bearing mice in which the tumor size was over 200 mm^3. After 24 hours, 10 μm frozen sections of tumors were prepared by cryostat (CM3050S, Leica Microsystems GmbH, Germany), and immunohistochemical staining with a rabbit anti-EGFR antibody (Abcam plc, UK) was performed. Frozen sections were incubated with the anti-EGFR antibody at 4 ºC for 18 hr after fixation with 4% paraformaldehyde. Then, the section was washed three times with PBS and was incubated at room temperature for 30 min with an Alexa Fluor 488-labeled goat anti-rabbit IgG antibody (Abcam plc, UK). Stained sections were observed by confocal laser scanning microscopy (LSM700, Carl ZEISS, Germany). The fluorescence intensity of $DiIC_{18}$ liposomes in cross section images was quantified with the image analyzing software ImageJ (https://imagej.net/Citing).

2.8. Statistical analyses

Statistical analyses were performed using one-way ANOVA followed by the Tukey–Kramer honest significant difference (HSD) test. *P* values < .05 were considered

significant. Data were evaluated using KaleidaGraph (Hulinks Inc., Japan).

3. Results and Discussion

3.1. Preparation of antibody-modified liposomes and physicochemical analyses of the nanoparticles

In this study, we prepared negatively-charged liposomes consisting of EPC and DPPG, an anionic lipid. Since the size of the liposomes was controlled by extrusion through membrane filters, the polydispersity index (PDI) was very small, indicating a narrow size distribution (Table 1). Both the liposome diameter and PDI increased after modification with DPACK. The size increase and reduction of homogeneity are consistent with modification of the liposomal surface with DPACK and antibody. In addition, an index of the charge of the nanoparticle surface, the zeta potential, decreased by 8 mV upon modification of liposomes with DPACK and antibody. It is likely that the decrease in surface charge is due not only to shielding of the charged liposomal surface by proteins but also to neutralization of phospholipid negative charges by positive charges of DPACK. These results are consistent with an electrostatic covering of the liposomal surface by the DPACK and antibody proteins. Regarding liposome preparation, we investigated for the preparation of immunoliposomes at different concentration of DPACK and antibody. The excess amount of antibody induced aggregation of liposomes, DPACK and antibody. Thus, we measured the size of liposomes after addition of DPACK and antibody at various amount ratios for evaluation of the aggregation as preliminary experiments. Based on the results of the preliminary experiments, we fixed the ratio of liposomes, DPACK and antibody as the best condition for in vitro and in vivo experiment.

Mixtures of liposomes, DPACK, and antibody were subjected to gel filtration in order to separate liposomes modified with DPACK and antibody from free liposomes, free DPACK, and free antibody. Turbidity of all fractions obtained by gel filtration was

measured at 750 nm. Obvious turbidity, which indicates the presence of liposomes, was observed in fractions 3 through 5 (Fig. 2a). Samples from each gel filtration fraction were treated with β-mercaptoethanol, and the proteins were separated by SDS-PAGE and stained with CBB (Fig. 2b). As shown in Figure 2b, bands corresponding to DPACK (13 kDa) and IgG (50 kDa (heavy chain) and 25 kDa (light chain)) were observed only in liposome-containing fractions 3 through 5. Moreover, protein band intensities correlated with the turbidities of liposomes in these fractions. These results are consistent with association of DPACK and IgG with liposomes in those fractions. The modification efficiencies of liposomes with DPACK and IgG as calculated by the band intensities displayed in Figure 2b were 76.3% and 84.1%, respectively (Table 2).

To compare the modification efficiency of the method developed in the present study with that achieved via a conventional method, we prepared antibody-modified PEG-liposomes using the NHS-mediated ligand conjugation method according to a published report (Ichikawa et al., 2013). The modification efficiency of PEG-liposomes with IgG achieved by the conventional chemical method was 14.0% (Table 2). In addition, the conventional method took approximately 17 hrs to complete, whereas the new method took approximately 0.5 hrs. Thus, the new method achieves a higher modification efficiency (6-fold) with a shorter time requirement (34-fold) relative to a conventional method.

3.2. Analysis of interactions among IgG, DPACK, and liposomes

To evaluate the interactions among IgG, DPACK, and liposomes, a binding reaction was performed as described in Materials and Methods. After addition of DPACK to biotinylated IgG fixed on surface of a bio-layer interferometry chip, a liposome

suspension was added to allow a binding reaction between liposomes and the IgG/DPACK complex. Dissociation of the complex was then measured in liposome-free buffer containing 0.1% BSA (Fig. 3). Binding of liposomes increased linearly with IgG/DPACK concentration. The dissociation rate constant (k_d) was 4.14×10^{-4} s^{-1}; this rate constant includes both k_{d1} (dissociation of IgG and DPACK) and k_{d2} (dissociation of DPACK and liposomes). From the k_d value in the absence of liposomes, k_{d1} was calculated to be 7×10^{-6} s^{-1}. Accordingly, k_{d2} was determined to be 4.07×10^{-4} s^{-1} .

From the sensorgram data, the rate constant of association (k_a) was determined to be 2.08×10^{2} $M^{-1}s^{-1}$. The dissociation constant (K_D) as calculated as the ratio of k_d to k_a, was 1.99×10^{-6} M. The sensorgram did not change significantly over the time period from 450 – 600 seconds, when a BSA solution was applied. This result suggests that binding among IgG, DPACK and liposomes might not be inhibited significantly by other proteins, such as serum albumin. Thus, DPACK and IgG are not expected to leave the surface of liposomes, even though the interactions between DPACK/IgG and liposomal surface are electrostatic, not covalent.

We examined the effect of pH and blood component serum on antibody binding on DPACK-modified liposomes. We prepared the immunoliposomes using fluorescein isothiocyanate (FITC) labeled antibody, and the Ab/DPACK/Lipo were incubated at 37 ^{o}C for 3hr in PBS, of which pH values were 6.5 and 7.4, respectively. After the incubation at different pH, unbound FITC-antibody in the liposome suspension was separated by gel filtration. The relative fluorescence intensity of liposome incubated at pH 6.5 was 0.68±0.28 against that at pH 7.4 (1.00). Although the difference was not statistically significant, the fluorescence intensity decreased by treatment with slightly acidic condition. It was suggested that slightly acidic condition (pH 6.5) such as intratumoral

environment might affect the modification of antibody to liposomes, although the effect of pH change was not significant. Furthermore, we incubated the liposomes at 37 ºC for 3hr in PBS containing 50% FBS without inactivation. Then, the mixture including liposomes was subjected to gel filtration for separation of unbound FITC-antibody from liposomes. As a result, the fluorescence intensity of liposome suspension after gel filtration did not decrease, suggesting that FITC labeled IgG bound with DPACK was not replaced to other antibodies in FBS. In addition, we measured the particle size of the Ab/DPACK/Lipo after incubation with 50% FBS and gel filtration. The size of liposomes did not change significantly even after incubation in 50% FBS solution (data not shown).

3.3. Modification of DPACK/Lipo with anti-EGFR antibody

To evaluate the function of antibodies associated with liposomes through DPACK, we prepared liposomes that can specifically recognize EGFR on cancer cells. In this study, we used the anti-EGFR antibody cetuximab, which is a chimeric antibody consisting of human IgG (65%) and murine IgG (35%). The affinity of cetuximab for EGFR is higher than that of the receptor's natural ligand (Mehra et al., 2008). To prepare the anti-EGFR antibody-modified liposomes, a solution containing cetuximab was incubated with a DPACK/Lipo suspension, and then gel filtration and SDS-PAGE were performed to estimate the modification efficacy (Fig. 4). Similar to the results obtained for association of liposomes with DPACK and non-specific human IgG (Fig. 2a), well-defined turbidity, indicating the presence of liposomes, was observed in fractions 3 through 5 (Fig. 4a). In the SDS-PAGE data (Fig. 4b), bands corresponding to DPACK (13 kDa) and anti-EGFR antibody (50 kDa (heavy chain) and 25 kDa (light chain)) were observed only in liposome-containing fractions (3 through 5). Thus, anti-EGFR antibody

and DPACK copurified with liposomes. The modification efficacies of liposomes with DPACK and anti-EGFR antibody calculated by the intensities of bands displayed in Figure 4b were 74.5% and 72.3%, respectively (Table 3). Clearly, the antibody modification efficacy of DPACK/Lipo is significant, regardless of the identity of the antibody. We attempted to calculate the number of DPACK and antibody on one liposome. At first, the number of liposomes formed by 1 mol of lipid was obtained based on the average surface area of one lipid molecule. Then, binding amounts of DPACK and antibody to liposomes were divided by the liposome number in 1 mol of lipid. As a result, the number of DPACK and antibody binding on one liposome were 314 and 6, respectively. Contrary to expectations, the binding number of antibody was very low.

We tried atomic force microscopy (AFM) imaging of the plane liposomes and immunoliposomes. From the AFM images of both liposomes, it was difficult to recognize the difference between un-modified liposomes and liposomes modified with DPACK and antibody (Ab(EGFR)/DPACK/Lipo) (Supplemental figure 1). Based on the calculation of the number of DPACK and antibody on the surface of liposomes, the number of those proteins on one liposome were around 300 and 6, respectively. Since the size of DPACK and antibody is too smaller than liposome, it was difficult to detect the modification of the liposomal surface by microscopic observation.

3.4. Quantification of Ab/DPACK/Lipo binding to and uptake by cancer cells

We evaluated the affinity of liposome assemblies associated with anti-EGFR antibodies (Ab(EGFR)/DPACK/Lipo) for A549 cells, a human lung cancer adenocarcinoma cell line in which EGFR is presented on the surface. A suspension of Ab(EGFR)/DPACK/Lipo containing fluorescently-labeled lipid was incubated with A549

cells for 1 hr at 37 ºC, and then the cells were observed by confocal laser scanning microscopy (Fig. 5a). Fluorescently-labeled liposomes consisting of EPC and DPPG without DPACK/Anti-EGFR antibody were used as a control. With control liposomes, very weak fluorescence was observed in the cells even after 1 hr incubation (Fig. 5a). On the other hand, significant liposome-specific fluorescence was observed in cells incubated with Ab(EGFR)/DPACK/Lipo (Fig. 5a). The liposomal fluorescence was distributed widely throughout all of the cells. The amount of fluorescently-labeled Ab(EGFR)/DPACK/Lipo in cells, as quantified with image analysis software, was significantly higher (about 9-fold) than that of unmodified control liposomes (Fig. 5b). From these results, it is clear that Ab(EGFR)/DPACK/Lipo has a high affinity for cancer cells that express cell surface EGFR, even though the zeta potential of Ab/DPACK/Lipo was found to be negative (Table 1). Thus, it is likely that the observed association is mediated by binding of the antibody to a specific target (i. e., EGFR) as opposed to a non-specific electrostatic effect.

We evaluated the modification efficiency and cellular uptake amounts of liposomes modified with different amount of antibody. We prepared DiI-labeled liposomes modified with DPACK and one-fifth amount of antibody (1/5Ab(EGFR)). The antibody modification efficiency of liposomes modified with 1/5Ab(EGFR) (1/5Ab(EGFR)/DPACK/Lipo) was 83.6%. We compared the relative fluorescence intensities of the cells after treatment with unmodified liposomes (Cont Lipo), 1/5Ab(EGFR)/DPACK/Lipo and antibody-modified liposomes shown in original manuscript (Ab(EGFR)/DPACK/Lipo). The fluorescence intensity of the cells treated with 1/5Ab(EGFR)/DPACK/Lipo was higher than that of Cont Lipo, while the values of 1/5Ab(EGFR)/DPACK/Lipo was lower than Ab(EGFR)/DPACK/Lipo (Supplemental

figure 2). Thus, the liposome-targeting potential was dependent on the antibody modification amount.

We examined the cellular uptake of Ab(EGFR)/DPACK/Lipo in the presence of an excess amount (10 times the amount of liposome modification) of antibody. Unexpectedly, fluorescence intensity of the antibody-modified liposomes in the presence of an excess amount of antibody was about 2.9 times higher than that of only the antibody-modified liposomes. In the presence of an excess amount of antibody (Ab(EGFR)/DPACK/Lipo + excess Ab(EGFR)), aggregated liposomes were observed in the cells (Supplemental figure 3). In this study, the small amount of antibody was used for the preparation of Ab(EGFR)/DPACK/Lipo. Since there might be DPACK Fc binding sites which are not occupied by the anti-EGFR antibody, it was guessed that liposomes bind with antibodies associating with antigen protein on the surface of cells, then form aggregation. Probably, as the aggregated liposomes were taken up by the cells, fluorescence intensity of the antibody-modified liposomes in the presence of an excess amount of antibody was significantly higher than that of only the antibody-modified liposomes. As a result, cellular uptake of Ab(EGFR)/DPACK/Lipo was not inhibited, but increased by an excess amount of antibody. However, we consider that the preparation condition for antibody-modified liposomes optimized in this study is appropriate for avoidance of liposome aggregation, even though the antibody amount modified on liposomes was low.

Although it was expected that the DPACK-modified liposomes can bind with much amount of antibody, we considered that the ratio of DPACK and antibody fixed in this study was optimum to avoid increase in particle size. In this study, we focus on the development of simple and quick technology for preparation of antibody-modified

liposomes. However, based on the analysis of the relationship between the amount of DPACK/antibody modification and the anticancer activity of the anticancer drug containing Ab (EGFR)/DPACK/Lipo, we would like to try more precise optimization of the amount of modification of DPACK and antibody in the future.

3.5. Accumulation of intravenously administered Ab(EGFR)/DPACK/Lipo in tumors in live mice

To examine the potential for active tumor targeting by antibody-modified liposomes *in vivo*, we evaluated intra-tumor accumulation of fluorescently labeled Ab(EGFR)/DPACK/Lipo administered via i.v. injection to A549 tumor-bearing mice. Cross sections of tumors from injected mice underwent immunohistochemical staining with an anti-EGFR antibody, and EGFR-positive cells and fluorescent liposome accumulation were observed with confocal laser scanning microscopy (Fig. 6). In tumors of mice treated with unmodified fluorescently labeled PEG-liposomes (Cont Lipo), very faint fluorescence signals were observed. On the other hand, significant red fluorescence was observed in the tumors after i.v. administration of Ab(EGFR)/DPACK/Lipo (Fig. 6a). The intensity of the red fluorescence (Ab(EGFR)/DPACK/Lipo) in the tumor cells was quantified with image analysis software. The fluorescence intensity in tumors of mice treated with Ab(EGFR)/DPACK/Lipo was approximately 6-fold higher than that of Cont Lipo (Fig. 6b).

Wide distribution of EGFR in tumors was confirmed by immunostaining with an anti-EGFR primary antibody and an Alexa Fluor 488-labeled goat anti-human IgG secondary antibody (Fig. 6a, green). In merged images, extensive yellow signal was observed, indicating colocalization of Ab(EGFR)/DPACK/Lipo and EGFR in the tumor.

These results suggest that Ab(EGFR)/DPACK/Lipo targets EGFR on cancer cells via the anti-EGFR antibody on the liposomal surface. These results are consistent with accumulation of Ab(EGFR)/DPACK/Lipo in tumors after i.v. injection by active targeting of EGFR by the associated anti-EGFR antibody.

We additionally compared the accumulation of DiI-labeled unmodified liposomes (Cont Lipo), non-specific antibody human IgG-modified liposomes (IgG/DPACK/Lipo) and Ab(EGFR)/DPACK/Lipo in mouse tumors after i.v. administration. IgG/DPACK/Lipo were also accumulated in the tumor like Cont-Lipo and Ab(EGFR)/DPACK/Lipo shown in Fig 6 (Supplemental figure 4). However, no significant difference was found between Cont Lipo and IgG/DPACK/Lipo.

Some publications have shown the similar tumor accumulation between non-modified liposomes and immunoliposomes via EPR effect. According to the previous paper (Kirpotin et al., 2006), it has been mentioned that both targeted and nontargeted liposomes achieved similar high levels of tumor tissue accumulation in HER2-overexpressing breast cancer xenografts. However, the localization of targeted and nontargeted liposomes was different in tumor, i.e., nontargeted liposomes were located predominantly in extracellular stroma or within macrophages, while anti-HER2 immunoliposomes achieved intracellular drug delivery via Mab-mediated endocytosis. In this study, we performed cardiac perfusion before collection of tumor tissue. Thus, non-modified liposomes existing in extracellular stroma would be washed out, while the Ab(EGFR)/DPACK/Lipo would remain on/in the cancer cells via antibody binding with EGFR. A recent publication (Shimizu et al., 2020) supports this consideration regarding the difference of tumor accumulation between nontargeted liposomes and targeted liposomes.

We attempted to evaluate the percentage of liposome accumulation in the solid tumor to injection dosage. Since we could not use radio isotope to trace the biodistribution of antibody-modified liposomes, we measured the accumulation amounts of liposomes labeled with DiI in tumor by in vivo imaging system (IVIS), although we recognized that the sensitivity and quantitative of IVIS is lower than that of radio isotope method. The accumulation percentages of control liposome, IgG-modified liposomes and anti-EGFR antibody-modified liposomes in the collected tumor calculated by fluorescence intensity measured by IVIS were around 0.1 %. Unexpectedly, the values were very low and almost the same, although fluorescence intensity of Ab(EGFR)/DPACK/Lipo in tumor cross section was higher than that of control liposomes in Figure 6. We considered the reason for this discrepancy. The very low accumulation percentages might be due to low delivery efficiency because the liposomes surface was not shielded with PEG. In addition, another possible reason would be the low sensitivity of IVIS to detect fluorescence present in organs. In fact, as shown in Supplemental figure 4, relative fluorescence intensities of these three liposomes were different, and Ab(EGFR)/DPACK/Lipo showed significant higher accumulation in tumor than control liposomes. Due to these reasons, even though there were difference of accumulation percentages among control liposomes, IgG-modified liposomes and anti-EGFR antibody-modified liposomes, the significant difference could not be recognized in this experiment using IVIS. Based on this experiment, we found that accumulation amounts of antibody-modified liposomes developed in this study would not enough for anti-cancer therapy, although the purpose of this study is development of simple and easy preparation method of antibody-modified liposomes. Thus, for application of the liposomes developed in this study to anti-cancer therapy, improvement of blood retention of the antibody-modified liposomes, such as

PEGylation, would be required in the future.

In addition, we examined the immunogenicity about the liposomes modified with DPACK and anti-EGFR antibody (Ab(EGFR)/DPACK/Lipo). We evaluated the amounts of inflammatory cytokines, interleukin (IL)-12 and tumor necrosis factor (TNF)-α, by ELISA in the serum 24 hr after i.v. administration of the Ab(EGFR)/DPACK/Lipo. A single administration of DPACK showed tendency of increase in the IL-12, although the change was not statistically significant (Supplemental Figure 5). While, the Ab(EGFR)/DPACK/Lipo did not show significant induction of IL-12. Furthermore, the amount of TNF-α in serum did not change after administration of DPACK only and the Ab(EGFR)/DPACK/Lipo, respectively (Supplemental Figure 5). Thus, it was confirmed that the Ab(EGFR)/DPACK/Lipo did not have immunogenicity, especially inflammation. Probably, since the surface of liposomes was covered with immunoglobulin via DPACK, the immunogenicity of DPACK was sealed by immunoglobulin shell.

The modification of liposomes with biocompatible molecule PEG is common method for in vivo administration. In this study, we consider that the Ab(EGFR)/DPACK/Lipo could reach into tumor by their blood retention due to the negatively surface charge. Thus, the immunoliposomes developed in this study would have some degree of biocompatibility. However, the liposome modification with PEG will be required for clinical use. For PEGylation of our immunoliposomes, the optimization of component ratio, modification timing and balance between targeting capability and biocompatibility are necessary. We would like to challenge the PEGylation of our immunoliposomes for clinical use in the future.

4. Conclusion

We have developed a simple, fast, orientation-controllable and effective technology for preparing antibody-modified liposomes by electrostatic interaction of positively charged DPACK with negatively charged liposomes. The modification efficacies of liposomes with DPACK and antibody were significantly higher than those of conventional methods, although the preparation time was only 35 min. The Ab(EGFR)/DPACK/Lipo exhibited high affinity to cancer cells expressing the target protein EGFR *in vitro* and achieved active targeting to mouse tumors following i.v. injection *in vivo*. The DPACK-mediated physical association of antibodies to liposomes is applicable not only to EGFR but also to other therapeutic antibodies. The technology developed in this study serves as a novel and effective method for preparing active drug delivery systems against cancer cells and other therapeutic targets.

Acknowledgements

This work was supported in part by a Tokushima University research program for the development of an intelligent Tokushima artificial exosome (iTEX).

Declaration of Interests

The authors declare no competing financial interests.

Author Contributions

K. Kogure conceived of and supervised the study, designed experiments, and wrote the manuscript; T. Fukuta and E. Majima supervised the study; Y. Hirata, R. Tashima, M. Ozono, S. Yoneda and N. Mitsuhashi performed experiments and analyzed data. All authors read and approved of the final manuscript.

References

Allen, T.M., Cullis, P.R., 2013. Liposomal drug delivery systems: from concept to clinical applications. Adv. Drug Deliv. Rev. 65, 36-48. https://doi.org/10.1016/j.addr.2012.09.037.

Bazak, R., Houri, M., E.l. Achy, S., Kamel, S., Refaat, T., 2015. Cancer active targeting by nanoparticles: a comprehensive review of literature. J. Cancer Res. Clin. Oncol. 141, 769-784. https://doi.org/10.1007/s00432-014-1767-3.

Amritkar, V., Adat, S., Tejwani, V., Rathore, A., Bhambure, R., 2020. Engineering Staphylococcal Protein A for high-throughput affinity purification of monoclonal antibodies. Biotechnol. Adv. 44, 107632. https://doi.org/10.1016/j.biotechadv.2020.107632.

Bertrand, N., Wu, J., Xu, X., Kamaly, N., Farokhzad, O.C., 2014. Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology. Adv. Drug Deliv. Rev. 66, 2-25. https://doi.org/10.1016/j.addr.2013.11.009.

Gülich, S., Uhlén, M., Hober, S., 2000. Protein engineering of an IgG-binding domain allows milder elution conditions during affinity chromatography. J. Biotechnol. 76, 233-244. https://doi.org/10.1016/s0168-1656(99)00197-2.

Ichikawa, K., Asai, T., Shimizu, K., Yonezawa, S., Urakami, T., Miyauchi, H., Kawashima, H., Ishida, T., Kiwada, H., Oku, N., 2013. Suppression of immune response by antigen-modified liposomes encapsulating model agents: a novel strategy for the treatment of allergy. J. Control. Release 167, 284-289. https://doi.org/10.1016/j.jconrel.2013.02.002.

Kim, I.Y., Kang, Y.S., Lee, D.S., Park, H.J., Choi, E.K., Oh, Y.K., Son, H.J., Kim, J.S., 2009. Antitumor activity of EGFR targeted pH-sensitive immunoliposomes encapsulating gemcitabine in A549 xenograft nude mice. J. Control. Release 140, 55-60.

https://doi.org/10.1016/j.jconrel.2009.07.005.

Kirpotin, D.B., Drummond, D.C., Shao, Y., Shalaby, M.R., Hong, K., Nielsen, U.B., Marks, J.D., Benz, C.C., Park, J.W., 2006. Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. Cancer Res. 66, 6732-6740. https://doi.org/10.1158/0008-5472.CAN-05-4199.

Lavasanifar, A., Lai, R., 2013. Anti-CD30 antibody conjugated liposomal doxorubicin with significantly improved therapeutic efficacy against anaplastic large cell lymphoma. Biomaterials 34, 8718-8725. https://doi.org/10.1016/j.biomaterials.2013.07.068.

Manjappa, A.S., Chaudhari, K.R., Venkataraju, M.P., Dantuluri, P., Nanda, B., Sidda, C., Sawant, K.K., Murthy, R.S., 2011. Antibody derivatization and conjugation strategies: application in preparation of stealth immunoliposome to target chemotherapeutics to tumor. J. Control. Release 150, 2-22. https://doi.org/10.1016/j.jconrel.2010.11.002.

Mehra, R., Cohen, R.B., Burtness, B.A., 2008. The role of cetuximab for the treatment of squamous cell carcinoma of the head and neck. Clin. Adv. Hematol. Oncol. 6, 742-750.

Molavi, O., Xiong, X.B., Douglas, D., Kneteman, N., Nagata, S., Pastan, I., Chu, Q., Nishikawa, K., Asai, T., Shigematsu, H., Shimizu, K., Kato, H., Asano, Y., Takashima, S., Mekada, E., Oku, N., Minamino, T., 2012. Development of anti-HB-EGF immunoliposomes for the treatment of breast cancer. J. Control. Release. 160, 274-280. https://doi.org/10.1016/j.jconrel.2011.10.010.

Muhamad, N., Plengsuriyakarn, T., Na-Bangchang, K., 2018. Application of active targeting nanoparticle delivery system for chemotherapeutic drugs and traditional/herbal medicines in cancer therapy: a systematic review. Int. J. Nanomedicine 13, 3921-3935. https://doi.org/10.2147/IJN.S165210.

Nishiyama, N., Matsumura, Y., Kataoka, K., 2016. Development of polymeric micelles for targeting intractable cancers. Cancer Sci. 107, 867-874. https://doi.org/10.1111/cas.12960.

Okamoto, A., Asai, T., Hirai, Y., Shimizu, K., Koide, H., Minamino, T., Oku, N., 2018. Systemic Administration of siRNA with Anti-HB-EGF Antibody-Modified Lipid Nanoparticles for the Treatment of Triple-Negative Breast Cancer. Mol. Pharm. 15, 1495-1504. https://doi.org/10.1021/acs.molpharmaceut.7b01055.

Santos, E.D.S., Nogueira, K.A.B., Fernandes, L.C.C., Martins, J.R.P., Reis, A.V.F., Neto, J.B.V., Júnior, I.J.D.S., Pessoa, C., Petrilli, R., Eloy, J.O., 2021. EGFR targeting for cancer therapy: Pharmacology and immunoconjugates with drugs and nanoparticles. Int. J. Pharm. 592, 120082. https://doi.org/10.1016/j.ijpharm.2020.120082.

Shimizu, K., Takeuchi, Y., Otsuka, K., Mori, T., Narita, Y., Takasugi, S., Magata, Y., Matsumura, Y., Oku, N., 2020. Development of tissue factor-targeted liposomes for effective drug delivery to stroma-rich tumors. J. Control. Release 323, 519-529. https://doi.org/10.1016/j.jconrel.2020.04.043.

Figure legends

Figure 1. a) Amino acid sequence of the C domain of protein A modified to contain a lysine cluster (DPACK). b) Schematic image showing the locations of the Fc binding regions on helices 1 and 2 of DPACK. c) The cluster of seven lysine residues in helix 3. d) The process for modification of liposomes with antibody via DPACK.

Figure 2. Analysis of association among antibody, DPACK and liposomes

a) Samples containing liposomes, DPACK, and antibody were subjected to gel filtration. Turbidity, indicating the presence of liposomes, was measured at 750nm. b) Fractions were analyzed by SDS-PAGE followed by CBB staining. The positions of molecular weight markers (MW) are indicated by arrows. The migration of free DPACK protein (DPACK), and free antibody IgG (Ab) are indicated. Bands corresponding to antibody (50 kDa: heavy chain and 25 kDa: light chain) and DPACK (13 kDa) in fractions No. 3 through -5 were surrounded by dotted lines.

Figure 3. Analysis of interactions among IgG, DPACK and liposomes

Interactions among IgG, DPACK and liposomes were measured by bio-layer interferometry as described in Materials and Methods. A solution containing DPACK was administered to IgG immobilized on a sensor chip from 150 to 300 s, and then association of liposomes to the antibody/DPACK complex was monitored from 300 to 450 s. a) Raw sensorgrams as measured by the BLItz System. b) Sensorgrams processed by subtraction of ligand binding and sign inversion. The association rate constant (k_a) and dissociation rate constant (k_d) were obtained from these sensorgrams. The dissociation constant (K_D)

was calculated as the ratio of k_d to k_a.

Figure 4. Analysis of association among anti-EGFR antibodies, DPACK and liposomes

a) Samples containing liposomes, DPACK, and anti-EGFR antibodies were subjected to gel filtration. Turbidity, indicating the presence of liposomes, was measured at 750nm. b) Fractions were analyzed by SDS-PAGE followed by CBB staining. Pure anti-EGFR antibody was analyzed in the lane labelled Anti EGFP Ab. Other abbreviations are as shown in Figure 2. The bands corresponding to antibody (50 kDa: heavy chain and 25 kDa: light chain) and DPACK (13 kDa) in fractions 3 through 5 are surrounded by dotted lines.

Figure 5. Evaluation of Ab(EGFR)/DPACK/Lipo affinity to A549 cancer cells expressing EGFR

a) Confocal laser scanning microscopic images of A549 cells after incubation with $DiIC_{18}$-labeled Ab(EGFR)/DPACK/Lipo and DAPI counterstaining. Red fluorescence and blue fluorescence indicate liposomes and nuclei, respectively. Cont Lipo denotes cells treated with $DiIC_{18}$-labeled liposomes without antibody or DPACK. The white bar indicates 50 μm. b) Relative fluorescence intensity of $DiIC_{18}$ in each image was quantified with ImageJ. The data are shown as the average ± SD obtained from at least three independent experiments. *$p< 0.05$.

Figure 6. Evaluation of Ab(EGFR)/DPACK/Lipo accumulation in mouse tumors after i.v. administration

a) Immunohistochemical images of tumor cross sections obtained 24 hours after i.v. administration of DiIC18-labeled unmodified liposomes (Cont Lipo) or Ab(EGFR)/DPACK/Lipo. Red fluorescence and green fluorescence indicate liposomes and EGFR, respectively. The white bar indicates 100 μm. b) Relative fluorescence intensity of DiIC18 in each image was quantified with ImageJ. The data are shown as the average ± SD obtained from at least three independent experiments. *$p < 0.05$.

Figure 1

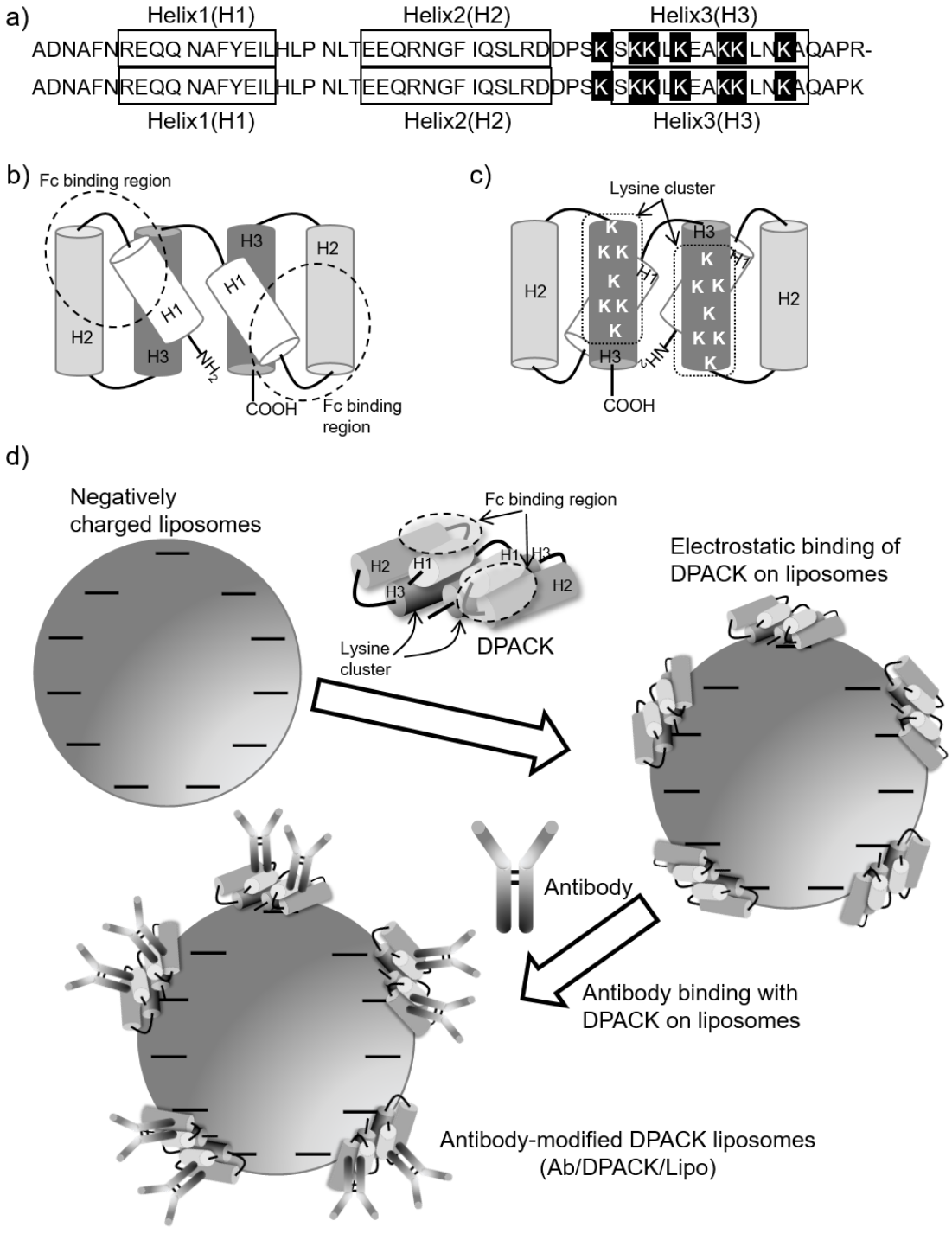

a)
Helix1(H1)
Helix2(H2)
Helix3(H3)
ADNAFNREQQ NAFYEILHLP NLTEEQRNGF IQSLRDDPSK SKKLKEAKK LNKAQAPR-
ADNAFNREQQ NAFYEILHLP NLTEEQRNGF IQSLRDDPSK SKKLKEAKK LNKAQAPK
Helix1(H1)
Helix2(H2)
Helix3(H3)
b)
Fc binding region
H2
H1
H3
H3
H2
H1
NH2
COOH
Fc binding region
c)
Lysine cluster
H2
K
K K
K
K K
K
H3
H1
H3
K
K K
K
K K
K
H1
H2
NH2
COOH
d)
Negatively charged liposomes
Fc binding region
H2
H1
H3
H1
H3
H2
Lysine cluster
DPACK
Electrostatic binding of DPACK on liposomes
Antibody
Antibody binding with DPACK on liposomes
Antibody-modified DPACK liposomes (Ab/DPACK/Lipo)

Figure 2

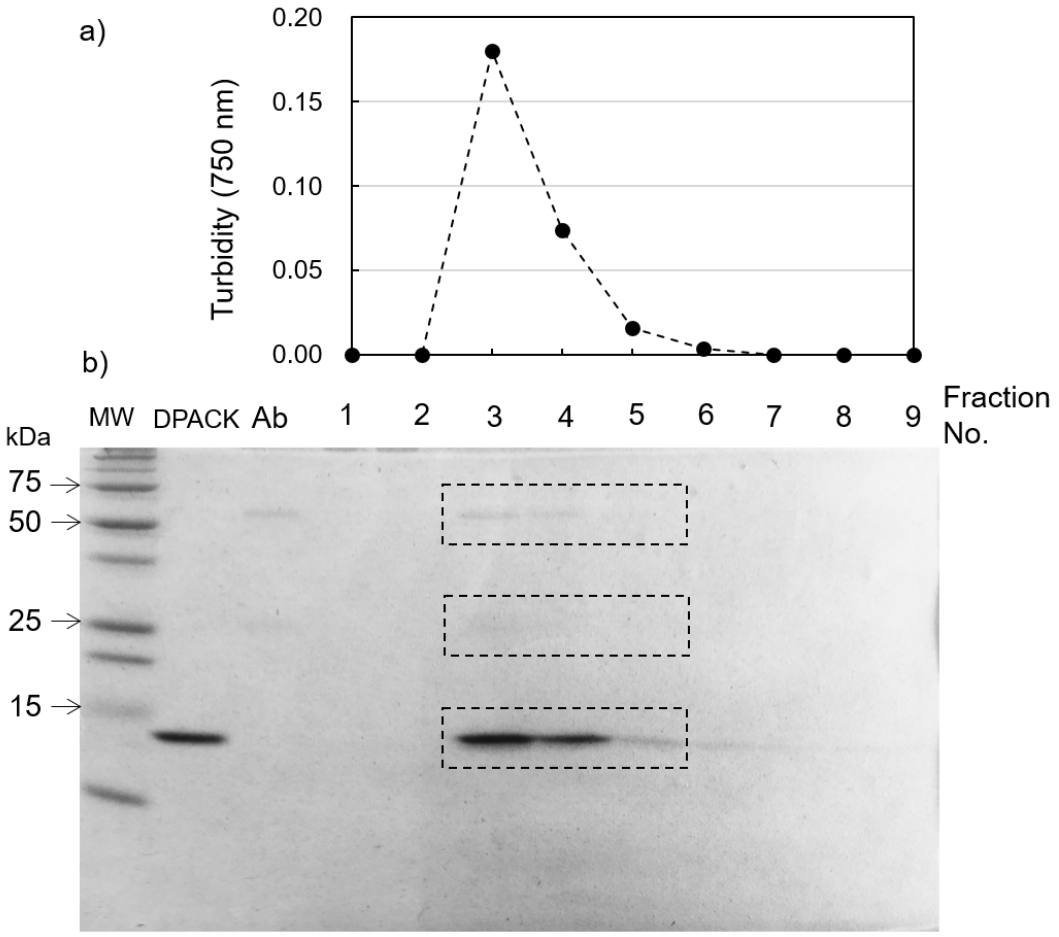

a)
Turbidity (750 nm)
0.20
0.15
0.10
0.05
0.00
b)
MW
DPACK
Ab
1
2
3
4
5
6
7
8
9
Fraction No.
kDa
75
50
25
15

Figure 3

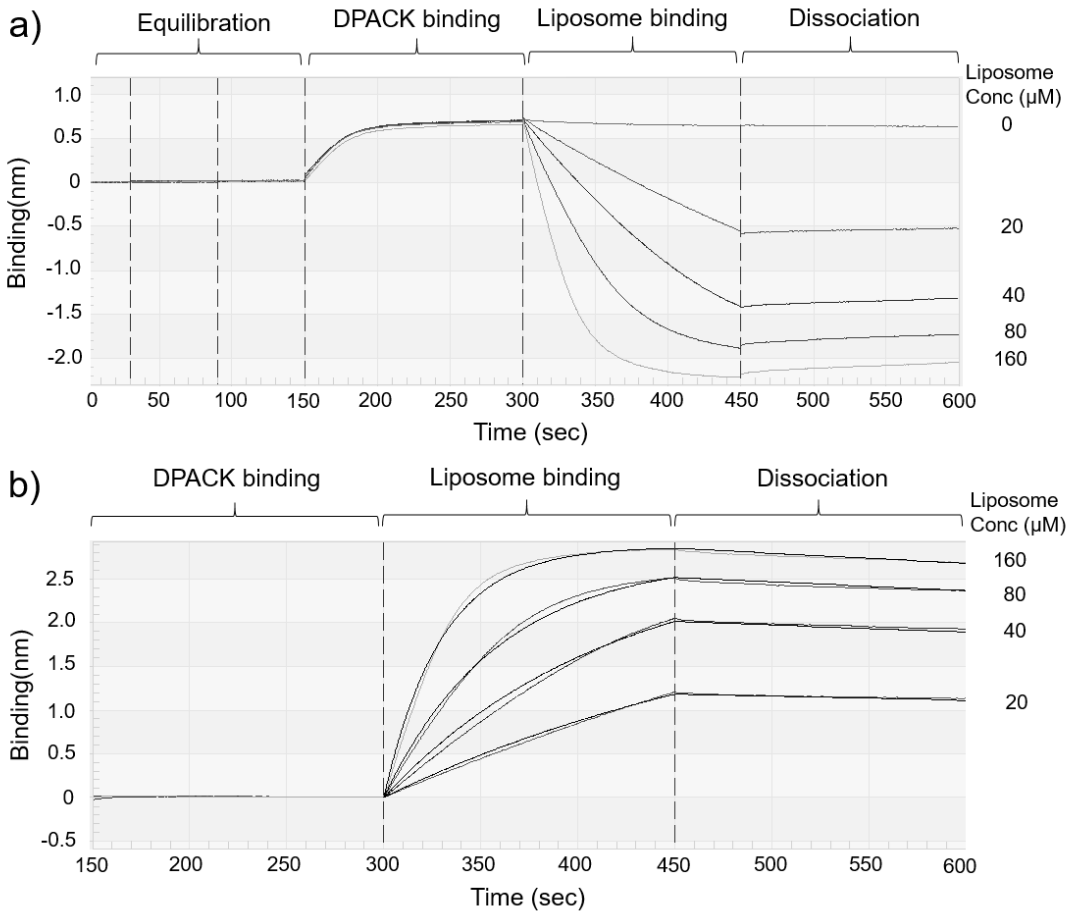

a)
Equilibration
DPACK binding
Liposome binding
Dissociation
Liposome Conc (μM)
0
20
40
80
160
Binding(nm)
Time (sec)
b)
DPACK binding
Liposome binding
Dissociation
Liposome Conc (μM)
160
80
40
20
Binding(nm)
Time (sec)

Figure 4

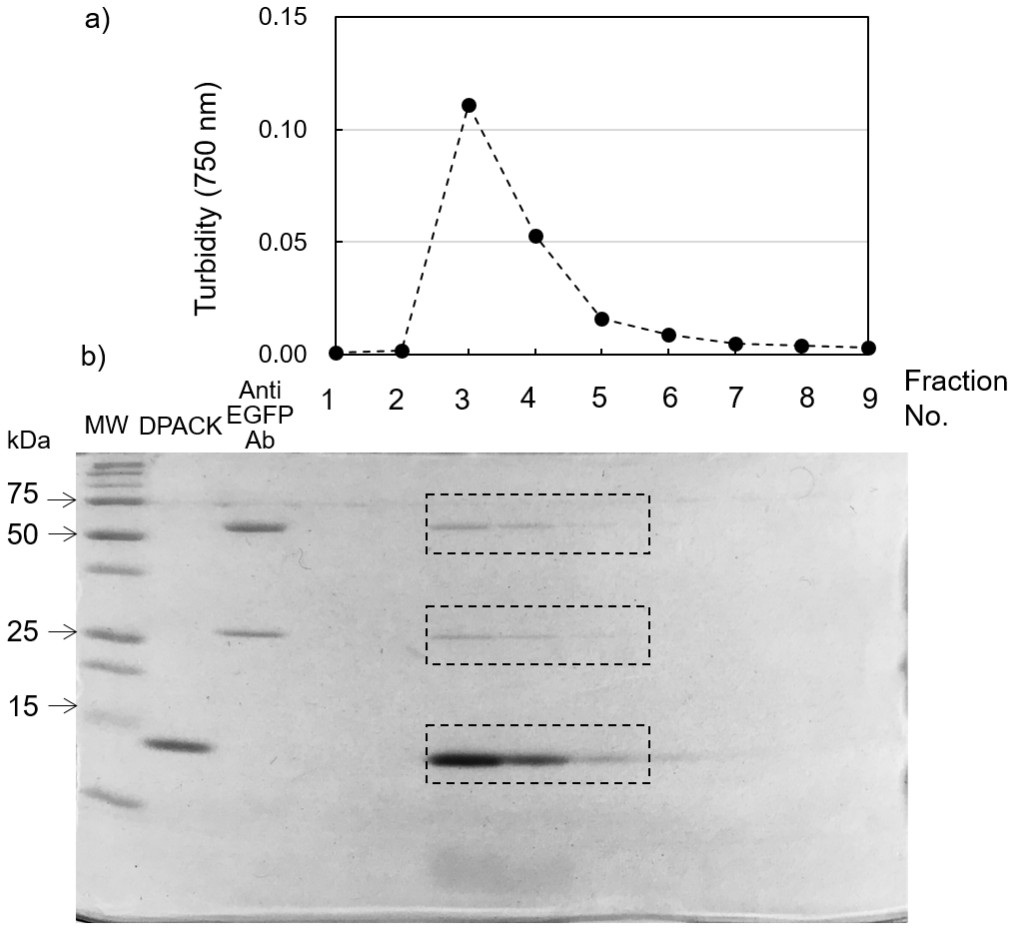

a)
0.15
0.10
0.05
0.00
Turbidity (750 nm)
1
2
3
4
5
6
7
8
9
Fraction No.
b)
MW
DPACK
Anti EGFP Ab
kDa
75
50
25
15

Figure 5

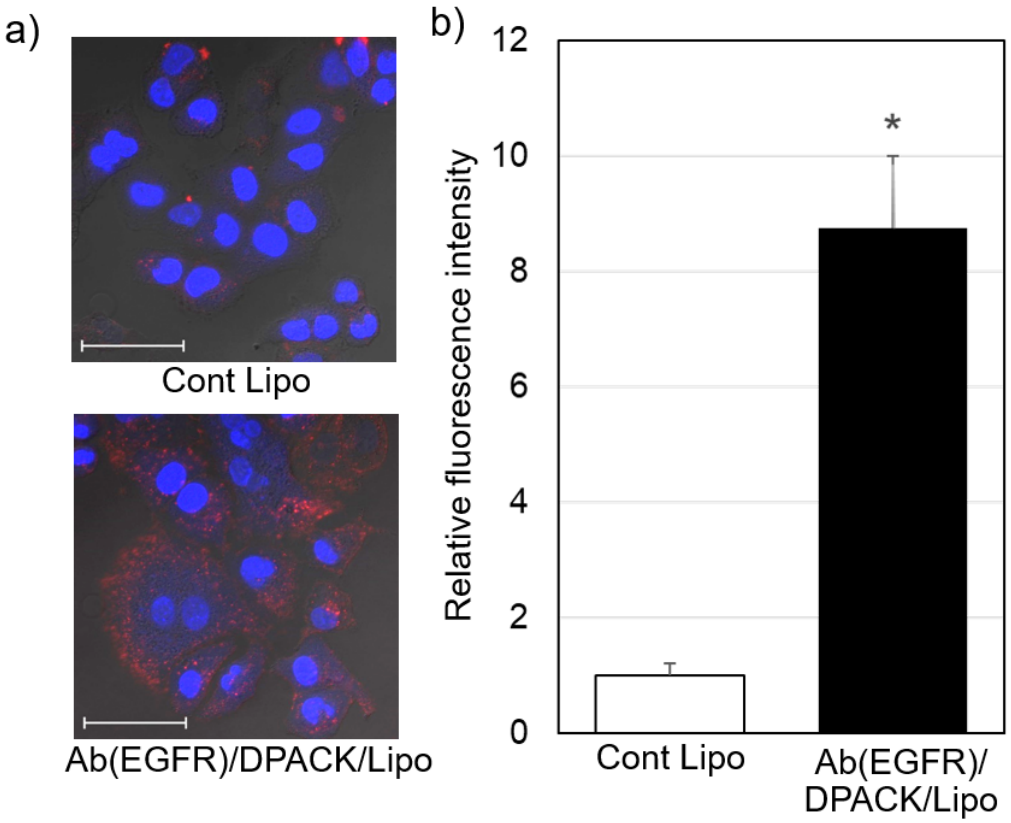
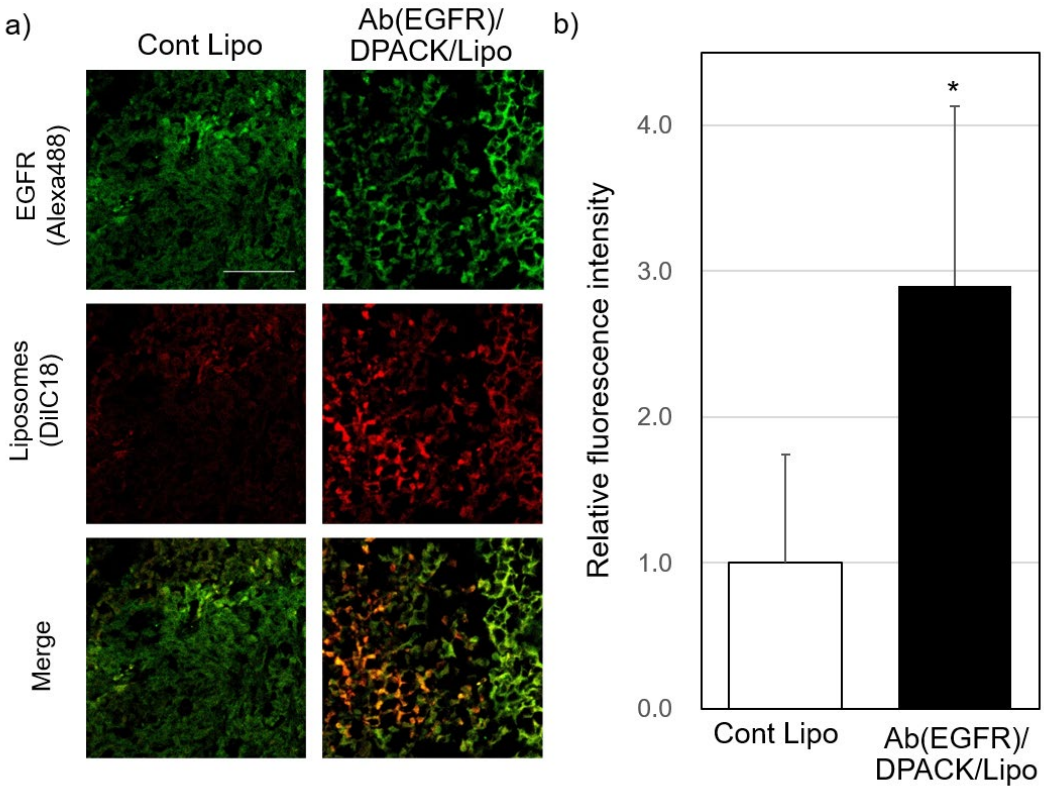

a)
Cont Lipo
Ab(EGFR)/DPACK/Lipo
b)
Relative fluorescence intensity
12
10
8
6
4
2
0
*
Cont Lipo
Ab(EGFR)/
DPACK/Lipo

Figure 6


a)
Cont Lipo
Ab(EGFR)/
DPACK/Lipo
EGFR
(Alexa488)
Liposomes
(DiIC18)
Merge
b)
Relative fluorescence intensity
4.0
3.0
2.0
1.0
0.0
*
Cont Lipo
Ab(EGFR)/
DPACK/Lipo