Elution Profiles of Antibody-Drug Conjugates in Preparative Chromatography

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Abstract Monoclonal antibody drug conjugate (ADCs) have received much attention as pharmaceutical agents for treating serious diseases such as cancer. However, it is difficult to separate them on the basis of the drug to antibody ratio, DAR. Hydrophobic chromatography (HIC) is commonly used for the analysis of the drug to antibody ratio, DAR. The retention of ADCs on HIC can be controlled by the hydrophobic nature of ADCs, depending on the mobile phase conditions. They are sometimes performed at the restricted conditions where the solubility is too low. Ion exchange chromatography (IEC) using electrostatic interaction is an orthogonal method to HIC. IEC is widely used because of its higher capacity than HIC. We investigated the retention behavior of the protein conjugated with surrogate drugs on IEC. The surrogate drugs employed are 7-diethylamino-3-(4'-maleimidylhenyl) 4-methylcoumarin (CPM), N-(1-pyrenyl) maleimide (NPM). Bovine serum albumin (BSA) was used as a model protein. The molar ratio (CPM and NPM to protein) was set to 3. The maleimide group of CPM and NPM reacts with the thiol group of the proteins. On the linear gradient elution experiments, the elution salt concentrations of the conjugated and non-conjugated proteins were measured to obtain chromatographic parameter of the number of binding sites, *B*.

1 Introduction

PEGylated proteins and monoclonal antibody drug conjugates (ADC) are considered as next generation biopharmaceuticals. The conjugated flexible PEG layers contribute to protect the proteins from the lytic actions of immune system cells and proteases (Abuchowski *et al*., 1977). Furthermore, the above chemically-modified proteins are large enough in their hydrodynamic diameters to escape from the kidney excretion system, which allows their long in vivo half-life. ADCs are designed to bind to cancer-specific antigens via antigen-antibody interaction causing the death of target cells effectively (Gordon *et al*., 2015).

The same chemical reactions can be employed in the preparation of PEGylated proteins and ADCs (Gordon *et al*., 2015; Fee and van Alstine, 2006). Lysine residue, which is one of the most abundant amino acids on the protein surface, is modifiable with acylating derivatives or ADCs which have activated functional groups such as succinimidyl carbonate and aldehyde (Bonora and Drioli, 2009). Since multiple lysine residues of proteins are involved in the reactions, the product is heterogeneous in the number of modified PEGs or drugs per protein. For the site-specific conjugation, the reactions between cysteine residues and the derivatives of malaimide and iodoacetamide, (Bonora and Drioli, 2009) can be employed since the number of cysteine residues on a protein surfaces is much smaller than that of lysine ones. On the other hand, the loss in the activity both of protein and drugs are undesirably observed even in the site-specific conjugation reactions. As for the PEGylated proteins with biological activity, the number of PEG conjugated to a protein molecule affects the activity. As for the ADCs, the drug to antibody ratio, DAR, significantly affects the potency, such as the binding capacity and cytotoxity. Therefore, the purification of these protein conjugates, PEGylated protein and ADCs is of critical importance in their production processes.

The protein molecules can be purified on the basis of their size, shape, surface charge and hydrophobicity. Ultrafiltration, diafiltration and chromatography can be applied for the purification of modified proteins. Ion exchange chromatography, IEC is the most common technique for the separation of PEGylated proteins (Fee and van Alstine, 2006). The retention time of PEGylated proteins on IEC becomes shorter than that of unmodified ones because of the charge shielding and steric hindrance effects caused by the conjugated PEG. The IEC separation can, therefore, offer the opportunity of the separation of protein isomers on the basis of the number and position of PEG conjugated (Yoshimoto and Yamamoto 2012; Yoshimoto *et al*., 2013). We reported that the number of binding sites, *B* to the ion exchange ligands is practically

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unchanged between the PEGylated and unmodified proteins even when the charge shielding effect of PEG chains weakens the electrostatic interaction between the PEGylated proteins and the ligand. For the purification of ADCs, hydrophobic interaction chromatography is commonly used because the proteins tend to become hydrophobic through the conjugation with drugs (Andris *et al.*, 2018). So far, IEC is rarely used for the purification of ADCs, as their charge properties are poorly understand.

In this study, the charge properties of ADCs were investigated based on their retention profiles on IEC. Bovine serum albumin (BSA), which has free cysteine residues on the surface, was employed as a model protein to be modified with drugs to give model ADCs. Pyren and coumarin were employed as surrogate hydrophobic drug for the preparation of ADC as reported previously (Andris *et al.*, 2018). The drug-modified BSA was characterized in terms of their number of binding sites and retention mechanism on IEC.

2 Experimental

2.1 Chemicals

Bovine serum albumin monomer (BSA monomer, SIGMA, #A1900) was used as model proteins. The surrogate drugs employed were 7- diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumar-in (CPM, ALDRICH, #C1484-25MG) and N-(1-pyrenyl) maleimide (NPM, ALDRICH, #P7908-500MG). L-cysteine (Nakaraitesuku, #10309-12) was used as a terminator of conjugation reactions.

2.2 Conjugation reaction

BSA monomer solution in 50 mM sodium phosphate buffer (pH7.2, buffer A) containing 10% dimethyl sulfoxide (DMSO) was prepared at a concentration of BSA of 2 mg/mL. The stock solution of CPM and NPM (Table 2) was prepared at a concentration of 1 mg/mL in DMSO. The BSA solution (5.1 mL) was mixed with a 0.187 mL of CPM solution or a 0.0934 mL of NPM solution to give the molar ratio (CPM and NPM to protein) of 3. The reaction solutions were mixed gently overnight at 25 °C. For the termination of the reaction, 0.1 mL of 10 mg/mL L-cysteine solution in 50 mM sodium phosphate buffer containing 10% of DMSO was added to the reaction mixture followed by mixing gently for 30 min at 25 °C.

2.3 Ion exchange chromatography

The ion exchange chromatography (IEC) analyses of the reaction mixtures were performed with AKTA pure 25M (GE Healthcare). The linear salt concentration gradient elution was carried out by changing NaCl concentrations from 0.03 to 1 M. Hitrap Q Sepharose HP (Table 1) (1 mL) was used as an anion exchange column. Sample volume was 0.1 mL and flow rate was 1 mL/min. Mobile

phase was 10 mM Tris-HCl buffer solution (pH 7.0) containing 30 mM or 1 M NaCl.
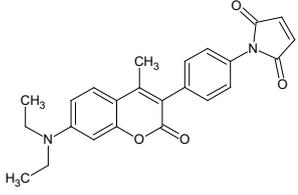
2.4 Size exclusion chromatography

Size exclusion chromatography was performed with AKTA pure 25M (GE Healthcare). Mobile phase was Tris-HCl buffer solution (pH 7.0) containing 100 mM NaCl (pH 8.0). TSK G3000 SW and TSK G3000 PW$_{XL}$ (Table 1) were used as stationary phase. The sample volume was 0.1 mL, and the flow rate was 0.5 mL/min.

Table 1. Characteristics of columns.

	Column	Z [cm]	dc [cm]	ε [-]	d_p [μm]	supplier
IEC	Q Sepharose HP	2.5	1.1	0.3	34	GE healthcare
SEC	TSK G3000SW	30	0.75		10	Tosoh
	TSK G3000PW$_{XL}$	30	0.78		7	Tosoh

Table 2. Surrogate drugs used.

| Surrogate drug	Chemical structure
CPM 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin	
NPM N-(1-pyrenyl) maleimide	
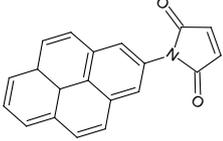
2.5 UV absorption measurement

The fraction was collected, and analyzed by using a mirco-plate reader at the wavelength ranging from 200 to 700 nm with Power Wave XS Microplate Reader (Bio Tek). UV-STAR® MICRO-PLATE, and 96 WELL (Bio-One, #655801) were used for measuring the absorbance of solutions at each volume of 0.2 mL.

3. Results and Discussion

3.1 Analysis of UV/VIS absorption spectra of conjugation reaction mixtures

Figure 1 shows the UV/VIS absorption spectra of native BSA and the reaction mixtures containing BSA and CPM or NPM. Absorption peaks are observed around 280 nm for both native BSA and the BSA-containing reaction mixtures. The additional peaks were observed around 400 nm with respect to BSA-NPM reaction mixture, and around 340 nm with BSA-CPM, respectively. Therefore, these wavelengths were empoyed to investigae the BSA conjugates elution profiles on the chromatography column in the following.
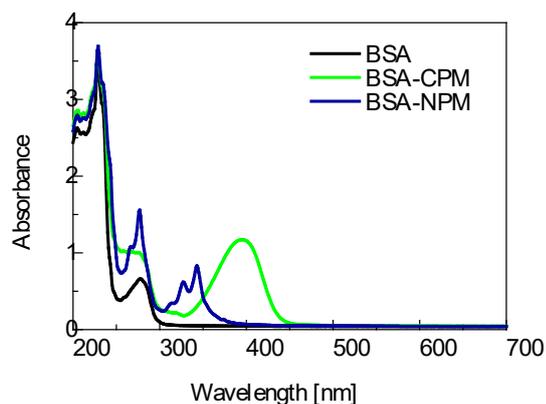
Figure 1. UV-VIS absorption spectra of native BSA, BSA - CPM and BSA – NPM conjugate

3.2 Analysis of the protein surface charge by ion exchange chromatography

### 3.2.1 Linear salt concentration gradient elution

Figure 2 shows that the elution curves of the linear salt gradient experiments (LGE) obtained with respect to BSA and CPM mixture containing cycteine used for terminating the reactions (a) and L-cysteine and CPM one without BSA (b). As shonwn in Fig. 1, UV signals both at 280 nm and at 400 nm are attributed to the conjugation of BSA or cycteine with CPM in the elution curves. In both Figures (a) and (b) the small elution peaks are seen at the elution volume of about 10 mL. The peaks correspond to cysteine-CPM conjugates. In Fig. 2(a), the later large peak conrresponds to the BSA-CPM conjugates. The elution volume of the large peak was slightly larger than that of native BSA (data not shown), and the volume increased as the gradient volume increased. The elution salt concentrations, I_R in LGE correlated with the normalized gradient volume GH ($= (I_F - I_0)/V_g \times V_t (1-\varepsilon)$, I_F and I_0 : final and initial salt concentrations in LGE, V_g : gradient volume, V_t : column volumu, ε : column porosity) (Figure 3). The correlation curve was fitted by using following equation,

$$GH = I_R^{(B+1)}/\{A(B+1)\} \qquad (1)$$

where *A* is a constant including equilibrium constant of ion exchange reaction, *B* is the number of binding site , K_e and ion exchange ligand density, Λ ($A = K_e \cdot \Lambda^B$). Similar elution profiles were observed with BSA-NPM reaction mixtures (data not shown). From the slopes of these correlation curves (GH-I_R curves, Yamamoto, et al., 2012 and 2013), the number of binding sites can be determined as shown in Table 3.

Table 3 The number of binding site, *B*.

	B
BSA	6.5
BSA-CPM	7.5
BSA-NPM	7.4

BSA dimer	11.9
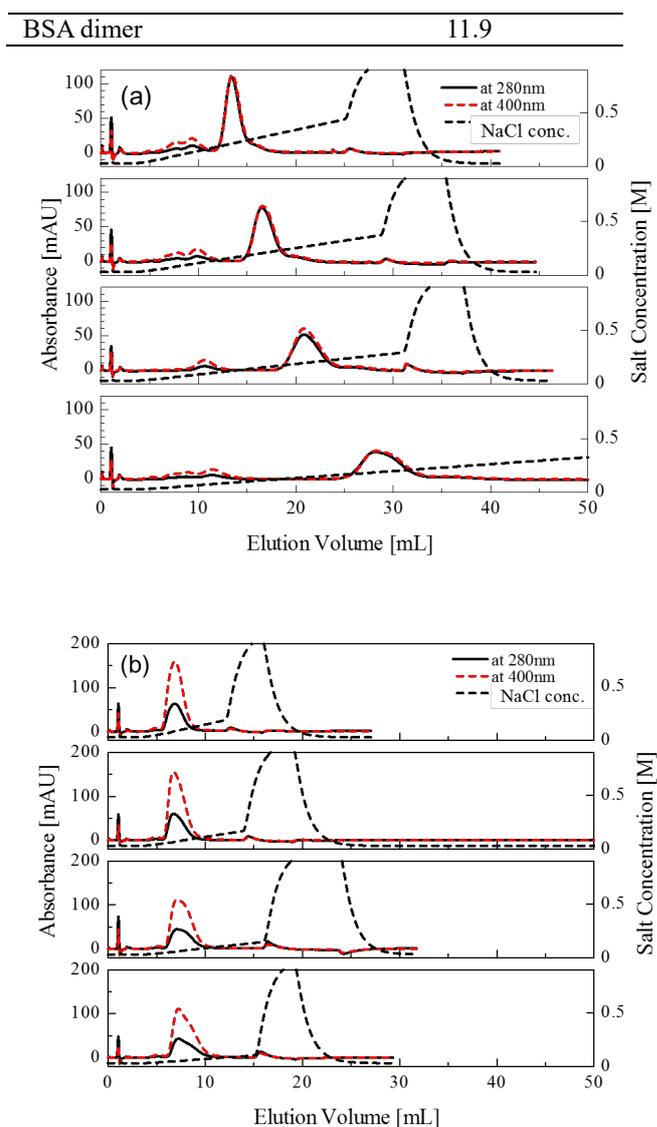
Figure 2. Elution curves of the linear salt gradient experiments with respect to the reaction mixture containing BSA and CPM (a) and L-cysteine and CPM (b). Gradient volumes, V_g were 50, 70, 100 and 150 mL from top to bottom in each panel.
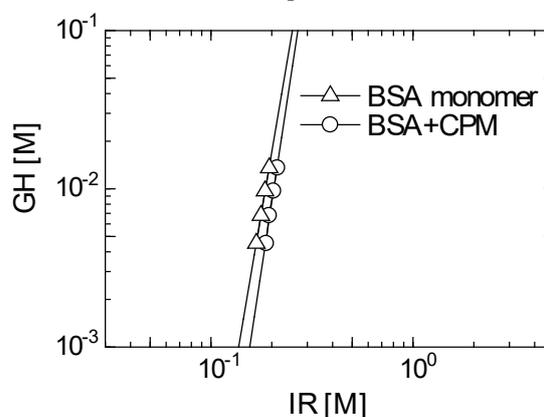
Figure 3. Relationship between the normalized gradient GH and the elution salt concentration I_R ($|R| >$ 0.99).

The B values correspond to the number of charged binding sites of the protein on the surface of the ion exchange resin. The B value of dimeric BSA was about two times larger than that of monomeric BSA. These results suggest that surrogate drugs (CPM and NPM) were successfully conjugated to thiol groups of BSA. Importantly, the conjugation of hydrophobic CPM and NPM caused the increase in the elution volume and the number of binding site. It might suggest that the hydrophobic interaction between proteins and resins is enhanced by the conjugation of hydrophobic surrogate drug to the proteins, which potentially promotes the electrostatic interaction between the binding sites of proteins and the resin. It is also suggested that the conjugates became dimer-like substances through π-π interaction between the surrogate drug molecules as a core, which caused the increase in the number of adsorption sites of the conjugates.

3.2.2 Size exclusion chromatography of conjugates

Figure 4 shows the elution curves of native BSA and the reaction mixture containing BSA-CPM or BSA-NPM on the size exclusion columns packed with TSK G3000 SW (a) and TSK G3000 PW$_{XL}$ (b). With the TSK G3000 SW column, the dimer and monomer BSA are separated into two peaks (Fig. 4 (a)). The intensities of the absorbance increased confirming the conjugation of CPM and NPM to BCA. However the aggregate of the conjugates is not seen. These results imply that the aggregate of conjugates was little formed in the SEC column.

4 Conclusion

Surrogate drugs (CPM and NPM) were successfully conjugated to thiol groups of BSA. In LGE experiments, the elution concentrations of the BSA-CPM and BSA-NPM conjugates were slightly increased than the concentration determined with respect to native BSA. The number of binding sites was 7.4 for BSA-CPM and 7.5 for BSA-NPM. The values were larger than the value of BSA (6.5), suggesting the aggregation of conjugates on the surface of ion exchange resin.

Acknowledgment

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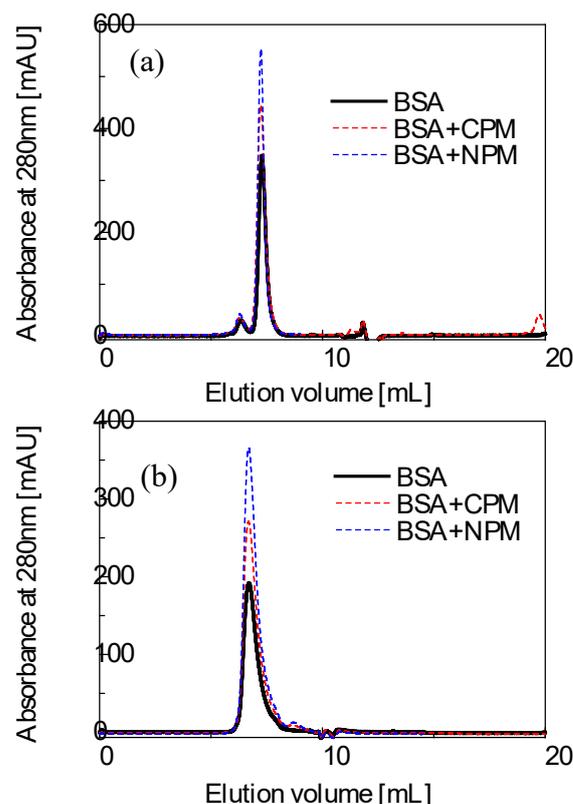
Figure 4. Elution curves of native BSA ant the reaction mixture of BSA-CPM or BSA-NPM on TSK G3000 SW (a) and TSK G3000 PW$_{XL}$ (b).