Simple PEG modification of DNA aptamer based on copper ion coordination for tumor targeting

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Short / Running Title:
Tumor targeting of aptamer modified with PEG

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Abstract

A simple modification of DNA aptamer with polyethylene glycol (PEG) based on metal coordination was developed. N,N-bis(carboxymethyl)-L-lysine (NTA) of a metal chalate residue was chemically introduced to one terminal of PEG. The NTA-introduced PEG (PEG-NTA) chelated Cu^{2+} ions to form a Cu^{2+}-chelated PEG (PEG-Cu). When the Cu^{2+}-chelated PEG was mixed with a DNA aptamer of anti-tumor activity (AS1411) in aqueous solution, the complex of PEG-Cu and AS1411 based on metal coordination was formed. The complex inhibited in vitro tumor growth in a dose-dependent manner. Body distribution study with tumor-bearing mice revealed that the PEG-Cu-AS1411 complexes intravenously injected showed a significant longer life-period in the blood circulation and 1.5-2.0 fold higher accumulation in the tumor tissue than free AS1411. Intravenous injection of complexes suppressed the in vivo growth of tumor mass to a significantly great extent compared with that of free AS1411. The Cu^{2+}-coordinated PEG modification is a simple and promising method to enhance the tumor accumulation of aptamer, resulting in the augmented anti-tumor effect.

Keywords

DNA aptamer; polyethylene glycol; metal coordination; tumor targeting; anti-tumor effect

Introduction

Drug Delivery System (DDS) is a technology available to enhance the therapeutic effect of drugs. As one DDS technology, chemical modification of drugs with various water-soluble polymers has been attempted [1, 2]. Polyethyleneglycol (PEG) modification of drugs, so-called PEGylation, has been widely carried out to improve their solubility and in vivo stability [3, 4]. PEGylation prevents the rapid renal clearance of drugs and their receptor-mediated uptake by the reticuloendothelial system, resulting in prolonging their in vivo half-time. In addition, passive tumor targeting of drugs can be achieved by their PEGylation. This can be explained by the enhanced permeability and retention (EPR) effect [5, 6], which is mainly governed by two factors: the disorganized pathology of angiogenic tumor vasculature with its discontinuous endothelium, leading to hyperpermeability to circulating polymers, and the lack of effective tumor lymphatic drainage, which leads to the consequent polymers accumulation. Based on this anatomical feature, it is well recognized that long-circulating polymers are passively accumulated in the tumor tissue. Such an enhanced accumulation to the site of action enables a drug to reduce the dose necessary for their therapeutic effect and consequently to minimize the side-effects caused by the high-dose and repeated administrations.

However, this PEGylation method needs the chemical conjugation of PEG with drugs. The chemical conjugation of drugs is sometimes of poor reproducibility and often causes the activity loss. Therefore, it is necessary to develop a new and simple conjugation method without using chemical coupling. We have developed a new conjugation method of drugs (protein or nucleic acid) and polymers (dextran, pullulan or PEG) based on metal coordination [7-10]. The objective of this paper is to prepare the drug conjugation with PEG based on the metal coordination. In this study, N,N-bis(carboxymethyl)-L-lysine (NTA) was used as the chelate residue which binds several types of metal ions such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Ni^{2+} or Zn^{2+}. The NTA has a low toxicity [11] and had been widely used for the biomedical application; ligand for the immobilized metal affinity chromatography (IMAC) which enables to purify a protein [12], oriented surface modification of substrate with the protein [13, 14], and sustained release of protein from the hydrogel [15]. Additionally, the NTA has one amino group, which is suitable to the chemical-modification with ease.

A DNA aptamer, AS1411 was used as the drug. AS1411 is a 26-mer guanosine-rich oligonucleotide (GRO) and inhibits the growth of tumor cells *in vitro* and human tumor xenograft *in vivo* [16-18]. AS1411 is known to form stable G-quartets structures and bind to nucleorin with a high affinity. Nucleorin is a multifunctional protein and its functions are ribosome biogenesis, signal transduction, DNA replication, protein trafficking, and mRNA stabilization [19, 20]. Nucleorin is present in the nucleus of normal cells. However, in tumor cells, the nucleorin expresses on the cell surface. Based on that, AS1411 has a specific affinity to the tumor cell, not to the normal cell.

The AS1411 internalized is transferred to the nucleus, followed by the inhibition of DNA replication and induction of S-Phase cell cycle arrest. It is reported that AS1411 induces the transcription inhibition of nuclear factor-κB (NF-κB) [20] and destabilization of Bcl-2 mRNA [21], resulting in showing an anti-tumor function.

In this paper, the nitrilotriacetic acid (NTA) of a chelating residue was chemically introduced to the terminal of PEG. The NTA-introduced PEG was conjugated to the AS1411 of DNA aptamer through Cu^{2+} coordination. *In vitro* and *in vivo* anti-tumor activity or tumor accumulation of PEG-AS1411 complexes with Cu^{2+} coordination was assessed. We physicochemically examined the Cu^{2+}-coordinated complexation between the NTA-introduced PEG and AS1411.

Materials and Methods

2.1. Materials

PEG with methoxy and N-hydroxy succinimide (NHS) group terminals (PEG-NHS, the weight-averaged molecular weight (Mw) 5,000 (linear) and 40,000 (branch, one terminal group)) was purchased from NOF Co., Tokyo, Japan. N,N-bis(carboxymethyl)-L-lysine (NTA) was purchased from Fluka Ltd., Tokyo, Japan. AS1411 (5'-GGTGGTGGTGGTTGTGGTGGTGGTGG-3') and AS1411-NH_2

(5'-Amino Modifier C6-GGTGGTGGTGGTTGTGGTGGTGGTGG-3') were purchased from Integrated DNA technologies, Inc., Coralville, IA.

2.2. NTA introduction to PEG

PEG-NHS with the Mw 5,000 or 40,000 was dissolved in 100 ml of dehydrated dimethyl sulfoxide (DMSO) at a final concentration of 1 mM, and then NTA was added to give a final concentration of 10 mM (showed scheme 1). The solution was agitated at 37 °C for 24 hr to allow NTA to couple to the NHS residue of PEG-NHS, and then dialyze against ultra-pure double-distilled water (DDW) for 3 days with a dialysis membrane (Spectra/Por® dialysis membrane MWCO 3,500, Spectrumlabs.com). The dialyzed solution was freeze-dried to obtain a NTA-introduced PEG (PEG-NTA). To characterize the PEG-NTA prepared, ^{1}H-NMR measurement was performed in D_2O including 0.05 wt% 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid, sodium salt on the NMR apparatus (JNM-EX, JEOL). The percent of NTA introduced was determined by the integration ratio of the CH_3O- peak (δ =3.4 ppm) from PEG to the -C-CH_2-C- (δ =1.6 ppm) peak from NTA.

2.3. Preparation and characterization of Cu^{2+}-chelated PEG-NTA

PEG-NTA with the Mw 5,000 or 40,000 was dissolved in 1 ml of DDW at a

final concentration of 10 mM. Then, 1 ml of 10, 30, 100, and 500 mM $CuCl_2$ aqueous solution was added to the PEG-NTA solution. Mixing molar ratio of Cu^{2+} ions to PEG-NTA was 1, 3, 10 or 50. The mixture was applied to a gel permeation chromatographic column (PD-10) (Sephadex G-25, column lengh = 10 cm, GE healthcare) with the elution solution of 10 ml DDW, and 0.5 ml of every fraction was collected. The concentration of Cu^{2+} ions in each fraction was determined by UV-Vis spectroscopy (800 nm, DU 800 UV/visible spectrophotometer, Beckman coulter). The concentration of Cu^{2+} ions was determined by use of the calibration curve which is prepared by $CuCl_2$ at pre-determined concentration. The number of Cu^{2+} ions chelated per one PEG-NTA molecule was calculated from the molar ratio of Cu^{2+} ions to PEG-NTA in high-molecular weight fraction.

2.4. Characterization of Cu^{2+} coordination to AS1411

Aqueous solution of $CuCl_2$ (1 ml, 100 mM) was applied to a chelating column (HiTrap^{TM} Chelating HP, GE Healthcare). After washed the column with phosphate-buffered saline solution (PBS, pH 7.4) to prepare a Cu^{2+} ions-immobilized column, 1 ml of AS1411 aqueous solution (1 μM) was applied. Additional 5 ml of PBS was applied to the column, and then the eluted solution (1 ml) was collected. Next, PBS

containing 50 mM imidazole was added while 1 ml of eluted solution was collected. Imidazole is a strong ligand to Cu^{2+} ions, and inhibits the coordinate bond of AS1411 to Cu^{2+} ions. The eluted solution was determined by UV-Vis spectroscopy (260 nm) to evaluate the concentration of AS1411. On the other hand, the mouse serum is added after AS1411 addition to the Cu^{2+}-immobilized column and incubated for various time periods. Then the same procedure was performed as described above. The concentration AS1411 eluted before imidazole addition was measured to evaluate the stability of Cu^{2+}-coordinated AS1411.

2.5. Preparation and characterization of PEG-Cu-AS1411 complexes

PEG-NTA with the Mw 5,000 or 40,000 with Cu^{2+} coordination (PEG-Cu) was dissolved in PBS, and the solution was mixed with PBS containing AS1411, followed by leaving for 15 min at room temperature to obtain 1 ml of solution containing complexes of PEG-Cu and AS1411 based on Cu^{2+} coordination. The final concentration of PEG-Cu was 60, 300 or 1200 μM while that of AS1411 was 60 μM. The mixing molar ratio of PEG-Cu to AS1411 was 1, 5 or 20. Following 1 ml of solution containing complexes was applied to an anion-exchange chromatographic column (HiTrap™ DEAE FF, GE Healthcare Amersham Biosciences KK, Tokyo, Japan), the eluted solution was collected

by applying 3 ml of phosphate-citrate buffer (0.1 M, pH 7.0). Then, phosphate-citrate buffer containing 0.3, 0.35, 0.4, 0.45, 0.5, 0.6, 0.7, and 1 M of NaCl was applied and the eluted solution was collected. The concentrations of PEG-Cu and AS1411 in the eluted solution were determined by high performance liquid chromatography (HPLC, LC-8020 model-Ⅱ, Tosoh, Tokyo, Japan) and UV-Vis spectroscopy, respectively. For the HPLC measurement, the samples were applied to HPLC equipped G3000SW_{XL} columns at 37 ℃ at a flow rate of 0.5 ml/min in 0.02 M phosphate-buffered solution pH 7.4 containing 0.3 M NaCl. The concentration of PEG-Cu was determined by use of the calibration curve. The concentration of PEG-Cu molecules complexed was calculated by subtracting the concentrations of PEG-Cu initially added from that of eluted. In the same way, the concentration of AS1411 complexed can be calculated. The number of PEG-Cu conjugated per one AS1411 molecule was calculated from by dividing the concentration of PEG-Cu complexed by that of AS1411.

2.6. *In vitro* anti-tumor activity of PEG-Cu-AS1411 complexes

Human lung cancer A549 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO) supplemented with 10 % heat-inactivated (55 ℃ for 30 min) fetal bovine serum, and 0.1 % penicillin and

streptomycin at 37 ℃ and 5 % CO_2-air atomosphere. A549 cells grown were seeded into each well of 96 multi-well cell culture plate (#3595, Corning Inc., Corning NY) at a density of 5 × 10^3 cells/well, and then 24 hr later PEG-Cu-AS1411 complexes were added at the final AS1411concentrations of 1, 3, 5, 7.5, and 10 μM (AS1411 amount; 0.1, 0.3, 0.5, 0.75, and 1.0 nmol, respectively) and at the mixing molar ratio of PEG-Cu to AS1411 of 0, 1, 5, and 20. After 6 days culture, the medium was changed (100 μl/well) and Cell Count Reagent SF (Nacalai Tesque, Inc., Kyoto, Japan) was added (10 μl/well). After 1 hr culture, the absorbance which reflect of each well at 450 nm was determined by a microplate reader (VERSA max, Molecular Devices Japan Co., Osaka, Japan) to evaluate the number of cells grown.

2.7. Tumor accumulation of PEG-Cu-AS1411 complexes

A549 cells (5 × 10^6 cells) suspended in 100 μl of PBS were inoculated into the left flank of BALB/c nude mice (6-week-old female, body weight = 18 g, Shimizu Laboratory Supplies Co. Ltd., Kyoto, Japan). When the tumor mass grew to 5-7 mm in average diameter about 10 days after inoculation, the tumor-bearing mice were used for the following *in vivo* experiments.

The tumor accumulation of AS1411 was evaluated by the conventional

fluorescent and radioisotope tracing methods. For the radiotracing, AS1411 was ^{125}I radioiodinated through the pyrimidine iodination in the presence of $TlCl_3$ [22]. Briefly, 5 μl of $Na^{125}I$ (740 MBq/ml in 0.1 M NaOH aqueous solution, NEN Reserch Products) solution was added into 20 μl of 0.3 mM Na_2SO_3 solution and left for 15 min in room temperature. Then, 100 μl of 0.1 M sodium acetate-40 mM acetic acid (pH 5.0) containing 10 mg/ml AS1411 solution was added into 100 μl of 4 mM $TlCl_3$. AS1411 solution was added into $Na^{125}I$ solution, and mixture was heated (60 ℃ for 50 min). Then, 0.1 ml of 0.1 M Na_2SO_3 and 0.9 ml of 50 mM tris buffer (pH 7) containing 0.1 M NaCl and 1 mM EDTA were added into the heated mixture. After heating at 60 ℃ for 30 min, the resulting mixture was passed through a PD-10 column to remove uncoupled ^{125}I molecules to obtain an aqueous solution of ^{125}I-labeled AS1411 (AS1411-^{125}I). The concentration of AS1411-^{125}I was determined with UV-Vis spectroscopy (260 nm).

Tumor-bearing mice received the intravenous injection of 100 μl of AS1411-^{125}I alone or PEG-Cu-AS1411-^{125}I complex. The amount of AS1411-^{125}I injected was 50 μg per mouse and the mixing molar ratio of PEG-Cu to AS1411-^{125}I was 20. The tumor tissue and blood samples were taken out 1, 6, and 24 hr later and the radioactivity was measured by a gamma counter (ARC-301B, Aloka Co. Ltd., Tokyo, Japan). From the radioactivity and weight of the tumor tissue, the amount of

accumulated AS1411-^{125}I (μg /g tumor) was calculated. On the other hand, from the blood radioactivity, the amount of AS1411-^{125}I remaining in the blood circulation was calculated under the assumption that the total blood weight in a mouse corresponds to 8% of its body weight and the blood density is 1.0. Each experiment group consists of 3 mice. For the fluorescent tracing, 800 μl of 0.1 M sodium carbonate and sodium biscarbonate-buffered solution (pH 9.3) containing 2 mg of AS1411-NH_2 was fluorescently labeled with Cy 5.5 (Cy 5.5 mono reactive dye pack, GE healthcare Amersham Biosciences KK, Tokyo, Japan) according to the manufacturer's instructions. Tumor-bearing mice received the intravenous injection of 100 μl of Cy 5.5-labeled AS1411 (AS1411-Cy 5.5) alone or PEG-Cu-AS1411-Cy 5.5 complex. The amount of AS1411 injected was 50 μg per mouse and the mixing molar ratio of PEG-Cu to AS1411-Cy 5.5 was 20. The *in vivo* fluorescence imaging was performed by LAS4000 (FUJI FILM Co. Ltd., Kanagawa, Japan) 1, 6, 24, and 48 hours after injection. From the fluorescent intensity at the tumor site, the mean gray value was recorded as photons per millimeter squared and was normalized by the autofluorescence to evaluate the time profile of tumor accumulation of AS1411. Each experiment group consists of 3 mice.

2.8. In vivo anti-tumor effect of PEG-Cu-AS1411 complexes

To evaluate the in vivo anti-tumor effect of PEG-Cu-AS1411 complexes, mice with the similar tumor tissue size (about 5-7 mm in diameter) received the intravenous injection of 200 μl of AS1411 alone or PEG-Cu-AS1411 complex every 3 days. The amount of AS1411 injected was 100 μg per mouse at each time and the mixing molar ratios of PEG-Cu to AS1411 was 20. The length of long axis (a) and short axis of tumor mass (b) were periodically measured and the tumor volume (V) was calculated according to the formula of $V=1/2\times a\times b^2$. On the 42 day, the tumor tissue was taken out to measure the wet weight. Each experiment group consists of 5 mice.

2.9. Statistical analysis

All the data were statistically analyzed by Post hocs Bonferroni /Dunn and expressed as the mean +/- the standard deviation of the mean. Statistical significance was accepted at $p < 0.05$.

Results

3.1. Preparation and characterization of PEG-NTA

Figure 1 shows the preparation scheme of PEG-NTA and the ^1H NMR spectra of NTA, PEG-NHS, and PEG-NTA. The NTA and PEG-NTA samples exhibited peaks

attributed to C-CH_2-C at 1.15-2.05 ppm, in contrast to the PEG-NHS. The percent NTA introduced to PEG with the Mws of 5,000 and 40,000 was 92.5 and 88.5 %, respectively.

3.2. Metal chelation of PEG-NTA

Figure 2 shows the gel permeation chromatographic patterns of PEG-NTA and PEG-NHS after mixing with Cu^{2+} ions. In the gel permeation chromatography, substances with high molecular weights (> 5,000) and low molecular weights were eluted in the flow volume ranges of 2-4 and 4-10 ml, respectively. For the mixture of PEG-NTA and Cu^{2+} ions, both the high-molecular and low-molecular weight peaks were detected. On the contrary, only the low-molecular weight peak was observed for the mixed PEG-NHS and Cu^{2+} ions. The amount of Cu^{2+} ions in the high-molecular weight peak was similar, irrespective of the mixing molar ratio of PEG-NTA to Cu^{2+} ions. One PEG-NTA molecule chelated about one Cu^{2+} ions for any mixed sample of Cu^{2+} ions and PEG-NTA with the molecular weight of 5,000 (**Figure 2B**). In the case of PEG-NTA with the molecular weight of 40,000, the molar ratio of Cu^{2+} ions to PEG-NTA increased with an increase in the amount of Cu^{2+} initially added (**Figure 2D**).

3.3. Characterization of PEG-Cu-AS1411 complexes

Figure 3 shows the elution pattern of AS1411 from the Cu^{2+}-chelated column in the absence or presence of serum. Most of AS1411 applied was eluted by the addition of imidazole (the flow volume was 6-10 ml) in the absence of serum. This means that the AS1411 bind to Cu^{2+} ions. On the other hand, when incubated with mouse serum, the AS1411 applied was partially eluted (the flow volume 2-5 ml) before the imidazole addition. This finding indicates that the PEG-Cu-AS1411 complex was dissociated in the presence of serum. The percentages of PEG-Cu-AS1411 complex dissociated after 1 and 24 hr incubation in the serum were calculated to be 30 and 57, respectively. **Figure 4** shows the elution patterns of AS1411 from anion-exchange chromatographic column. The PEG-Cu-AS1411 complex was eluted in low salt concentration conditions compared with free AS1411. **Figure 5** shows the number of PEG-Cu conjugated per one AS1411 molecule. The numbers of conjugated PEG-Cu per one AS1411 molecule became higher as the ratio of PEG-Cu / AS1411 increased. The number of PEG-Cu complexed per one AS1411 molecule was constant when the mixing molar ratio was more than 5. PEG-Cu prepared from high molecular weight PEG (Mw 40,000) showed one-third lower number of PEG-Cu complexed than that of low molecular weight PEG (Mw 5,000).

3.4. *In vitro* anti-tumor activity of PEG-Cu-AS1411 complexes

Figure 6 shows the absorbance of cells cultured with or without PEG-Cu-AS1411 complexes. The absorbance reflects the mitochondria activity which is assumed to correspond to cell number. At 1 and 3 μM of AS1411 concentration, the absorbance was as high as that of cells without any complexes, irrespective of the mixing molar ratio of PEG-Cu to AS1411. At 10 μM of AS1411 concentration, the absorbance was low for any PEG-Cu-AS1411 complex. At the 5 and 7.5 μM AS1411 concentration, the absorbance increased with the increase in a mixing molar ratio.

3.5. Tumor accumulation and blood circulation profiles of PEG-Cu-AS1411 complexes

Figure 7 shows the amount of AS1411-^{125}I accumulated in the tumor site and the time profiles of blood circulation. The AS1411-^{125}I modified with PEG-Cu was accumulated in the tumor site at significantly higher (1.5-2 fold) amounts than free AS1411-^{125}I 6 and 24 hr after injection. In addition, the AS1411-^{125}I modified was retained in the blood circulation for longer time periods. When evaluated, the distribution percentages in the organ of reticuloendothelial system (RES, liver and

spleen) 24 hr after injection were 5.8 ± 0.03 and 0.20 ± 0.02 for free AS1411, 6.5 ± 0.28 and 0.20 ± 0.06 for AS1411 complex with PEG-Cu of a molecular weight of 5,000, and 6.5 ± 0.40 and 0.19 ± 0.04 for AS1411 complex with PEG-Cu of a molecular weight of 40,000.

Figure 8 shows the imaging photograph and intensity patterns of AS1411-Cy5.5 in the tumor site. The mice injected with PEG-Cu-AS1411-Cy5.5 complex showed significantly stronger (1.5-2 fold) fluorescence at the tumor site than those injected with free AS1411-Cy5.5.

3.6. *In vivo* anti-tumor therapeutic effect of PEG-Cu-AS1411 complexes

Figure 9 shows the *in vivo* anti-tumor activity after intravenous injection of PBS, AS1411 alone or PEG-Cu-AS1411 complex to tumor-bearing mice. Both the tumor volume and weight of complex-injected mice were significantly smaller (10 and 30% for tumor volume and weight, respectively) than those of mice injected with PBS or free AS1411.

Discussion

The present study demonstrates that AS1411 was complexed to PEG-NTA with

Cu^{2+}-coordination only by their simple mixing. The Cu^{2+}-coordinated PEG modification enabled AS1411 to prolong the life-time in the blood circulation and enhance the tumor accumulation. It is highly conceivable that this in vivo fate of complexes contributed to their enhanced in vivo anti-tumor effect.

It has been previously demonstrated that the body distribution profiles after intravenous injection of drug modified with a polymer by different methods (covalent bond [23] vs. metal coordination [24]) was similar to each other. Therefore, the PEGylation based on metal coordination is a simple and feasible method to actively change the body distribution of drugs. It was revealed that the percentages of PEG-Cu-AS1411 complex dissociated after 1 and 24 hr incubation in the serum were 30 and 57, respectively (**Figure 3**). It is possible that the metal coordination bond was stable enough to allow the complex to circulate in the blood for modification of the body distribution.

NTA was selected as the chelating residue of metal ions [25, 26], because it has been previously used to prepare a chelating residue-introduced PEG [13]. Copper ions (Cu^{2+} ions) were selected as the metal ions because of the high stability constant to NTA (Cu^{2+}; 12.96) compared with another metal ions (Ca^{2+}; 6.41, Mg^{2+}; 5.46, Fe^{2+}; 8.84, Ni^{2+}; 11.54, Zn^{2+}; 10.66). The NMR and chromatographic studies showed that the

PEG-NTA had a chelating ability for Cu^{2+} ions **(Figures 1 and 2)**. The number of Cu^{2+} coordinated per one PEG-NTA molecule was not influenced by the mixing molar ratio of PEG-NTA to Cu^{2+}. Cu^{2+} ions were not bound to PEG-NHS without any NTA residue. Taken together, it is likely that Cu^{2+} ions was chelated to the NTA residue introduced to PEG in a theoretical fashion. In addition, it is shown that the 50% lethal dose (LD50) of is 87 mg/kg in a mouse. Since the body weight of mice used in this study is around 20 g, the LD50 for the mice is 1.74 mg. The amount of Cu^{2+} injected in this study is calculated to be 15.5 μg, it is possible, therefore, that the toxicity of Cu^{2+} used in this study is quite low although the toxicity study should be done for the accurate evaluation.

In this study, an aptamer was selected as a therapeutic drug [27]. Aptamer of nucleic acid has an artificial and short sequences of DNA or RNA and has a specific affinity for various materials. Thus, aptamer recoginizes biologically to a substance, similarly to antibody. In addition, it has many advantages over the antibody, such as the low cost, the shorter time or easer of synthesis, and the non-immunogenicity (called super antibody). The aptamer synthesis method is called "SELEX" [28] and various therapeutic aptamer has been synthesized [29, 30]. Various aptamers have been investigated to demonstrate their therapeutic activity of anti-tumor drugs [31, 32].

In the anion-exchange chromatographic study, the elution peak of AS1411 shifted to the direction to lower salt concentration fractions by mixing with PEG-Cu **(Figure 4)**. This can be explained in terms of reduced negative charge of AS1411. It is conceivable that the negative charge of AS1411 was reduced by the complexing with PEG based on Cu^{2+} coordination, resulting in decreased interaction force between the AS1411 and the column. The number of PEG-Cu complexed per one AS1411 molecule was constant when the mixing molar ratio was more than 5 **(Figure 5)**. When the ratio of PEG-Cu / AS1411 became higher, the frequency of PEG-Cu to encounter the AS1411 increased. This increment would result in the enhanced ratio. However, the steric hinderance of PEG chains suppresses the Cu^{2+}-coordinated conjugation. It is conceivable that the balance brings about the constant PEG-Cu/AS1411 ratio. PEG-Cu prepared from high molecular weight PEG (Mw 40,000) showed lower number of PEG-Cu complexed than that of low molecular weight PEG (Mw 5,000). It is likely that the high molecular weight and branched shape of PEG cause their larger steric hindrance than the linear and low Mw of PEG, which results in reduced complexation.

The in vitro anti-tumor activity tended to increase as the AS1411 concentration become higher **(Figure 6)**. It is reported that AS1411 itself can be recognized by nucleolin present on the surface of tumor cells [19, 33]. It is possible that the AS1411

was covered by PEG-Cu with an increase in the PEG-Cu/AS1411 ratio, resulting in the reduced extent of AS1411 to be recognized. As the result, the anti-tumor activity of AS1411 would decrease. As lower amounts of AS1411, even though the covering effect by PEG-Cu conjugation was high, the anti-tumor activity was low. However, at the higher amounts, the AS1411 showed higher anti-tumor effects even although it was conjugated. The anti-tumor action of AS1411 is strong enough to suppress the in vitro growth of tumor cells. As the result, higher doses of AS1411 can biologically function the tumor cells for their growth suppression. The preliminary experiment demonstrates that the viability of A549 incubated with a scrambled DNA aptamer (20 μM) or PEG-Cu (20, 100, and 400 μM) was similar to that of cells without any treatment (data not shown). This result indicates that the DNA aptamer sequence is important to recognize a nucleorin expressed on the cell surface and that the Cu^{2+} coordinated does not affect the cell viability.

The retention time of AS1411 in the blood circulation was prolonged by the Cu^{2+}-coordinated PEG modification **(Figure 7)**. The reason can be explained in terms of PEGylation effect. The PEGylation prevents not only the rapid renal clearance of drugs but also the uptake by the reticuloendothelial systems. In the present study, it was shown that the AS1411 showed a relatively longer circulation time in the blood (**Figures 8)**

compared with the general aptamer [34], which well corresponds to other papers [35]. It is conceivable, therefore, that the prolonged life-time of AS1411 by the modification is due to the prevention of uptake by the reticuloendothelial systems or non-desired cells.

It is likely that the prolonged blood circulation consequently enhanced the tumor accumulation **(Figures 7 and 8)**. It is known that the coordination bond is weaken when the pH decreases (data not shown). In addition, the pH in the tumor tissue is lower than that of normal tissue [36]. It is possible that when the complex is accumulated in the tumor tissue, AS1411 is dissociated from the complex. We experimentally confirmed that the elution of AS1411 from Cu^{2+} chelating column was promoted at lower pHs. The AS1411 dissociated in the tumor tissue would be recognized by nucleolin on the tumor cell surface effectively, resulting in enhanced the anti-tumor activity. Therefore, the in vivo anti-tumor effect of AS1411 was enhanced by even although the activity of AS1411 was inhibited by the molecular covering through PEG-Cu complexation **(Figure 6)**.

In conclusion, the present study demonstrates that Cu^{2+} coordination enabled AS1411 to modify by the simple mixing of PEG. The AS1411 modified with PEG based on Cu^{2+} coordination was retained in the blood circulation for longer time periods, resulting in the increased tumor accumulation and the consequently enhanced in vivo

anti-tumor effect.

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Figure Legends

Figure 1. Preparation of PEG-NTA by chemical coupling PEG-NHS with NTA. (A) Reaction scheme and (B) ^{1}H-NMR spectra of NTA, PEG-NHS, and PEG-NTA. (C) The ^{1}H-NMR spectra of PEG-NTA enlarged.

Figure 2. Gel permeation chromatographic patterns of Cu^{2+} ions and PEG-NTA mixtures ((A) and (C)) and the molar ratio of Cu^{2+} ions coordinated to the PEG-NTA ((B) and (D)) after mixing PEG-NTA with Cu^{2+} ions at Cu^{2+} / PEG-NTA molar ratios of 1 (○), 3 (△), 10 (●), and 50 (▲). (□) is control (PEG-NHS and Cu^{2+} ions). The Mw of PEG is 5,000 ((A) and (B)) or 40,000 ((C) and (D)).

Figure 3. An elution pattern of free AS1411 from a Cu^{2+} ions chelating column in the absence (●) or presence of mouse serum incubated for 1 (▲) or 24 hr (■).

Figure 4. Elution patterns of free AS1411 and PEG-Cu-AS1411 complexes from anion-exchange column at different salt concentrations. The PEG-Cu /AS1411 molar ratios are 0 (○) 1 (△), 5 (●), and 20 (▲). The Mw of PEG is 5,000 (A) or 40,000 (B).

Figure 5. The number of PEG conjugated per one AS1411 molecule. The Mw of PEG is 5,000 (□) or 40,000 (■)

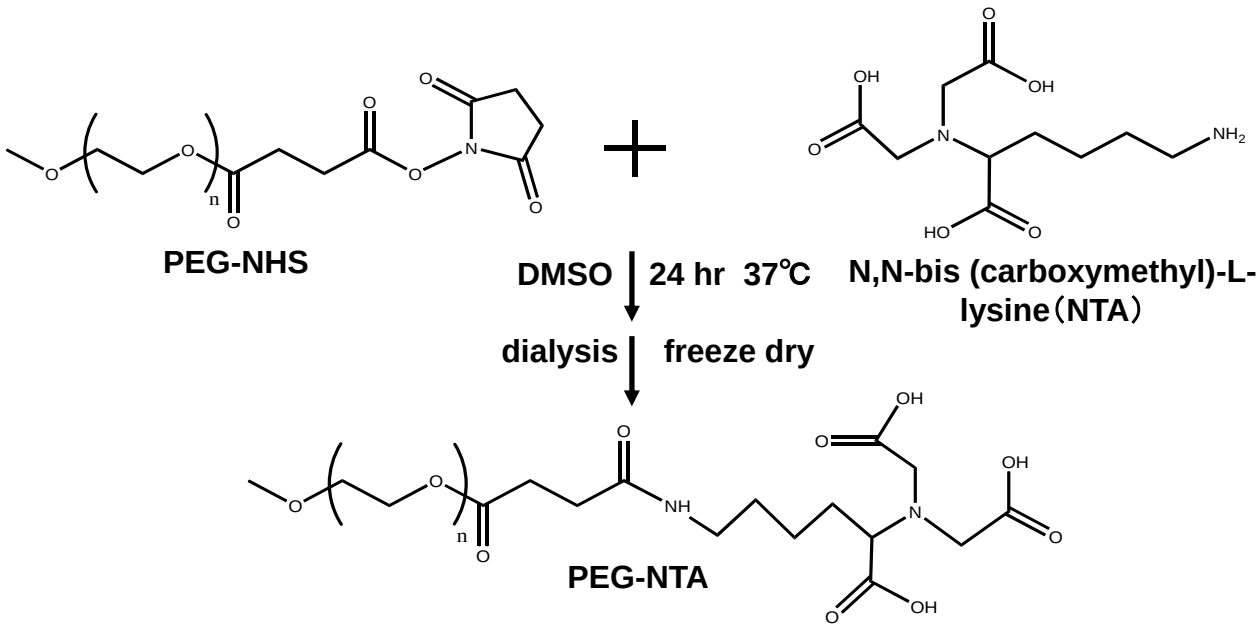
Figure 6. Viability of A549 cells after incubation with PEG-Cu-AS1411 complexes. Final concentration of AS1411 is 1 (○), 3 (△), 5 (●), 7.5 (▲) and 10 μM (□). The

Mw of PEG is 5,000 (A) or 40,000 (B).

Figure 7. Time course of AS1411 accumulation in the tumor site (A) and blood circulation (B) after the intravenous injection into tumor-bearing nude mice with free AS1411 (□) and PEG-Cu-AS1411 complexes prepared from PEG with Mws of 5,000 (▢) and 40,000 (■). The amount of AS1411 injected is 50 mg per a mouse. *:$p<0.05$, significant against the accumulation amount of tumor-bearing mice injected with free AS1411 at the corresponding time. N.S.; not significant

Figure 8. Time course of fluorescence imaging photograph (A) and intensity in the tumor site (B) after the intravenous injection into tumor-bearing nude mice with free AS1411 (□) and PEG-Cu-AS1411 complexes prepared from PEG with Mws of 5,000 (▢) and 40,000 (■). *:$p<0.05$, significant against the accumulation amount of tumor-bearing mice injected with free AS1411 at the corresponding time. N.S.; not significant

Figure 9. Tumor volume (A) and tumor weight (B) of tumor-bearing mice after intravenous injection of PBS (○), free AS1411 (●), and PEG-Cu-AS1411 complexes prepared from PEG with Mws of 5,000 (△) and 40,000 (▲). Free AS1411 or the complexes were administered at a dose of 50 μg /mice every 3 days. The tumor weight was measured 42 days after injection. *, $p<0.05$; significant against the tumor volume or tumor weight of tumor-bearing mice injected with free AS1411 at the corresponding time. N.S.; not significant



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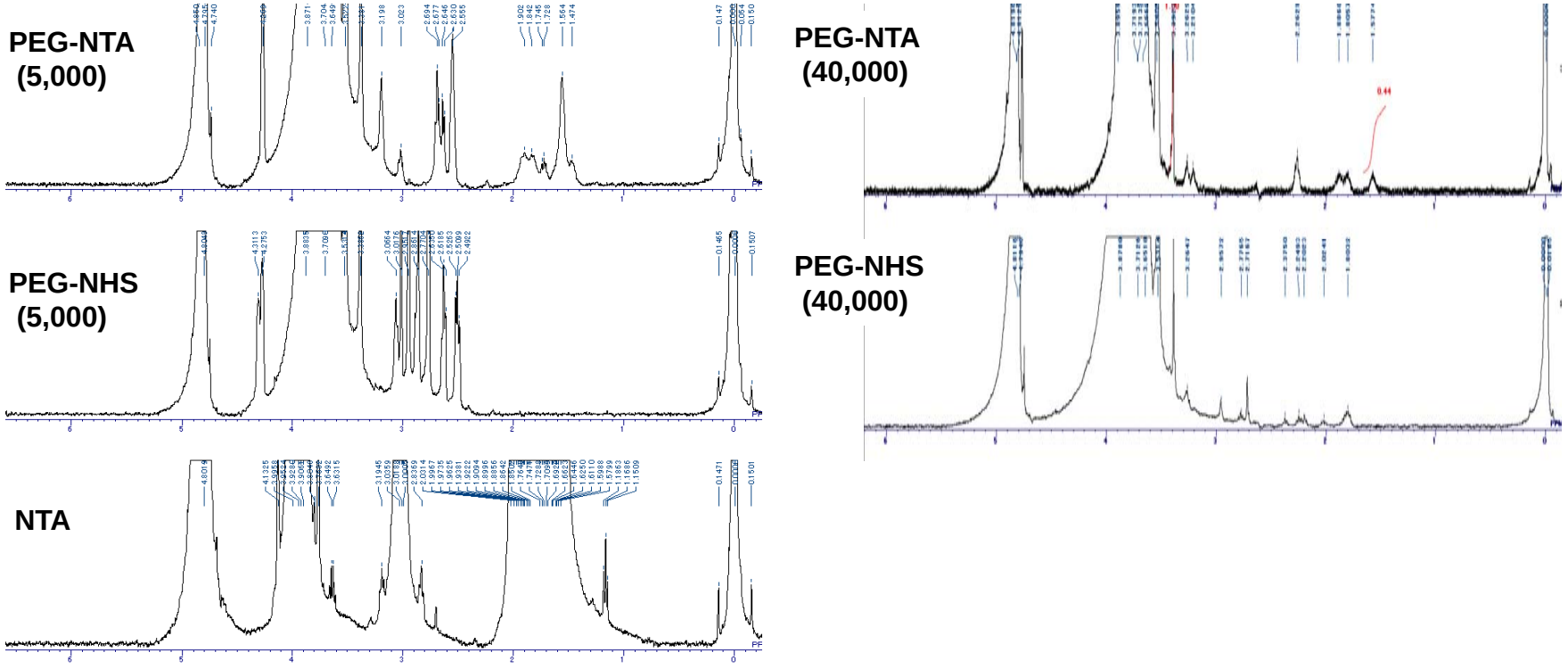

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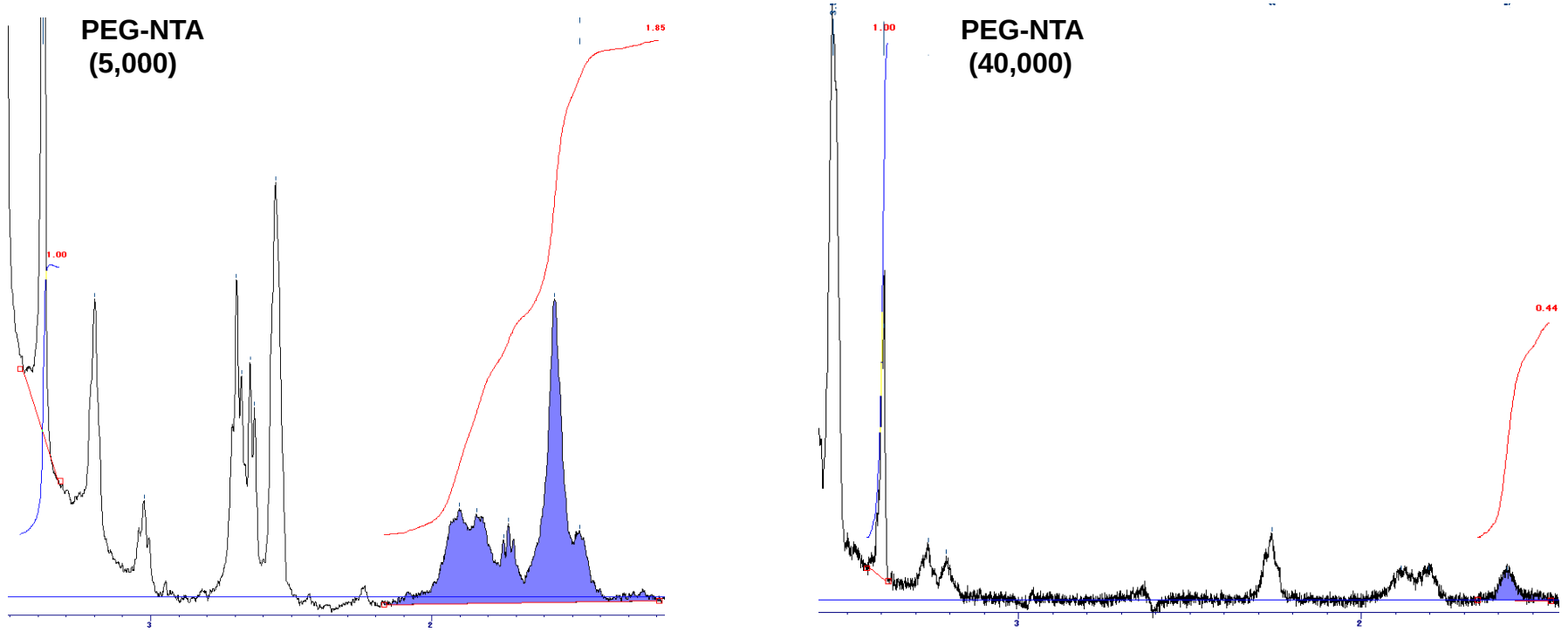
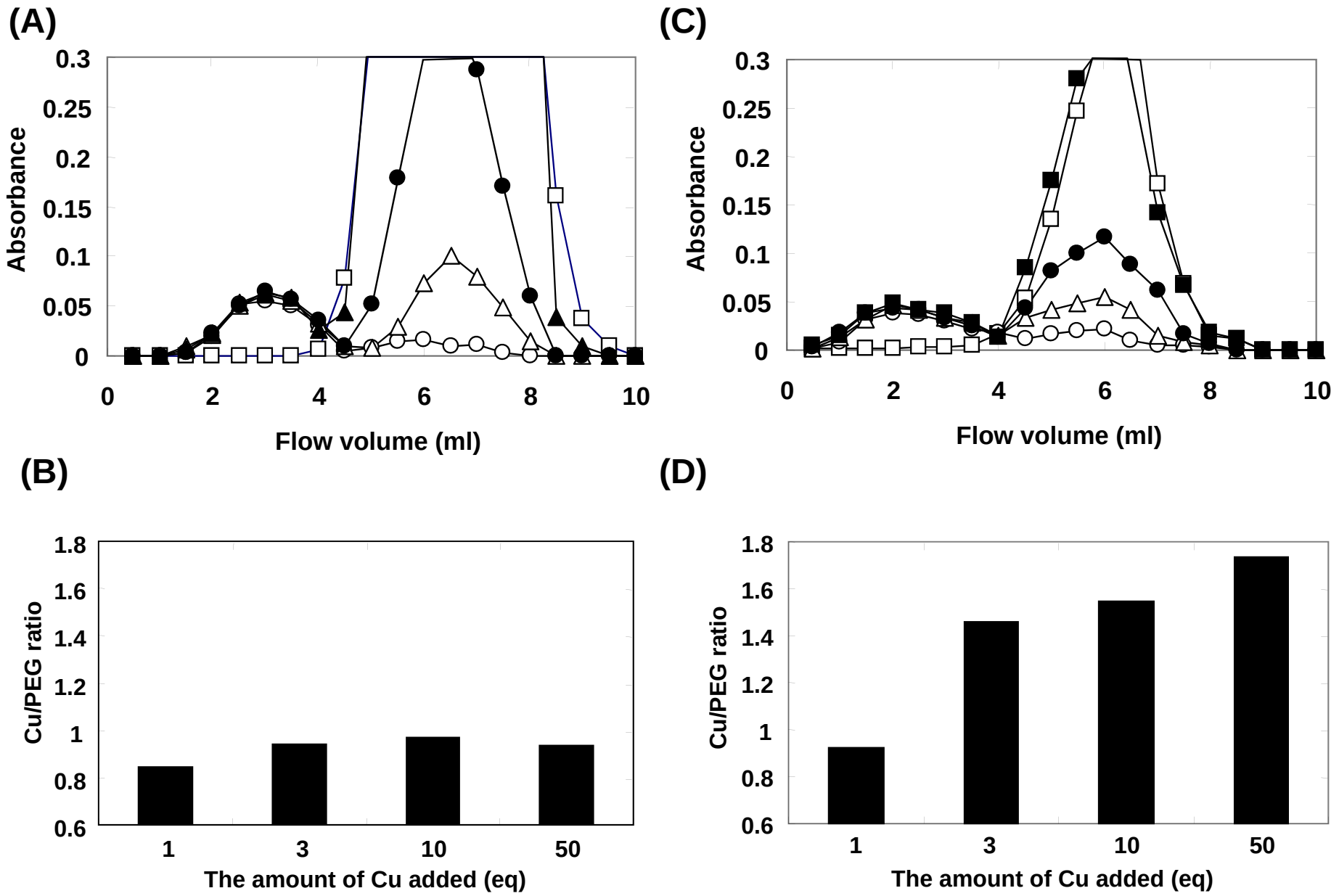


Figure 1(C). Takafuji et al.



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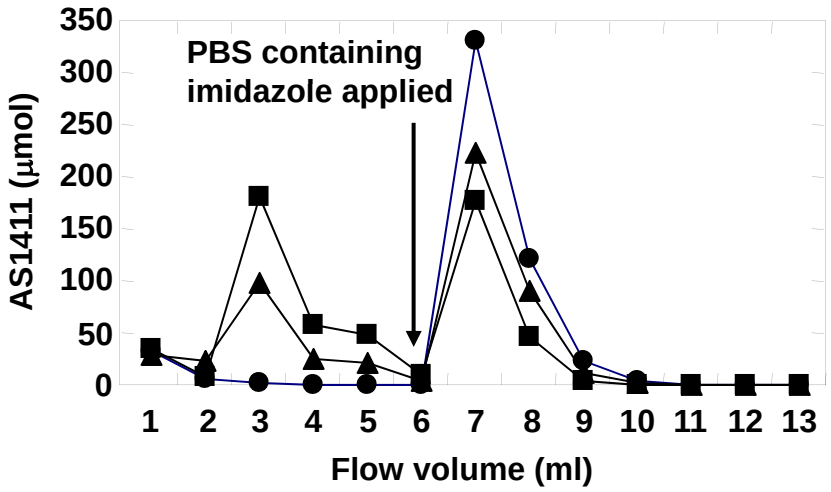

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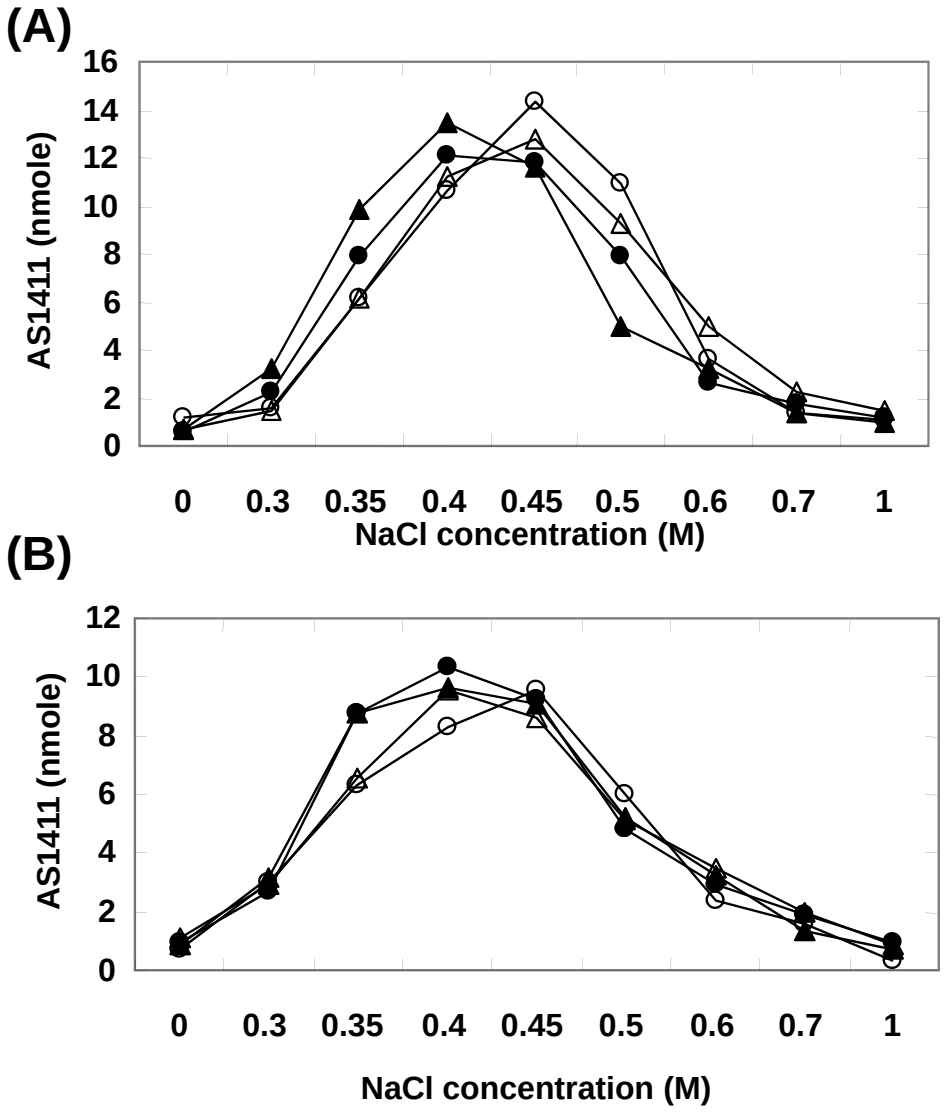

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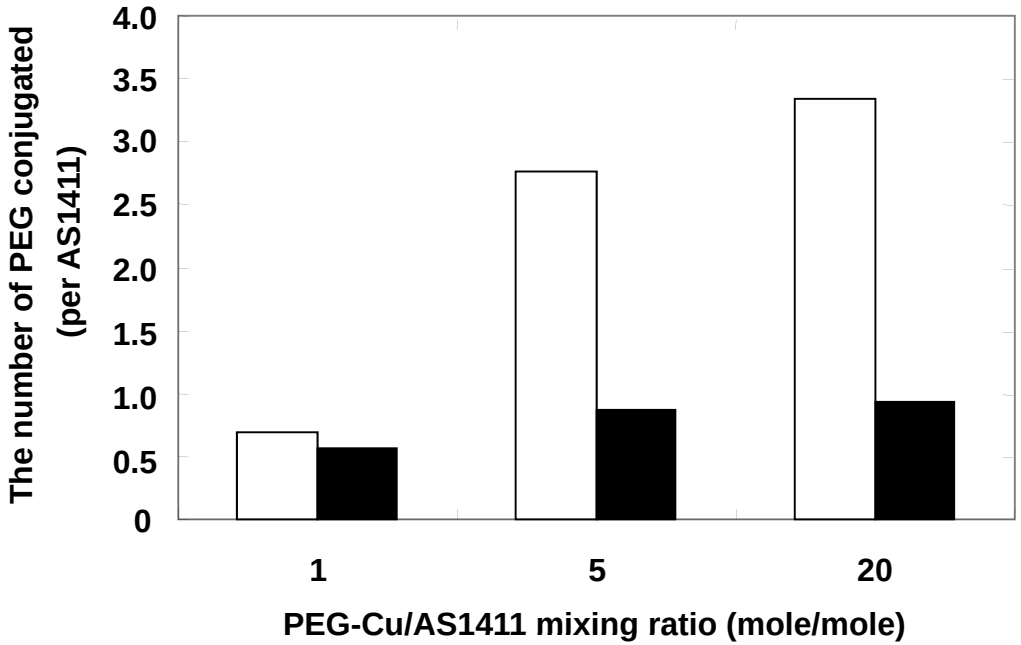

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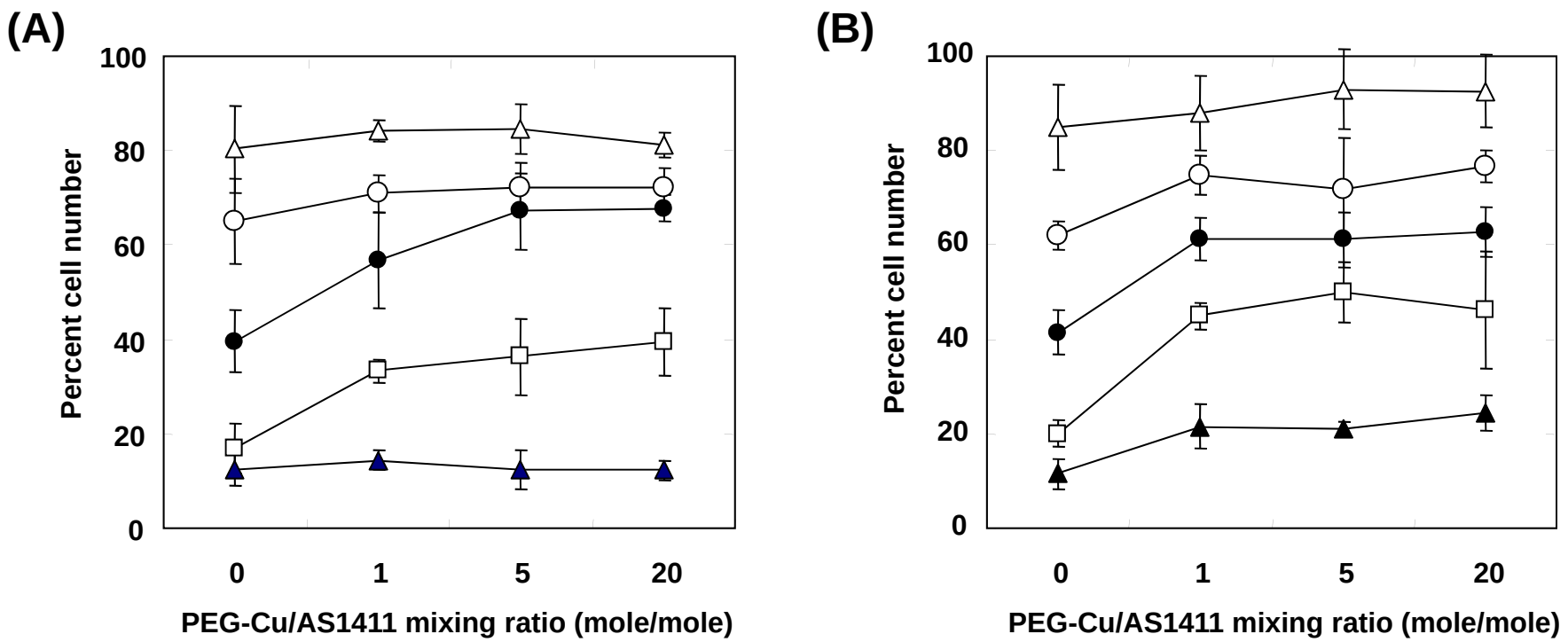

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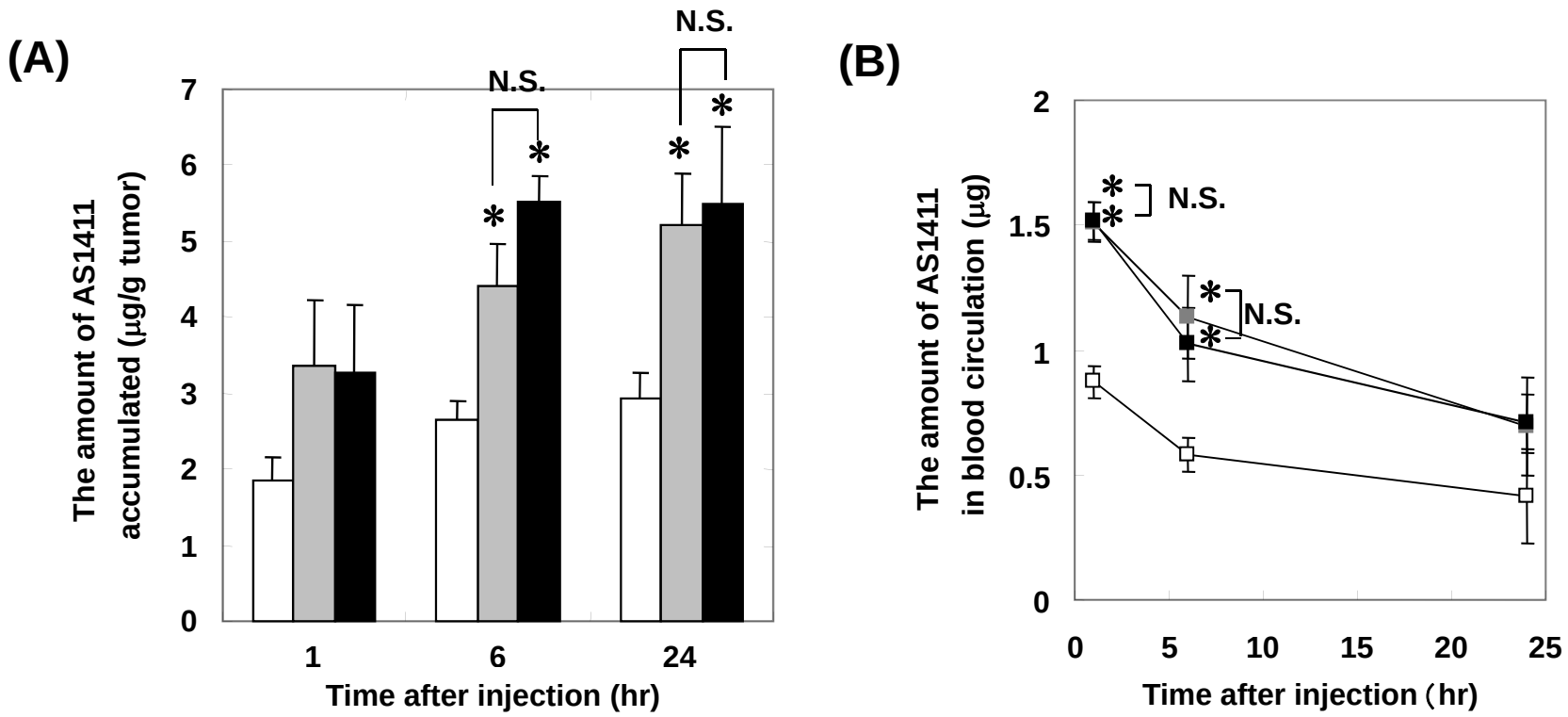

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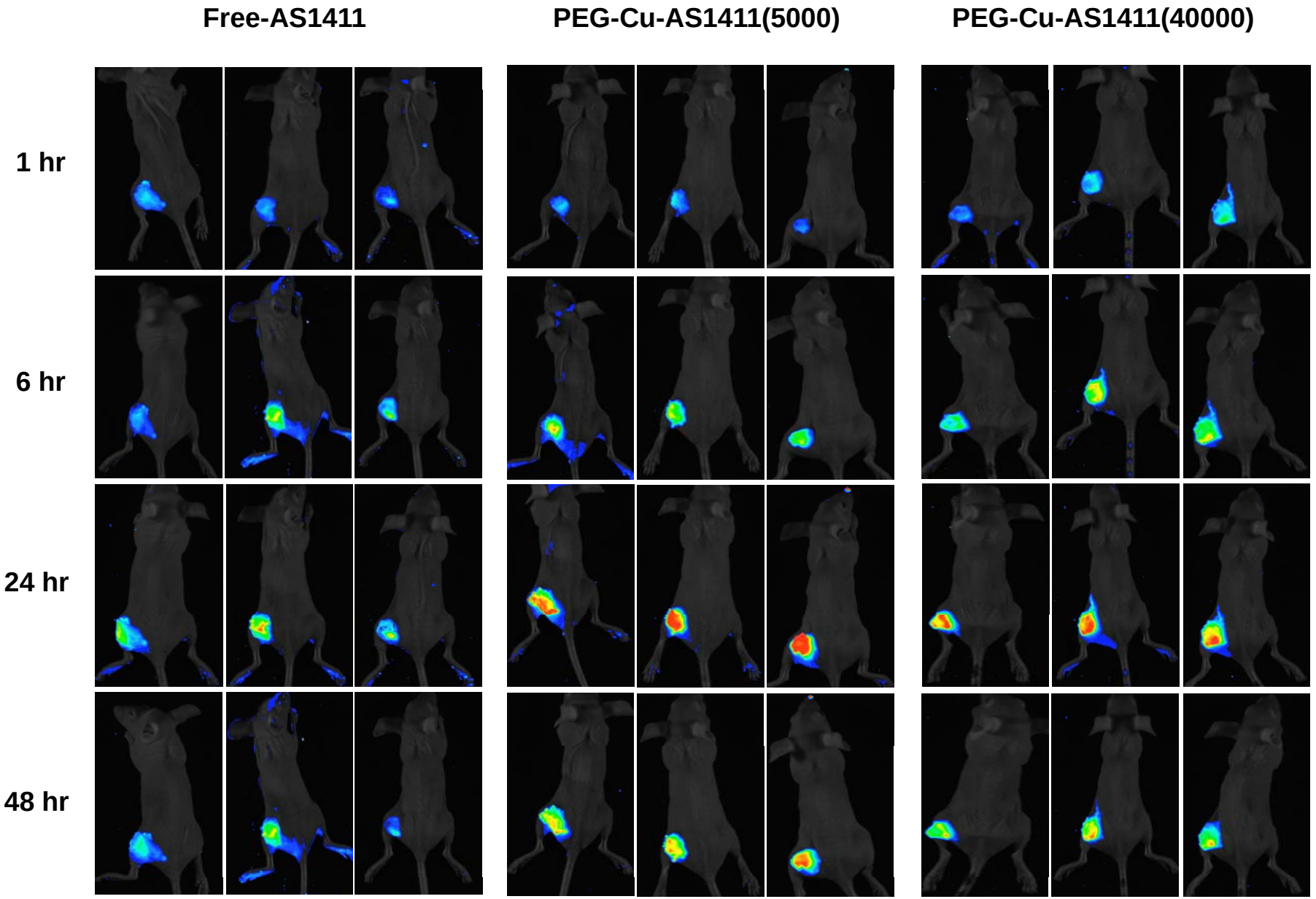
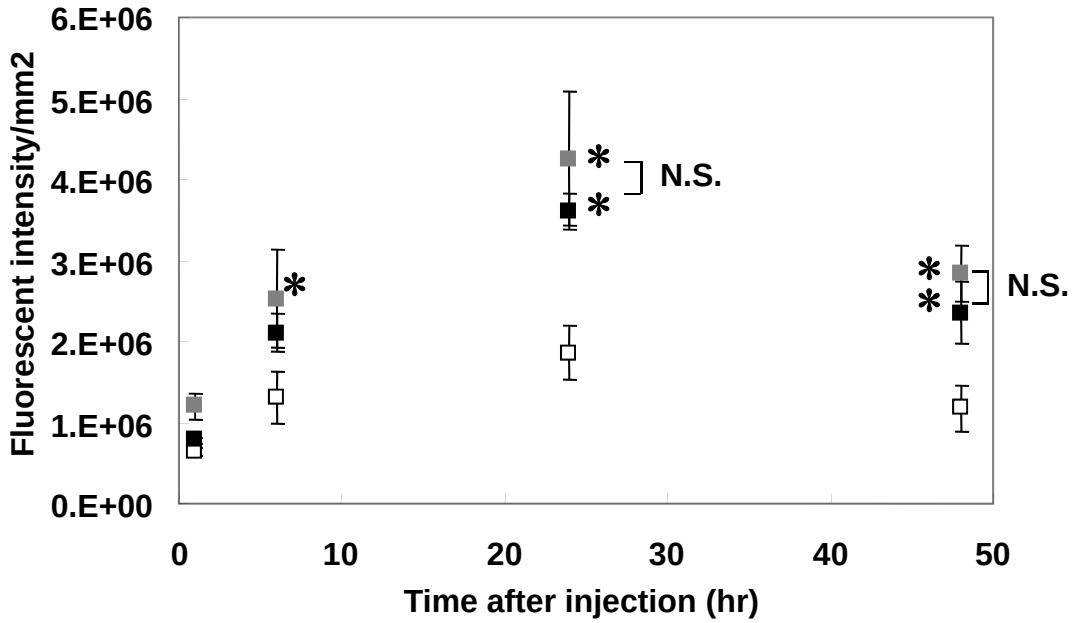


Figure 8(A). Takafuji et al.



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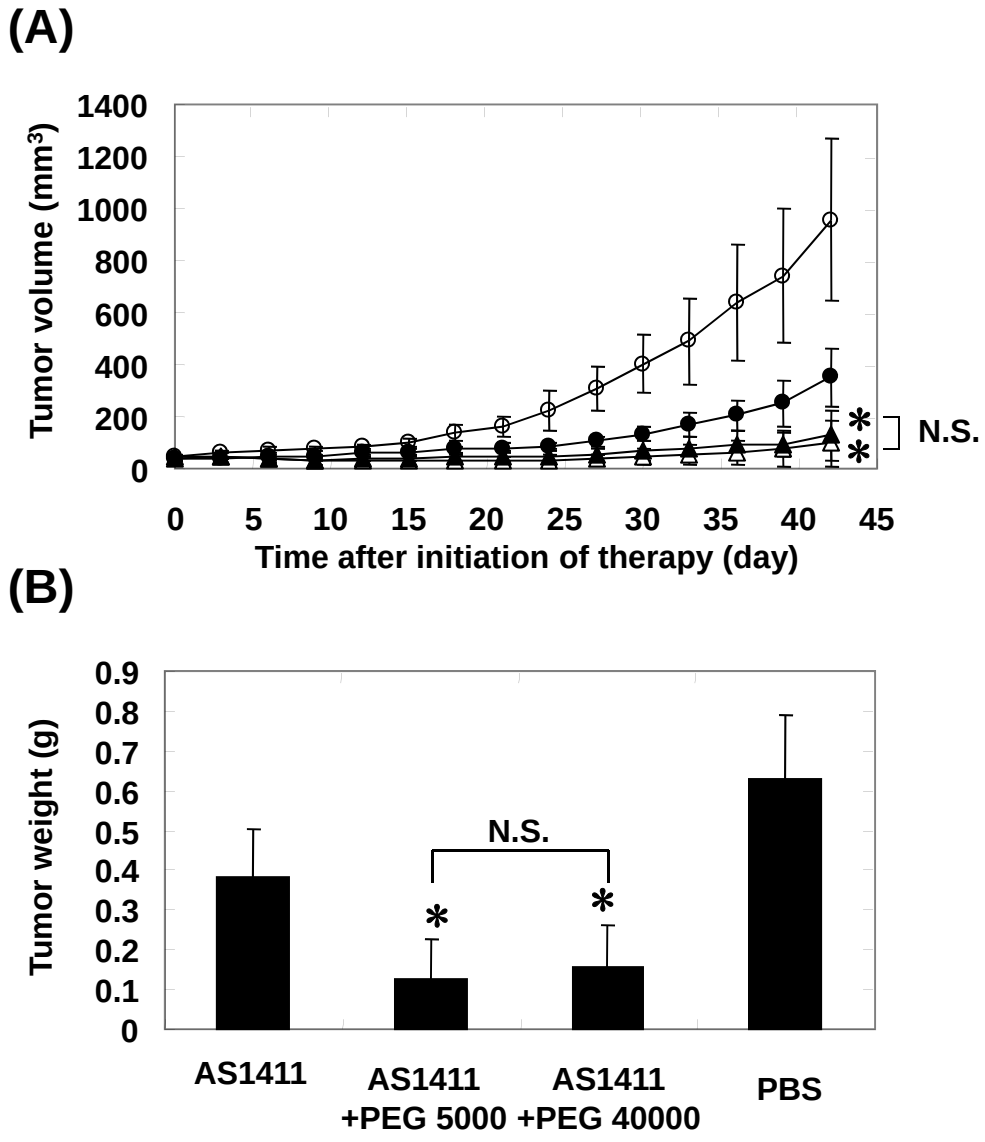

Figure 9. Takafuji et al.