Intermolecular interactions and conformation of antibody dimers present in IgG1 biopharmaceuticals

Received September 2, 2013; accepted October 11, 2013; published online October 22, 2013

Takafumi Iwura1,2, Jun Fukuda2, Katsuysahi Yamazaki2, Shuji Kanamaru1 and Fumio Arisaka1,*

1Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 B-9 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8501; and 2Bio Process Research and Development Laboratories, Production Division, Kyowa Hakko Kirin Co., Ltd.; 100–1 Hagisawa-machi, Takaasaki, Gunma 370–0013, Japan

*Fumio Arisaka, 4259 B-9 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8501, Japan. Tel/Fax: +81-45-924-5713, email: farisaka@bio.titech.ac.jp

Intermolecular interactions and conformation in dimer species of Palivizumab, a monoclonal antibody (IgG1), were investigated to elucidate the physical and chemical properties of the dimerized antibody. Palivizumab solution contains ~1% dimer and 99% monomer. The dimer species was isolated by size-exclusion chromatography and analysed by a number of methods including analytical ultracentrifugation-sedimentation velocity (AUC-SV). AUC-SV in the presence of sodium dodecyl salt; AUC-SV, analytical ultracentrifugation-sedimentation velocity; CD, circular dichroism; DSC, differential scanning micro-calorimetry; DTT, dithiothreitol; HC, heavy chain; IAM, iodoacetamide; LC, light chain; MALLS, multi-angle laser light scatter- ing; NPM, N-(1-pyrenyl)maleimide; PBS, phosphate-buffered saline; SD, standard deviation; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography.

Keywords: immunoglobulin G/aggregation/intermo-

Featured Article

Soluble proteins in solution can aggregate under normal conditions with relatively small perturbations such as thermal or physical agitation (1–3). Pharmaceutical proteins are not the exception; in fact, they may aggregate during production, isolation, purification, storage and delivery (4), and the aggregation may affect their activity and immunogenicity (5, 6). Therefore, characterization of aggregation is of great importance in pharmaceutical industry. In monoclonal antibodies which are the fastest growing class of biopharmaceuticals, many studies have been conducted to acquire physical and chemical information regarding antibody aggregations (7–10). When a monoclonal antibody is prepared as a biopharmaceutical, it is mostly monomer (complex of two heavy chains (HC) and two light chains (LC), H2L2) in solution; however, ~1% or lower amount of dimer (dimerized monomer, (H2L2)2) is always present. Although the amount of dimer may appear insignificant, in the field of biopharmaceuticals, it is highly important to elucidate the nature of dimerization for the safety and prevention of dimerization or further association and to determine whether the dimer fraction increases during storage.

In the previous study on IgG dimer, mass measurement of the dimer molecules after papain digestion has indicated that dimer formation can occur between any possible combination of domains (i.e. F\textsubscript{ab}, F\textsubscript{ab}–Acetyl-Cysteine; ANS, 8-Anilino-1-naphthalenesulphonic acid ammonium salt; AUC-SV, analytical ultracentrifugation-sedimentation velocity; CD, circular dichroism; DSC, differential scanning micro-calorimetry; DTT, dithiothreitol; HC, heavy chain; IAM, iodoacetamide; LC, light chain; MALLS, multi-angle laser light scattering; NPM, N-(1-pyrenyl)maleimide; PBS, phosphate-buffered saline; SD, standard deviation; SDS–PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEC, size-exclusion chromatography. In the present article, we focused on the dimer species in the pharmaceutical antibody, Palivizumab, which is a humanized monoclonal antibody (IgG1) used to prevent respiratory syncytial virus infections (11). Dimer species of Palivizumab have been fractionated by SEC and studied by a number of methods to evaluate the intermolecular interactions and conformation. The fractionated dimer was analysed by AUC-SV in the absence or presence of SDS, and by SDS–PAGE in order to investigate the intermolecular interactions in the dimer. Since AUC-SV has higher
resolution capacity than SEC, we employed AUC-SV to quantify the amount of non-covalently and covalently associated dimer species in the present study. SDS–PAGE under reducing conditions showed that remaining dimer in the presence of SDS dissociated into the HC, LC and slight amount of irreducible species. The sulphydryl contents were also measured to confirm the possibility that disulphide bond is involved in the dimerization. Furthermore, fluorescence from dityrosine was measured to investigate the irreducible species. Furthermore, limited proteolysis of the dimer by Lys-C and mass spectrometry for the resultant products indicated that the dimer species were mainly formed by $F_{\text{abs}}-F_{\text{c}}$ or $F_{\text{abs}}-F_{\text{ab}}$ interactions. To evaluate the conformation of the dimer species, surface hydrophobicity and thermal stability were measured in addition to the secondary and tertiary structures, which were evaluated in previous reports (7, 8).

Materials and Methods

Materials and Methods

Sodium phosphate, SDS, IAM, acetonitrile, 2-propanol and trifluoroacetic acid (Wako Pure Chemicals, Tokyo, Japan), NPM, Ac-Cys and ANS (Sigma-Aldrich, MO, USA), Novex 4–12% Tris–Glycine Gel and NuPAGE sample reducing solution (0.5 mol l$^{-1}$ DTT solution) Mark 14$^{\text{th}}$ generation standard (Life Technologies, NY, USA), Durbecco’s PBS (Nissui Seiyaku, Tokyo, Japan), guanidine hydrochloride (MP Biochemicals, CA, USA), N-glycosydase F (New England Biolabs, MA, USA) and endoproteinase Lys-C (Roche Diagnostics, Basel, Schweiz) were used in this study. Commercially available Synagis$^\text{®}$ injection containing Palivizumab (AbbVie Inc., Illinois, USA) was purchased through alfresa Co., Ltd. (Tokyo, Japan).

Preparation of dimer fraction of Palivizumab

A Synagis$^\text{®}$ injection was reconstituted in water and the solution was analysed by SEC to collect the monomer and marginally contained dimer of Palivizumab. SEC experiments were performed with an Agilent 1100 chromatography system at 25°C using Tosoh TSKgel G3000SWXL with a mobile phase containing 0.05 mol l$^{-1}$ sodium phosphate and 0.5 mol l$^{-1}$ sodium chloride of pH 7. Elution in MALLS analysis and fractionation was monitored by UV detector at a wavelength of 280 nm, whereas the wave length of 215 nm was used when rechromatography was carried out to re-examine the elution profile. In order to prepare dimer and monomer fractions, ~2 mg of Palivizumab was injected into the column per one chromatography, and the chromatography was repeated many times to isolate the necessary amount of dimer species. After the fractionation of the monomer and dimer peaks, their solvents were substituted by ultrafiltration with 0.01 mol l$^{-1}$ sodium acetate and 0.1 mol l$^{-1}$ sodium chloride at pH 5.5 in order to prevent absorbance due to L-histidine in CD measurement and confer appropriate ion strength for AUC-SV. The monomer and dimer solutions were stored under refrigerated conditions until each examination.

SEC–MALLS

MALLS analysis of each peak was performed on the SEC column eluate of injected Palivizumab protein of 0.1 mg. A DAWN-EOS MALLS photometer was connected in series with an Optilab T-REX refractive index detector (both from Wyatt Technologies, CA, USA). Data analysis was conducted with Astra software (version 6) using 1.53 (mg/ml)$^{-1}$ cm$^{-1}$ as an extinction coefficient at 280 nm and 0.185 as a refractive index increment. The extinction coefficient was calculated from the theoretical amino acid sequence (13).

AUC-SV in the presence of SDS

To investigate intermolecular covalent interactions in the dimer of Palivizumab, the dimer and monomer fractions were measured by AUC-SV in the absence or presence of SDS. The AUC-SV sample solutions were prepared by dilution to 0.16 mg ml$^{-1}$ protein concentration and heating at 65°C for 10 min with or without 0.3% SDS. AUC-SV was performed on a Beckman Coulter XL-A analytical ultracentrifuge using 12-mm Epon charcoal-filled double-sector centrepieces and absorbance detection at 280 nm. The rotor speed and rotor temperature were set for 40,000 rpm and 20°C, respectively. The data analysis was carried out using a continuous ($\alpha$) distribution model in the software program SEDFIT (version 11.8) (13, 14). When the obtained sedimentation coefficient of each peak was corrected for $s_{20w}$, the calculated specific volume of Palivizumab and the measured density and viscosity of solvent were used. The specific volume of Palivizumab, 0.7249 cm$^3$ g$^{-1}$, was obtained by calculating from the theoretical amino acid sequence, and the value of protein–SDS complex, 0.8125 cm$^3$ g$^{-1}$, was calculated by using 0.875 cm$^3$ g$^{-1}$ as the specific volume of SDS and 1.4 as grams of bound SDS per gram of protein (15). The density and the viscosity were measured by an Anton Paar 4.500 and an Anton Paar AMVc, respectively. The density of 10 mmol l$^{-1}$ sodium acetate and 100 mmol l$^{-1}$ sodium chloride of pH 5.5 without or with 0.3% SDS was 1.0027 g cm$^{-3}$ or 1.0031 g cm$^{-3}$, respectively. The viscosity of the solvent without or with 0.3% SDS was 1.0048 mPa s or 1.0103 mPa s, respectively.

SDS–PAGE

SDS–PAGE was performed to investigate intermolecular covalent interactions in the dimer species of Palivizumab. Non-reduced and reduced sample solutions were loaded onto a Novex 4–12% Tris–glycine gradient gel and run under 125 V until the dye front reached the end of the gel. Both sample solution types were prepared by heating at 65°C for 10 min in the presence of 0.025 mol l$^{-1}$ IAM or 0.05 mol l$^{-1}$ DTT. Protein bands were visualized by staining the gel with a Coomassie Brilliant Blue solution, and the relative amount of each band was analysed with FUJIFILM Multi Gauge software (version 2.2).

Measurement of sulphydryl content

Sulphydryl contents of the dimer and monomer were measured by the method of quantitation of non-covalently and covalently associated dimer species in the present study. SDS–PAGE was performed with a Beckman Coulter XL-A analytical ultracentrifuge using 12-mm Epon charcoal-filled double-sector centrepieces and absorbance detection at 280 nm. The rotor speed and rotor temperature were set for 40,000 rpm and 20°C, respectively. The data analysis was carried out using a continuous ($\alpha$) distribution model in the software program SEDFIT (version 11.8) (13, 14). When the obtained sedimentation coefficient of each peak was corrected for $s_{20w}$, the calculated specific volume of Palivizumab and the measured density and viscosity of solvent were used. The specific volume of Palivizumab, 0.7249 cm$^3$ g$^{-1}$, was obtained by calculating from the theoretical amino acid sequence, and the value of protein–SDS complex, 0.8125 cm$^3$ g$^{-1}$, was calculated by using 0.875 cm$^3$ g$^{-1}$ as the specific volume of SDS and 1.4 as grams of bound SDS per gram of protein (15). The density and the viscosity were measured by an Anton Paar 4.500 and an Anton Paar AMVc, respectively. The density of 10 mmol l$^{-1}$ sodium acetate and 100 mmol l$^{-1}$ sodium chloride of pH 5.5 without or with 0.3% SDS was 1.0027 g cm$^{-3}$ or 1.0031 g cm$^{-3}$, respectively. The viscosity of the solvent without or with 0.3% SDS was 1.0048 mPa s or 1.0103 mPa s, respectively.

Fluorescence spectrum measurement from dityrosine

The fluorescence spectra were determined for the dimer and monomer fractions to investigate whether dityrosine formation was involved in dimerization via covalent bonds. These measurements were made with a Tecan spectrophotometer, M1000. The fluorescence spectra were determined for 2 mg ml$^{-1}$ sample solutions by excitation at ~320 nm for dityrosine (17, 18), and emissions were recorded in the range of 350–500 nm.

Limited Lys-C digestion and mass spectrometry

The dimer and monomer fractions were diluted to 1 mg ml$^{-1}$ with 0.08 mol l$^{-1}$ Tris solution (pH 8) and treated overnight with N-glycosydase F at 37°C to eliminate N-glycans. After the treatment, endoproteinase Lys-C was added to the treated solution for a final enzyme/protein ratio of 1:800, and the sample solution was incubated at 37°C for 30 min to achieve limited digestion in the hinge region. The digested sample was analysed on a ZORBAX 300SB-C8 (Agilent Technologies) chromatography column with an acetonitrile and 2-propanol gradient run on a Waters UPLC with an inline Waters Synapt G2 HDMS mass spectrometer set at an ionization region of 350–3,000 m/z. Spectra were derived from multiply charged ions and deconvoluted with MassLynx software (version 4.1).

CD spectrum

CD spectra were determined to investigate the conformations of the monomer and dimer of Palivizumab. The spectropolarimeter was a Jasco J-820 equipped with a CDT-426L, Peltier thermal control unit. CD spectra in the 200–260 nm (far-UV) and 250–320 nm
(near-UV) ranges were determined for 0.2 mg ml\(^{-1}\) sample solutions at 25°C using quartz cells having path lengths of 1 and 10 mm, respectively.

**Fluorescence spectrum**
The fluorescence spectra were determined to investigate the conformations of the monomer and dimer of Palivizumab. The measurements were made with a Tecan spectrophotometer M1000. The fluorescence spectra were determined for 0.2 mg ml\(^{-1}\) sample solutions by excitation at 280 nm and emissions were recorded in the range of 300–450 nm.

**Measurement of surface hydrophobicity of protein**
To investigate the conformation of the monomer and dimer of Palivizumab, the surface hydrophobicity of the sample solutions was assessed by use of ANS reagent. ANS is a non-covalent extrinsic fluorescence dye that can characterize protein surface hydrophobicity (19). The monomer and dimer solutions were mixed with ANS solution for a protein/ANS ratio of 1:100, and then the mixtures were reacted for 2 h at room temperature. The fluorescence from ANS was measured by excitation at 380 nm, and emissions were recorded in the range 400–600 nm.

**DSC analysis**
DSC analysis was conducted to evaluate the conformations of the monomer and dimer species of Palivizumab on a MicroCal capillary DSC platform. Sample solutions of 0.5 mg ml\(^{-1}\) were used for the experiment. The temperature range of the scan was 5–100°C, and the scan rate was set at 1°C min\(^{-1}\). The data analysis was performed with Origin software (version 7). The thermogram was background-corrected and normalized to the molar concentration as the monomer antibody. The melting temperature of each peak was determined by integration of the each endothermic peak.

**Results**

**Preparation of dimer fraction in Palivizumab**
Palivizumab was separated into two peaks by SEC as shown in Fig. 1A. The eluted peaks at ~6.6 or 7.9 min corresponded to dimer and monomer, respectively, based on MALLS analysis. Dimer species constituted ~1% of the total protein. The chromatogram in preparation of dimer and monomer fractions is presented in Fig. 1B. The dimer and monomer peaks were sufficiently separated under the condition for the preparation of each peak fraction. Rechromatography of the isolated dimer fraction (Fig. 1C) revealed that some fraction of the dimer was dissociated into monomer and a small fraction of a fragment, and the resultant percentages of the dimer, monomer and fragment were 85%, 12% and 3%, respectively.

**AUC-SV of the dimer fractions in the absence or presence of SDS**
The collected dimer and monomer fractions were analysed by AUC-SV in the absence and presence of SDS. Both in the presence and absence of SDS, the samples were incubated at 65°C for 10 min. The obtained c(s) are shown in Fig. 2A and B. In the absence of SDS, c(s) indicated that the dimer fraction from the SEC contained ~4% oligomers larger than dimer, 77% dimer species and 18% monomer species. As compared with the result from SEC, the amount of dimer was lower, whereas the amount of monomer was higher. The cause for the difference in abundance between the two methods is likely due to the partial dissociation of the dimer to monomer upon sample dilution and incubation for AUC measurement. In the presence of SDS, the larger oligomers, dimer, monomer and fragment were ~2%, 48%, 46% and 4% in c(s), respectively. This result indicated that 48% of the fractionated dimer molecules were covalently linked, whereas 46% of the original fractionated dimers were shown to consist of non-covalently associated monomers.

**SDS–PAGE of fractionated dimer in Palivizumab**
The collected dimer and monomer fractions were analysed by SDS–PAGE under non-reducing and reducing conditions. The result is shown in Fig. 3. In the
non-reducing conditions (lane 1 and 2), bands of larger oligomers, dimer, monomer, and fragments were observed in the dimer fraction (lane 2). Their relative abundance was estimated to be 2%, 48%, 45% (including thin bands above and below the monomer) and 4%, respectively, which was almost the same as in the presence of SDS (c). Under reducing conditions, the dimer band disappeared as shown in lane 4 of Fig. 3. Under reducing conditions, the dimer is involved in the dimerization. The sulfhydryl content was measured in the dimer and monomer species to confirm the conclusion that disulfide bond is involved in the dimer formation in the next section. Furthermore, some bands with molecular weights between the monomer and the HC were observed, and they were apparently irreducible. The fraction of irreducible bands was estimated to be 15% of the whole protein on the basis of scanning the band densities in lane 4 of Fig. 3. This indicates that covalent bonds other than disulfide bonds are involved in the dimerization.

**Sulfhydryl content in Palivizumab dimer**

Sulfhydryl contents were measured in the monomer and dimer fractions, and the results are shown in Table I. This results showed that sulfhydryl contents of the monomer and dimer were 4.2 and 3.8 mol mol\(^{-1}\) as mole of sulfhydryl per mole of monomer IgG, respectively. A native monomer molecule of Palivizumab contains 16 intra- and interchain disulfide linkages and two sulfhydryl groups, which means that the native monomer of Palivizumab has 2 mol mol\(^{-1}\) of sulfhydryl. The discrepancy of 2.2 sulfhydryl per monomer Palivizumab is likely to have arisen by reduction of one of the native disulfide bonds. Since the sulfhydryl content in the dimer species was lower than that in the monomer, it is very likely that disulfide bond formation is at least to some extent involved in covalent dimerization which is reducible.

**Detection of dityrosine in Palivizumab dimer by fluorescence**

Fluorescence from dityrosine was measured for the collected fractions of the dimer and monomer to investigate the possibility that dityrosine was involved in the formation of covalently linked dimer. The Palivizumab molecule contains 16 and 10 tyrosine residues on the HC and LC, respectively. A differential fluorescence spectrum from Palivizumab between dimer and monomer.
monomer is shown in Fig. 4. The spectrum showed a broad convex curve with a positive maximum ~400 nm, suggesting the possibility that dityrosine was contained in the dimer fraction (17, 18). Thus, it was likely that the dityrosine formation was involved in the covalent dimerization in addition to the disulphide bond formation. The dityrosine formation might engage in irreducible bond formation in the dimer species as shown in SDS–PAGE (lane 4 in Fig. 3).

**Limited Lys-C digestion of Palivizumab dimer and analyses of the products by mass spectrometry**

The dimer and monomer fractions were analysed by mass spectrometry after limited digestion by Lys-C to determine the region involved in dimerization of Palivizumab. Lys-C has been used to cleave IgG specifically in the hinge region (20, 21). As the limited Lys-C digestion results in production of the F\text{ab} and F\text{c} fragments of Palivizumab, it is expected to help determine which part of the IgG is involved in dimerization on the basis of analyses of the resulting fragments.

The total ion chromatograms in the reversed phase mode are shown in Fig. 5A and B. The results of mass analysis for each peak are listed in Table II. In the chromatogram of the digested monomer sample, two groups of peaks were observed (Fig. 5A). The mass of the peak eluted at ~15.5 min was 50,407 Da, which coincided with the calculated masses of the F\text{ab} fragment. Also, the mass of 50,425 Da was observed in the peak eluted at ~15.2 min. The difference in mass indicated that the 15.2 min species is the oxidized form of F\text{c} that eluted at 15.5 min. On the other hand, the mass of 47,347 Da at 18.8 min corresponds to the calculated mass of 47,348 Da for the F\text{ab} fragment.

In the digested dimer sample, significant alterations of the chromatogram were observed at ~18.0 and 19.5 min (Fig. 5B). The mass of the peak eluted at 18.0 min was 97,755 Da, which corresponded to the calculated mass of 97,756 Da for a F\text{ab}–F\text{c} complex formed via a covalent interaction including a disulphide or a dityrosine bond and/or 97,758 Da for the complex formed via a non-covalent bond. Also, the mass of eluted at 19.5 min was 94,695 Da. The value corresponded to the calculated mass of 94,699 and/or 94,701 Da for a F\text{ab} homodimer formed via a covalent (a disulphide or a dityrosine bond) or a non-covalent interaction. These result showed that the dimer species were formed by F\text{ab}–F\text{c} interaction or F\text{ab}–F\text{ab} interaction. F\text{c}–F\text{c} interaction was not found.

**Comparison of secondary and tertiary structure of the dimer and monomer**

*CD spectrum.* Far- and near-UV CD spectra of the dimer and monomer fractions were measured to investigate their conformation. The intrinsic fluorescence, surface hydrophobicity and thermal transition property of the protein were also measured to evaluate the conformation, as described in the following sections. As shown in Fig. 6A, the far-UV CD spectrum of the dimer fraction was identical to that of the monomer fraction, with a negative minimum at 218 nm, which was derived from the β-sheet structure. Furthermore, the near-UV CD spectra of the monomer and dimer fractions were almost identical, with positive peaks at 257, 265 and 291 nm, which were attributable to aromatic amino acid residues (Fig. 6B). These results indicated that the secondary and tertiary structure of Palivizumab did not significantly change upon dimerization.

*Fluorescence spectrum.* Fluorescence spectra of the dimer and monomer fractions are shown in Fig. 6C. A wavelength of 280 nm was used for excitation. The fluorescence spectrum of the dimer fraction was identical to that of the monomer fraction, with a positive maximum at 334 nm attributable to tryptophan residues. This result was in agreement with that of CD measurement, where the near-UV spectrum was identical between monomer and dimer. It indicated that the tertiary structure of the dimer and monomer is very similar.

**Assessment of surface hydrophobicity of the dimer and monomer**

Fluorescence spectra from ANS on the dimer and monomer fractions are shown in Fig. 6D. No blue
shift of the fluorescence maximum was observed in the spectra of both fractions. It is known that the blue shift of ANS fluorescence is associated with binding to a hydrophobic cluster of proteins (19, 22). Hence most likely, neither dimer nor monomer has any significant hydrophobic region on the protein surface.

DSC analysis of the dimer and monomer. DSC analysis can evaluate the thermal transition of proteins. It is thus expected that the technique may detect some difference in thermal stability between the dimer and monomer. Thermograms of the dimer and monomer are presented in Fig. 6E. The DSC curves for dimer

---

**Table II. Experimental and calculated masses of fragments resulting from limited Lys-C digestion.**

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Experimental mass (Da)</th>
<th>Expected structure from mass</th>
<th>Calculated mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.2</td>
<td>50,425</td>
<td>Oxidized Fc</td>
<td>50,423 (2K-)a, b</td>
</tr>
<tr>
<td>15.2</td>
<td>50,553</td>
<td>Fc</td>
<td>50,551 (K-)a, b</td>
</tr>
<tr>
<td>15.5</td>
<td>50,407</td>
<td>Fc</td>
<td>50,407 (2K-)a</td>
</tr>
<tr>
<td>15.5</td>
<td>50,535</td>
<td>Fc</td>
<td>50,535 (K-)a</td>
</tr>
<tr>
<td>18.8</td>
<td>47,347</td>
<td>FaFb</td>
<td>47,348</td>
</tr>
<tr>
<td>18.0</td>
<td>97,755</td>
<td>FaFb</td>
<td>97,756 (Covalent)</td>
</tr>
<tr>
<td>18.0</td>
<td>97,755</td>
<td>FaFb</td>
<td>97,758 (Non-covalent)</td>
</tr>
<tr>
<td>19.5</td>
<td>94,695</td>
<td>FaFb</td>
<td>94,699 (Covalent)</td>
</tr>
<tr>
<td>19.5</td>
<td>94,701</td>
<td>FaFb</td>
<td>94,701 (Non-covalent)</td>
</tr>
</tbody>
</table>

aThe mass of oxidized Fc is calculated as that of the Fc fragment which is oxidized at a single amino acid residue.
b(K-) and (2K-) represent the Fc fragment of one or both C-terminal lysine residues are processed.
cThe mass with ‘(covalent)’ is calculated as 2 Da-subtracted mass, based on an intermolecular binding such as a disulphide or a di-tyrosine formation. (See A and B in Fig. 5 for retention times and the corresponding peaks.) (Raw mass spectrum with series of charge state and deconvoluted mass spectrum are shown in supplementary Fig. 1.)
and monomer superimposed, where two transitions at \( \sim 68 \, ^\circ \text{C} \) and \( 87 \, ^\circ \text{C} \) are observed. The first peak at \( 68 \, ^\circ \text{C} \) corresponds very likely to the denaturation of the C12 region of the \( F_c \) domain, and the second peak at \( 87 \, ^\circ \text{C} \) is for the \( F_{ab} \) domain based on the observed temperature and magnitude of each peak in the previous reports (22–24). The overall transition curves from the two association states are superimposed. Whether the slight difference in the first transition has any importance remains to be elucidated.

**Discussion**

In the present article, we have focused on the existing dimer in Palivizumab, a monoclonal antibody (IgG1), in order to understand the nature and behaviour of the dimer in solution. The information obtained is expected to be useful in evaluating the safety of Palivizumab as a pharmaceutical and also for the future study of protein pharmaceuticals.

**Intermolecular interactions in Palivizumab dimer species**

Palivizumab solution contains \( \sim 1\% \) dimer and 99% monomer. The size-exclusion chromatography was repeated more than 100 times and the dimer fractions were collected. The rechromatography of the collected dimer fractions revealed that some portion of the dimer was dissociated into monomer and fragment (Fig. 1C), indicating that part of the isolated dimer is in equilibrium with monomer. A small amount of detected fragment is considered to be a \( F_{ab} - F_c \) form, based on the previous report (25, 26). AUC-SV measurement of incubated sample in the presence of 0.3% SDS at \( 65 \, ^\circ \text{C} \) for 10 min indicated that monomer and dimer were present with percentages of 46% and 48% in \( c(s) \), respectively (Fig. 2B). It was thus shown that approximately half of the dimer of Palivizumab was stabilized by covalent bonds and the others were associated with non-covalent bonds. It was previously reported that the SEC-isolated dimer species of Epratuzumab, a monoclonal antibody (IgG1), remained mostly dimerized after rechromatography by SEC, whereas 70% of the dimer species remained dimer after treatment with SDS and the remaining 30% were dissociated into monomer (7). Thus, both covalent and non-covalent dimers are present in the SEC-isolated dimer fraction. However, since the nature of dimers are considered to vary even among structurally similar antibodies on the basis of the differences in the amount of fragment and the ratio of covalent and non-covalent dimers, it is important to elucidate the nature of dimer species in each antibody sample.

Disulphide bond and dityrosine formation is likely to be involved in the covalent dimerization of Palivizumab. Sulphhydryl content decreased from 4.2 to 3.8 mol mol\(^{-1}\) due to the dimerization. On the basis of decreased content of sulphhydryl group upon the dimerization, approximately one-tenth of the dimer was formed by intermolecular disulphide bond. On the other hand, dityrosine is irreducible, and is known to be formed by oxidative stress due to reactive oxygen species, UV irradiation and so on (27, 28). Dityrosine might have been formed by peroxidase derived from disrupted cell during cultivation and/or by UV irradiation during chromatography for purification.

Limited proteolysis of the dimer IgG by Lys-C and mass spectrometry for the resultant products indicated that the dimer species were formed by \( F_{ab} - F_c \) or \( F_{ab} - F_{ab} \), \( F_c - F_c \) interactions. \( F_c - F_c \) interactions were not found. It is thus likely that the \( F_{ab} \) region is preferentially involved in the intermolecular interactions of Palivizumab rather than the \( F_c \) region. A \( F_{ab} \) domain of a certain IgG1 was previously reported to mainly participate in reversible intermolecular associations in solution (29). Palivizumab molecules in the present study are also likely to associate via \( F_{ab} \) domain in solution. On the other hand, the dimer of Epratuzumab was formed by \( F_c - F_c \) interaction in addition to \( F_{ab} - F_c \) and \( F_{ab} - F_{ab} \) interactions (7). Thus, the intermolecular interactions in the Epratuzumab dimer are more complicated than those in the Palivizumab dimer.

Also, the binding activity to F-protein of RS virus, an antigen of Palivizumab, was measured in the dimer and monomer fractions by enzyme-linked immunosorbent assay (ELISA). As expected from our experience, both dimer and monomer bound to F-protein in a dose-dependent manner (Supplementary Fig. 2), indicating that the dimer retained activity. Since dimer IgG should retain at least two free \( F_{ab} \) domains and the conformation is the same as in the monomer as will be described later, it was surmised that the dimer could bind to F-protein via the free \( F_{ab} \) domains.

For quantitation of covalently and non-covalently associated dimers, SEC in the presence and absence of SDS has been commonly utilized. In the presence of SDS, however, the separation between monomer and dimer tends to be poor due to the increased Stokes radius of the protein due to SDS-binding and unfolding (8). The column used in the presence of SDS may not be used any longer in the absence of SDS due to the fact that it is not easy to completely remove the SDS. In the present study, AUC-SV was employed to quantitate the amount of covalently and non-covalently associated dimer species. AUC-SV has higher resolution capacity than SEC and the separation between the monomer and dimer species in AUC-SV was scarcely affected by the presence of SDS (Fig. 2B). In addition, SDS does not affect the AUC cells as AUC does not utilize carrier or gel. If an adequate amount of sample for AUC-SV measurement is obtainable, AUC-SV in the presence of SDS has advantages in quantitation of covalently associated species compared with SDS–SEC.

**Conformation of Palivizumab dimer species**

The conformations of the dimer species were evaluated by a number of methods including far- and near-UV CD spectroscopy, intrinsic fluorescence spectroscopy and measurement of surface hydrophobicity of the protein. All the results indicated that the conformation of dimer species was almost identical to that of monomer species (Fig. 6A–D). This finding is consistent
with the result observed in the Epratuzumab dimer species by UV and CD (7). In the present study, DSC analysis was also employed to assess the conformation of dimer species. Although we expected that the technique might detect the difference in thermal stability between the dimer and monomer, no significant difference was observed by DSC (Fig. 6E). All together, these results indicated that the dimer was very similar to the monomer in higher order structure and thermal stability. Therefore, the dimer species is likely to have no significant effect on immunogenicity and stability of biopharmaceutical during storage. Under the conditions of the present study, no significant amounts of trimer or larger aggregates were observed. Larger aggregates of antibody have been reported to be generated under thermal or physical stress; in such cases, altered conformation has been observed (9, 10). Therefore, it is important to minimize stress during the manufacturing process to avoid aggregation.

Conclusion
In the present study, we investigated the intermolecular interactions and conformation in the dimer species of Palivizumab, a monoclonal antibody (IgG1), in order to elucidate the physical and chemical properties of the dimerized pharmaceutical antibody. The results indicate that approximately half of the dimer is non-covalently associated, whereas the other half is dimerized through covalent bond including disulphide bond and dityrosine. Dimerization is likely to occur mainly via the $F_{ab}$ region. Furthermore, the conformation and thermal stability of the dimer turned out to be very similar to those of the monomer. This study indicated that although major features of dimerization appear to be common among the IgG1 antibodies, the details in intermolecular interactions can vary; for example, Palivizumab dimerization takes place mostly via the $F_{ab}$ region, whereas Epratuzumab dimerization occurs through $F_{c}$ as well as $F_{ab}$.

Supplementary Data
Supplementary Data are available at JB Online.

Funding
This work was supported by Grant-in-Aid for Scientific Research (C) (No. 23570190) from the Ministry of Education, Culture, Sports, Science and Technology of Japan to F.A.

Conflict of interest
None declared

References