Characterization of mAb dimers reveals predominant dimer forms common in therapeutic mAbs

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ABSTRACT

The formation of undesired high molecular weight species such as dimers is an important quality attribute for therapeutic monoclonal antibody formulations. Therefore, the thorough understanding of mAb dimerization and the detailed characterization mAb dimers is of great interest for future pharmaceutical development of therapeutic antibodies. In this work, we focused on the analyses of different mAb dimers regarding size, surface properties, chemical identity, overall structure and localization of possible dimerization sites. Dimer fractions of different mAbs were isolated to a satisfactory purity from bulk material and revealed 2 predominant overall structures, namely elongated and compact dimer forms. The elongated dimers displayed one dimerization site involving the tip of the Fab domain. Depending on the stress applied, these elongated dimers are connected either covalently or non-covalently. In contrast, the compact dimers exhibited non-covalent association. Several interaction points were detected for the compact dimers involving the hinge region or the base of the Fab domain. These results indicate that mAb dimer fractions are rather complex and may contain more than one kind of dimer. Nevertheless, the overall appearance of mAb dimers suggests the existence of 2 predominant dimeric structures, elongated and compact, which are commonly present in preparations of therapeutic mAbs.

Introduction

Monoclonal antibodies (mAbs) are a growing class of therapeutic proteins that have a substantial impact in the treatment of oncology, inflammatory and immunological disorders and neurodegenerative diseases. During manufacturing, storage and distribution, the antibody is exposed to different stress conditions such as mechanical stress, light exposure, temperature differences, pH variations and contact to different surface materials. Numerous studies investigated the effect of these stress conditions on the formation of aggregates in biopharmaceutical formulations. Despite extensive efforts to minimize negative effects on the molecules, the formation of undesired high molecular weight species (HMWs) and aggregates cannot be avoided completely. Protein aggregation is therefore an important quality attribute that may have an effect on potency and pharmacokinetics. The presence of protein aggregates in pharmaceutical formulations is often discussed as a potential risk factor for the generation of immune responses. However, a recent study in transgenic mice suggests that the immunogenic potential of small soluble oligomers is rather low and correlates with the degree of chemical modifications within the primary sequence as only high levels of chemical alterations in combination with oligomerization induced a strong immune response in the present mouse model. Thus, a better understanding of the structure and function of small oligomers and aggregates is generally required for future development of therapeutic proteins.

Protein aggregation is initiated by self-association of 2 monomers forming a dimer, which may further accumulate into larger HMWs and aggregates. These protein-protein interactions can be either reversible or irreversible. For example, reversible dimerization of rhuMAb VEGF was described as an equilibrium reaction between native monomer and dimer.

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Abbreviations: AUC, analytical ultracentrifugation; CE-SDS, capillary electrophoresis with sodium dodecyl sulfate; D_max, maximum particle size; DTT, dithiothreitol; HIC, hydrophobic interaction chromatography; HMW, high molecular weight; IEC, ion exchange chromatography; IgG1, immunoglobulin G1; mAb, monoclonal antibody; MW, molecular weight; R_g, radius of gyration; r_h, hydrodynamic radius; RT, retention time; SAXS, small angle X-ray scattering; SEC-MALS/QELS, size exclusion with multi angle light scattering/quasi elastic light scattering; TEM, transmission electron microscopy

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In contrast, the formation of irreversible aggregates is often induced by non-native monomeric forms susceptible to self-association.\textsuperscript{17} The loss of native protein structure is related to external stress and might lead to exposure of hydrophobic patches, which are normally buried in the interior of the native protein.\textsuperscript{18,19} Upon formation of aggregates of various size and shape, these aggregation-prone areas are shielded from the aqueous environment by inter-molecular interactions between monomer molecules.\textsuperscript{15,20}

Dimeric forms of IgG molecules and mAbs have been described previously in literature. Recently, dimers of IgG1 and IgG2 have been found to occur naturally in human serum, although in low amounts.\textsuperscript{21} Several studies investigated the nature of dimer formation in therapeutic mAb formulations. For palivizumab dimers, the co-existence of non-covalent and covalent dimers involving both Fab and Fc domains was demonstrated.\textsuperscript{22} Heterogeneity concerning the mode and domain of interaction was also shown for the dimeric form of epratuzumab, another IgG1.\textsuperscript{23} Further studies have confirmed the presence of both covalently and non-covalently linked dimers in different therapeutic mAbs.\textsuperscript{24} In addition, more homogenous mAb dimer fractions were described as the result of exclusively non-covalent interactions via the Fab domains.\textsuperscript{24-26} These divergent findings indicate a complex nature of dimer formation depending on different factors such as primary structure, protein concentration, formulation conditions and the nature of stress conditions.

In this study, dimer fractions of several IgG1 mAbs were isolated from unstressed and temperature-induced bulk material by a set of chromatographic techniques. The dimer samples were characterized separately with regard to size, surface properties, chemical nature and overall structure. The findings indicate that mAb dimer populations are more complex than previously thought, and may contain more than one dimeric mAb species. Based on our results, elongated and compact dimers that represent predominant dimeric structures common in therapeutic IgG1 mAb preparations were identified. Elongated dimers were mainly detected as process-by-products or due to temperature induction, whereas compact dimers were found in bulk material stored at $-20^\circ$C for long periods. The combination of limited proteolysis and single particle analysis allowed for localization of putative dimerization areas on the Fab-domains of dimeric mAb fragments.

**Results**

**Heterogeneity of mAb dimers**

Three clinically tested IgG1 mAbs (mAb\textsubscript{A}, mAb\textsubscript{B} and mAb\textsubscript{C}) were measured by tandem-size-exclusion chromatography (SEC) for homogeneity of HMWs. Fig. 1a shows the obtained elution profiles and the zoomed view into the region of interest where HMWs are eluting (RT: 22–31 minutes). The peaks in the retention time window between 26 and 29 minutes represent mAb dimers. The asymmetric peak shapes with merging shoulders and several peak maxima indicate heterogeneity within the dimer fractions. Here, the dimers are called dimer1 and dimer2 according to their elution order in SEC. The ratio between the 2 dimers varied between the different mAbs: in mAb\textsubscript{A}, the early eluting dimer1 was the predominant species whereas mAb\textsubscript{B} and mAb\textsubscript{C} showed a larger content of late-eluting dimer2. The total dimer content differed between mAbs in the range of 0.9% for mAb\textsubscript{C} and 2.8% for mAb\textsubscript{A}. In the case of mAb\textsubscript{A}, the peak around 24–25 minutes retention time represented larger oligomers. To provide insights into the structural characteristics of dimer populations, the crude dimer mixtures of mAb\textsubscript{C} were separated from monomer and analyzed with transmission electron microscopy (TEM; Fig. 1b). The sample showed non-uniform distributions of mainly dimeric structures besides a minor population of monomers and higher oligomers (Fig. 1b, left panel). The observed dimers exhibit more elongated...
Physico-chemical characterization of mAb dimer species

Dimers of mAb_A and mAb_B were isolated from bulk solution containing a mixture of monomer, dimers and other species using preparative SEC (data not shown). As this method was not able to separate the individual dimers to high purity, an analytical ion exchange chromatography (IEC) was performed with the isolated dimer mixtures. Narrow fractionation windows were chosen to obtain solutions of the different dimers with highest possible purity. For purity assessment, dimer1 and dimer1* can be discriminated from dimer2 on a tandem-SEC by slightly different retention times (Fig. 2a and Table 1). According to SEC, purity between 70% and 90% was achieved for all dimers. As all dimer samples show only small monomeric content after reinterception of the purified dimer solutions on the SEC column (Fig. 2a), the stability of dimers is sufficient for purification under various conditions. The structural integrity of the dimer samples after purification was verified by SEC analysis, where the purified dimers exhibit the same retention times as the dimers in the starting material. The corresponding chromatograms of mAb_B dimer1 and dimer2 are shown in Fig. S3.

Different analyses were performed to further assess dimer1 and dimer2 of mAb_A and mAb_B. Peptide mapping did not reveal any significant chemical modifications of the different dimers compared to the corresponding monomeric mAbs (data not shown). Due to material limitations, capillary electrophoresis with sodium dodecyl sulfate (CE-SDS) analysis was only performed for dimer1, dimer1* and dimer2 of mAb_B. Dimer1 and dimer2 exhibited mainly non-covalent interactions as they dissociated predominantly into monomeric units when analyzed by non-reduced CE-SDS (Fig. S4 and Table S1). In contrast, dimer1* was only dissociable in the presence of SDS and the reducing agent dithiothreitol (DTT), and still exhibited a residual amount of species that was non-dissociable under these conditions. This finding indicates that dimerization in the temperature-induced dimer1* occurs mainly via disulfide-linkage of 2 monomers, whereas the association of dimer1 and dimer2 is mainly non-covalent.

SEC-MALS/QELS

Tandem-SEC coupled to multi-angle light scattering was used to measure the absolute molecular weight of the isolated dimers (Fig. 2a). All dimers were found to have a molecular weight in the range of about 300 kDa, yielding 2 possible explanations for the different retention times in SEC: 1) different interactions with the stationary phase; and 2) different hydrodynamic properties. To address this question, quasi-elastic light scattering was used to compare the hydrodynamic properties of the proteins analyzed (Table 1). For mAb_A, the hydrodynamic radius of dimer1 was larger than for dimer2 (r_h of 6.4 nm vs 5.4 nm), indicating a more extended molecule shape. The same tendency was reported for mAb_B with dimer2 (r_h of 5.8 nm) exhibiting a smaller hydrodynamic radius than dimer1 or dimer1* (r_h of 6.1 nm and 6.8 nm, respectively). This referred to a more compact overall shape for dimer2 and a rather elongated form for dimer1/1*. These results explain the observed elution order on the SEC.

IEC

Ion exchange chromatography was conducted to further assess the surface charge properties of the different dimers (Fig. 2b). All dimer samples showed one main peak indicating the presence of a major dimer species that exhibited more basic features compared to the corresponding monomeric mAbs. For mAbs A and B, dimer1 eluted earlier than dimer2 in IEC, which implies a more basic character of dimer2 compared to dimer1. The temperature-induced dimer1* of mAb_B showed the same elution behavior in IEC as the original dimer1. The elution profiles of all dimer samples indicated the presence of small impurities such as monomeric mAb. This might be due to insufficient purification procedures or minor dissociation of dimers under the conditions applied.

Hydrophobic interaction chromatography

The surface hydrophobicity of the dimers was analyzed by hydrophobic interaction chromatography (HIC; Fig. 2c). For both mAbs, the corresponding monomers eluted first in HIC, followed by dimer1/1* and dimer2, indicating the most hydrophobic surface properties for dimer2. A main peak representing the corresponding dimer species was identified for each dimer sample. However, small amounts of monomer and other impurities were detected in HIC profiles. This slight heterogeneity might result from incomplete dimer isolation. In addition, the high-salt conditions used in HIC might impair the stability of dimers, leading to changes in quaternary structure such as dissociation into monomers.

Analytical ultracentrifugation

The different dimer samples were further characterized by analytical ultracentrifugation (AUC; Fig. 2d). For mAb_A and mAb_B, the composition of all dimer species was comparable to the values obtained by SEC (Table 1). The sedimentation coefficients $s_{20,w}$ and the frictional ratios $f/f_0$ clearly differed for dimer1 and dimer2.
in both mAbs. As the molecular mass is identical for various dimeric assemblies of a given sample, the observed different frictional ratios can be attributed to different shapes of the 2 dimers. For both mAbs, dimer2 had the highest sedimentation coefficient in AUC experiments followed by dimer1 and dimer1/C3 and the corresponding monomers. Dimer2 of mAb_A and mAb_B exhibited the most compact dimensions with the smallest frictional ratios. In contrast, dimer1 showed the highest frictional ratio for both mAbs indicating the most elongated molecular shape. For dimer1/C3 of mAb_B, slightly different values were obtained which was likely due to greater sample heterogeneity.

**Structural characterization of mAb dimers**

**TEM**

The isolated dimers of mAb_A and mAb_B were analyzed with TEM to gain further insights into their specific structural
properties (Fig. 3). For both mAbs A and B, the main species detected on the raw images of the dimer1 samples are elongated dimers, with a small amount of monomers and other higher oligomers (Figs. 3a and 3c, left and middle panel). The class averages showed several elongated dimeric sub-classes that were all formed by connecting 2 monomeric subunits via one dimerization site at the tip of one mAb domain (Figs. 3a and 3c, right panel). Most sub-classes showed a linear linkage; however, some slightly kinked dimeric structures were also observed, implicating sample heterogeneity and domain flexibility. For mAb_B dimer1/C3, similar elongated dimeric forms were identified in the class averages besides a small sub-class that revealed 2 contact sites (Fig. 3e). For dimer2 of mAb_A and mAb_B, more compact and entangled dimer structures were detected in the raw images (Figs. 3b and 3d, left and middle panel). Monomers, some elongated dimeric structures and other oligomers were found as minor impurities, which correlates with the slight heterogeneity observed in IEC and HIC. Several sub-classes for these compact dimeric structures that differ slightly in their morphology (Figs. 3b and 3d, right panel) became evident during class averaging. In all sub-classes, 6 domains representing

### Table 1. Parameters of mAb_A and mAb_B dimers in SEC-MALS/QELS and AUC.

| mAb_A          | Dimer1 | Dimer2 | Dimer1/C3 |
|----------------|--------|--------|-----------|
| RT [min]       | 33.0   | 29.2   | 26.9      |
| Area [%]       | 94     | 89     | 80        |
| MW [kDa]       | 150.0  | 299.7  | 274.5     |
| r₄ [nm]        | 4.3    | 5.4    | 6.8       |
| Area [%]       | 91     | 87     | 77        |
| s_{20,w} [S]   | 6.55   | 10.11  | 9.00      |
| f/f₀           | 1.53   | 1.49   | 1.54      |
| r₄ [nm]        | 5.3    | 6.3    | 6.3       |

| mAb_B          | Monomer| Dimer1 | Dimer1* |
|----------------|--------|--------|---------|
| RT [min]       | 32.2   | 26.8   | 26.9    |
| Area [%]       | 95     | 80     | 80      |
| MW [kDa]       | 141.5  | 281.3  | 274.5   |
| r₄ [nm]        | 4.5    | 6.1    | 6.8     |
| Area [%]       | 99     | 82     | 77      |
| s_{20,w} [S]   | 6.45   | 9.21   | 9.00    |
| f/f₀           | 1.56   | 1.62   | 1.54    |
| r₄ [nm]        | 5.4    | 6.9    | 6.3     |

![Figure 3](image-url)
2 single mAbs could be clearly identified; however the interaction point(s) of the 2 molecules could not be assigned exactly and are probably not limited to a single dimerization site. In some sub-classes of mAb_A dimer2, the localization of the Fc domains could be estimated due to slightly different appearances of the individual domains (Fig. 3b, right panel, arrows).

**Small angle X-ray scattering**

The adsorption of the sample for TEM analyses leads to preferred molecule orientations on the carbon grid by maximizing the contact surface. Therefore, in solution small angle X-ray scattering (SAXS) analysis was performed as a complementary method (Fig. 4). Due to material limitations, the experiment was conducted with the isolated dimers 1* and 2 of mAb_B. The overall parameters derived from the scattering profiles are summarized in Table S2. The molecular weight estimates (320±50 kDa) agreed well with the estimation of the SEC-MALS measurement. Although the scattering curves of dimer1* and dimer2 overlapped well with each other at higher angles (s > 1.5 nm⁻¹), significant differences were revealed at lower angles (Fig. 4a). This observation suggests that the quaternary structures of the dimers differ from each other, whereas the tertiary structures with 2 monomers building up each dimer are similar. The differences between the dimers became even more evident in the distance distribution functions (Fig. 4b). Both the radius of gyration $R_g$ and the maximum particle size $D_{max}$ of dimer1* exceeded significantly those of dimer2 (Table S2). In addition, the dimensionless Kratky plot of dimer2 showed a bell-shaped curve with a peak position at $q = \sqrt{3}$, typical for globular particles, whereas the Kratky plot of dimer1* exhibited a plateau in this q range, as expected for extended and possibly flexible proteins (Fig. 4c).

*Ab initio* shape analysis was performed to gain further insights into the structural properties of the 2 dimers 1* and 2 of mAb_B in solution (Fig. 4d). As apparent from the TEM figures for dimer1* and dimer2 (Fig. 3d and 3e), it is feasible to impose a P2 symmetry for calculating SAXS models of the different dimers (Fig. 4d). The averaged reconstruction of dimer1* appeared to be very elongated, looking nearly like a stick with a protrusion, whereas the shape of dimer2 was much more compact. As evident from the rotated view, both models looked rather flat and displayed approximately the same thickness. The obtained unbiased models of dimer1* and dimer2 are consistent with the specific low resolution structures visualized by TEM analyses of mAb_B and with the overall shape parameters from...
light scattering and AUC, such as $r_h$ or $f_0$. In addition, the larger values for $R_g$ compared to $r_h$ are in agreement with the elongated and potentially flexible structures of both dimers.

**Localization of the dimerization site**

Dimers 1, 1* and 2 of mAb_B were analyzed concerning their specific dimerization sites. All dimers were digested with papain, resulting in 100 kDa and 50 kDa species in SEC. The isolation of the 100 kDa species followed by ESI-MS-analysis clearly revealed the presence of Fab domains in the dimerized fragments, whereas Fc domains were rarely identified (data not shown).

To obtain more detailed information about the localization of the dimerization sites on the Fab domains, limited proteolysis followed by negative-stain TEM of the isolated dimerized fragments was performed. Due to material limitations, only dimer1* and dimer2 of mAb_B were digested with FabRICATOR® and papain (Figs. 5 and 6, respectively). The use of FabRICATOR® produced dimerized F(ab')2-F(ab')2 fragments that clearly differ for dimer1* and dimer2 (Figs. 5a and b, respectively). F(ab')2 of monomeric mAb_B is shown for comparison in Fig. 5a. In the raw image of dimer1*, elongated F(ab')2-F(ab')2 fragments were identified that all show one dimerization site at the tip of one Fab domain (Fig. 5a). These structures could also be identified in the full-length dimer1* structures (see Fig. 3e). Class averaging was not possible for this sample due to flexibility of the individual domains mediated by the hinge regions. For dimer2, the dimeric F(ab')2-F(ab')2 fragments exhibit several conformations. Several propeller-like structures that are probably connected by the hinge regions were detected (Fig. 5b, marked with yellow box). A second type of dimeric F(ab')2-F(ab')2 fragments showed 2 F(ab')2 subdomains interacting in an antiparallel manner via the base of 2 Fab domains (Fig. 5b, marked with blue box). Furthermore, a third type of dimeric F(ab')2-F(ab')2 fragments was identified that adopts a cross-like form (Fig. 5b, marked with red box). Class averaging was possible indicating greater rigidity of dimeric F(ab')2-F(ab')2 fragments of dimer2 compared to the elongated structures of dimer1*.

In the case of papain-digested dimer1*, class averaging was feasible, identifying several sub-classes of elongated dimeric Fab-Fab structures with the 2 Fab subunits interacting either in linear or in kinked fashions (Fig. 6a). Fig. 5b shows a single Fab domain for comparison. All dimeric Fab-Fab fragments of dimer1* were formed involving the tips of the Fab domains similar to the F(ab')2-F(ab')2 fragments obtained with FabRICATOR® (see Fig. 5a) and the full-length structures of dimer1* (see Fig. 3e). For the dimeric Fab-Fab fragments generated from papain digest of dimer2, compact structures were observed in the raw image, producing several sub-classes during class-averaging (Fig. 6b). In contrast to the elongated Fab-Fab fragments of dimer1*, the compact Fab-Fab structures of dimer2 involve the base of the individual Fab domains, which are connected in an antiparallel manner. These antiparallel dimeric Fab-Fab motifs were present in some of the F(ab')2-F(ab')2 fragments (see Fig. 5b, blue box); however, they were difficult to localize within the full-length compact dimer2 structures (see Fig. 3d).

**Discussion**

All analyses presented here were carried out using fractions of dimer1 and dimer2 of mAb_A and mAb_B and a temperature-induced dimer1* of mAb_B, which were all sufficiently stable in non-denaturing buffer solutions such as SEC mobile phase to be purified. Although all dimer samples exhibit minor impurities of monomer, other dimers and oligomers in varying amounts, purity was sufficient for detailed characterization based either on size (SEC, QELS, AUC, SAXS), surface properties (IEC, HIC), chemical identity (MALS, MS, CE-SDS) or structure of single particles (TEM). The rather complex dimer samples made the analyses challenging and required cross-validation with a set of orthogonal methods. Table 2 provides a qualitative summary of selected dimer properties.

Two dimer forms, dimer1 and dimer2, were identified; these are sufficiently different in molecule size and molecule
The existence of overall elongated and compact dimers observed in different mAbs of the IgG1 might be responsible for the overall elongated and compact framework residues probably contribute to dimerization and structural methods. To form such compact dimer structures, the individual domains of the mAb monomers have to be bent enormously. This internal flexibility is mediated by the hinge region, which in turn might be involved in dimerization (see discussion below) as published previously.24 Compact dimers2 of mAb_A and mAb_B were identified in bulk material stored for a long time at −20°C. During freezing, local elevated concentrations of the mAb itself or of excipients, salts or buffer components might induce protein aggregation as described in Liu et al.27 Furthermore, the storage temperature of −20°C might favor the crystallization of trehalose present in formulation buffer out of a supersaturated state, which has been shown to accelerate aggregate formation.28,29 However, also other unidentified factors might influence the formation of compact dimers.

The presence of elongated dimers in different mAb formulations was described previously by different studies. An elongated dimer was assigned a probable solution in SAXS measurements of dilute mAb solutions.30 Furthermore, molecular dynamics simulation detected an elongated dimer as lowest energy minimum under certain conditions.31 Elongated mAb dimers that are similar to those described here and were generated by process and light stress have been analyzed previously using TEM analysis.35 As elongated dimer structures are observed after exposure to different stresses, the formation of elongated mAb dimers is rather common in various mAbs and under various conditions.

Interestingly, the temperature-induced dimer1 of mAb_B exhibits similar surface properties and similar elongated overall shapes as the dimer1 that is generated as a process-byproduct (see Figs. 2 and 3). The only identified difference between these 2 elongated dimer structures is the nature of the chemical linkage: dimer1 is associated mainly non-covalently whereas dimer1 is mainly formed by disulfide bridges. The cysteines involved in dimerization of dimer1 could not be identified as profiles of non-reducing peptide mapping of dimer1 were comparable to the corresponding monomer (data not shown). It is likely that the known cysteine residues bridge 2 different monomers instead of the same monomer, resulting in an intramolecular Cys-Cys linkage that would mimic the typical intramolecular bonding in a monomeric mAb. Remarkably, the formation of disulfide bonds within dimer1 does not seem to alter the overall molecule form compared to dimer1 as both dimers exhibit similar elongated molecule dimensions in electron microscopy. Due to this, the use of dimer1 as a mimic for dimer1 in some of the analyses presented here is justifiable. As dimer1 is generated upon storage at 25°C for several weeks without any exposure to light, it is feasible that disulfide bridges might break and reform under these accelerated degradation conditions. In addition, especially dimer1 contains other covalent species that are stable even in the presence of SDS and DTT. The co-existence of covalent and non-covalent mAb dimer species has been reported previously,22–24 and contributes to the complex nature of mAb dimer solutions.

### Table 2: Qualitative summary of different dimer properties.

| Species    | Basicity | Hydrophobicity | r, f, f | s_{20,W} | f/f_0 |
|------------|----------|----------------|--------|----------|-------|
| Monomer    | +        | +              | +      | +        | +     |
| Dimer1/*   | ++       | ++             | ++++   | ++       | ++++  |
| Dimer2     | +++      | +++            | ++     | +++      | +     |

Figure 6. Structural analyses of the dimeric Fab-Fab fragments obtained by papain digestion of mAb_B (A) dimer1 and (B) dimer2 by negative stain transmission electron microscopy. Left, overview image showing well distributed particles, 4 of which are highlighted (white boxes). Insets: First column, 2x enlarged views of the 4 representative particles highlighted in the corresponding overview. Second column, 4 representative class averages, each containing about 200 particles. (A) For the Fab-Fab fragments of dimer1, the projections are 16 nm long and are connected by the tip of the individual Fab domains. (B) The Fab-Fab fragments of dimer2 exhibit compact shapes with dimensions of 13 nm in length and are connected via the base of the Fab domains in an antiparallel manner. Scale bar: 100 nm; all inset boxes are 30 by 30 nm.
The morphology in TEM analyses of dimer1/1* clearly differs from dimer2 for mAbs A and B and allows the assignment of overall elongated and compact dimer forms, respectively, in agreement with the solution data obtained in SEC-MALS/QELS, AUC and SAXS measurements. In contrast to these bulk methods, which analyze the averaged ensemble of molecules in solution, the use of transmission electron microscopy is able to display single particles, and thus can provide greater sample details. The thorough analyses detected several TEM sub-classes for the elongated and compact dimer structures (Figs. 1b and 3). This might at least partly be due to internal flexibility between Fab and Fc domains of a monomeric mAb molecule, but might also be attributed to intrinsic dimer heterogeneity that becomes even more evident after protease cleavage and subsequent TEM analysis of the different fragments needed for dimerization (see discussion below). As several different interaction sites are identified for the elongated as well as for the compact dimers, it is likely that the formation of dimers is not a completely selective process, which would result in only one unique interaction site. On the basis of TEM analyses, schematic models of several possible elongated and compact dimer forms are shown in Fig. 7a.

**Localization of the dimerization site**

The use of limited proteolysis is an efficient procedure to reduce rather complex mAb dimer molecules to smaller interacting subunits by depleting fragments not involved in dimerization. The subsequent electron micrograph imaging of the interacting subunits leads to a more detailed view of dimerization areas, although molecular detail is still missing. To our knowledge, this is the first report showing that approximate dimerization site(s) on Fab domains can be localized on different dimerized F(ab')2-F(ab')2 and Fab-Fab structures visualized with TEM (see Figs. 5 and 6). Despite sample heterogeneity, all elongated dimeric subunits obtained by protease digestion of dimer1* exhibit one interaction site at the tip of the Fab domain. It is likely that the observed kinked and linear fragments show a slightly different molecular basis for dimerization; however, it might be located within or near the complementarity-determining regions, as described for 2 other IgG1 dimers.18,26 This position might influence the bioactivity and antigen-binding of full-length elongated dimers, and remains to be further investigated.

Analyses of dimeric Fab-containing fragments of dimer2 display several different overall structures and at least 2 different dimerization sites. The propeller-like dimeric F(ab')2-F(ab')2 sub-units are connected by the hinge region and are lost after papain digest. Hydrogen-deuterium exchange experiments coupled to mass spectrometry with monomeric mAb_B as reference detected the hinge region as a possible interaction site for full-length dimer2 (data not shown). In agreement with our results, the hinge region was identified as a putative dimerization site in another IgG1 antibody.24 The second dimerization site is conserved during papain digestion and is presumably located within the constant regions comprising the base of individual Fab domains. In contrast to dimer1*, the tip of the Fab domains seem to be freely accessible in dimer2; however, the exact molecular basis and the impact on bioactivity require further evaluations. Schematic representations of possible dimeric F(ab')2-F(ab')2 and Fab-Fab subunits of elongated and compact dimers are depicted in Fig. 7b and c.

In summary, dimerization of mAbs results in a limited number of predominant overall structures, namely elongated and compact dimers. As these elongated and compact conformations are
detected in different mAbs of the IgG1 type, it is likely that they represent common overall dimer forms, which can develop under various relevant conditions. The thorough characterization of these mAb dimers concerning size, surface properties, chemical identity and overall structure requires the combination of bulk and single particle methods to address the entire complexity of mAb dimer fractions. Both elongated and compact dimer fractions display certain heterogeneity with regard to particle flexibility and dimerization sites. The involvement of conserved regions of the Fab domains and the hinge region in dimerization is apparently required in order to form these favored dimeric overall structures, although molecular details remain to be elucidated. The impact of these different dimer species on safety and efficacy is of great interest for future pharmaceutical development of therapeutic antibodies. The evaluation of defined and intensively characterized dimer fractions with regard to their bioactivity, pharmacokinetics and immunogenicity would greatly contribute to understanding of the potential criticality of dimers in therapeutic mAb formulations.

Materials and methods

Description of the mAbs

The three mAbs A, B and C used in this study are humanized IgG1s. The antibodies show high sequence identities between heavy and light chain (at least 87% and 79%, respectively). The Fc regions are identical, whereas the Fab domains exhibit sequence identity of at least 77%. The individual amino acid content of the Fab domains does not indicate any uncommon distribution (Table S3). The structural stability of all 3 mAbs assessed with differential scanning calorimetry lies within the range of a conventional IgG1 (data not shown). mAb_A, mAb_B and mAb_C contain the same number of cysteine residues that are all involved in the typical disulfide bridging of an IgG1. No additional cysteine residues are present in the complementarity-determining regions.

Purification of mAb dimers

mAb_A dimer was purified from monomer as described in Paul et al.25 The dimer content of individual fractions after preparative SEC (mobile phase 51 mM NaH₂PO₄, pH 6.2) was evaluated by SEC as described in the SEC-MALS/QELS section below. Fractions containing dimer1 or dimer2 as major components were pooled separately and concentrated for IEC (VivaSpin2, MWCO 50 kDa, Sartorius) as specified in the Ion exchange chromatography section below. Fractions were collected using MAPII robot (Bruker Daltonics) coupled on-line to an Alliance 2695 HPLC system (Waters).

mAb_B bulk solution was concentrated by ultrafiltration/diafiltration using a Centrimate T-Series cassette (190 cm², Pall Life Science) and an Omega polyethersulfone membrane (MWCO 30 kDa, Pall Life Science) on an ÅKTA crossflow system (GE Healthcare) to a concentration of 100 mg/mL in mobile phase A (20 mM His, 200 mM NaCl, pH 6.0). For the crude separation of mAb_B dimer from mAb_B monomer several preparative SEC runs on a HiLoad 26/600 Superdex 200 pg with mobile phase A were carried out on an ÅKTA Explorer system (GE Healthcare). Individual fractions were tested for their dimer content by SEC similarly as described for SEC-MALS/QELS (see section below) before fractions containing dimer1 or dimer2 as major components were pooled separately. Both dimer samples were prepared for IEC using buffer exchange by concentration (VivaSpin2, MWCO 50 kDa, Sartorius) and addition of mobile phase A (50 mM MOPS, pH 6.8). The conditions and parameters of IEC separation of dimer1 and dimer2 are described in detail under Ion exchange chromatography in this section. Fractions were collected using MAPII robot (Bruker Daltonics) coupled on-line to an Alliance 2695 HPLC system (Waters).

To induce dimer formation, mAb_B bulk solution was stored at 25°C for 20 weeks under protection from light. Dimer1 was then purified from the crude mixture as described before by using preparative SEC and IEC. Dimer1 obtained from the temperature stressed mAb_B solution is named dimer1*.

Bulk solution of mAb_C was loaded on preparative SEC column (HiLoad 16/600 Superdex 200 pg, GE Healthcare) to a concentration of 100 mg/mL in mobile phase A (20 mM His, 200 mM NaCl, pH 6.0) from 2% to 48% over a time range of 26 minutes. Medium scale separation of dimer1 from dimer2 was done by several injections of 200 µg of dimer mixture collecting fractions using a MAPII robot (Bruker Daltonics). For analytical assessment of surface properties, 50 µg of each purified dimer sample was injected and eluted as indicated above.

The samples of mAb_B were analyzed using a strong cation exchange column (PL-SCX 1000 A, 5 µm, 4.6 mm × 50 mm, Polymerlabs/Agilent Technologies) by diluting 1:5 with mobile phase A (50 mM MOPS, pH 6.8). The flow rate was set to 1 mL/min at a column temperature of 40°C. Elution was done on a Waters Alliance 2695 separations module by linearly increasing salt concentration to 23% mobile phase B (50 mM NaH₂PO₄, 500 mM NaCl, pH 6.0) from 2% to 48% over a time range of 26 minutes. Medium scale separation of dimer1 from dimer2 was done by several injections of 200 µg of dimer mixture collecting fractions using a MAPII robot (Bruker Daltonics). For analytical characterization of the purified dimer samples, 15 µg of dimer1 and 30 µg of dimer1* and dimer2 were applied to IEC and eluted with the gradient indicated.

Ion exchange chromatography

For mAb_A, both dimer samples were diluted 1:5 with mobile phase A (20 mM NaH₂PO₄, pH 6.0) before applying on a weak cation exchange column (ProPac WCX-10, 10 µm, 4 mm × 250 mm, Dionex/Thermo Scientific). Samples were eluted with a flow rate of 1 mL/min at a column temperature of 25°C by increasing mobile phase B (20 mM NaH₂PO₄, 500 mM NaCl, pH 6.0) from 2% to 48% over a time range of 26 minutes. Measured scale separation of dimer1 from dimer2 was done by several injections of 200 µg of dimer mixture collecting fractions using a MAPII robot (Bruker Daltonics). For analytical assessment of surface properties, 50 µg of each purified dimer sample was injected and eluted as indicated above.

Size exclusion chromatography with multi angle light Scattering and quasi elastic light scattering

SEC-MALS/QELS was performed as follows: 50 µg of each mAb sample was applied to 2 SEC columns (BioSuite™ HR, 250 Å, 5 µm, 7.8 mm × 300 mm, Waters) connected in a row. Separation was performed using an Agilent System (1100 Series) and the mobile phase 200 mM KH₂PO₄, 250 mM KCl,
Hydrophobic interaction chromatography
HIC analysis was prepared by diluting each mAb sample 1:5 to 1 mg/mL in mobile phase A (20 mM Tris, 1.5 M (NH₄)₂SO₄, pH 7.0). Thirty μg of dimer1* and 15 μg of dimer1 and dimer2 were injected on a TSKgel® Butyl-NPR column (Tosoh Biosciences) and separated using a Waters Alliance 2965 HPLC system. The start condition of 80% mobile phase A was reduced linearly over 36 minutes to 100% mobile phase B (20 mM Tris, pH 7.0). Flow rate was set to 0.5 mL/min at a column temperature of 25°C.

Analytical ultracentrifugation
Sedimentation velocity experiments were performed at 50,000 rpm (An60-Ti rotor) and 20°C on a XLI analytical ultracentrifuge (Beckman Coulter) with SedVel60k centerpieces (SpinAnalytical). The samples contained 0.8 mg/ml protein in the irradiated sample. For both dimers 5% (v/v) glycerol was added to the sample to minimize the radiation damage.25 20 successive exposures of 30 ms exposure time were collected and compared to detect and discard the radiation damage effects. The data were normalized to the intensity of the transmitted beam and radially averaged; the scattering of the buffer was measured at 280 nm. The data were analyzed with the program Sedfit32 to calculate c(s) sedimentation coefficient distributions. In the case of data set 2 of mAb_B of dimer1 and dimer2 were collected using the automated data pipeline SASFLOW.36

Transmission electron microscopy
For TEM experiments, the antibody stock solutions of non-digested and digested dimers were diluted to 3.5 μg/ml and 2 μg/ml respectively with D-PBS (Gibco Life Technologies). Four μl of diluted samples were adsorbed for 60 s to glow-discharged parlodion carbon-coated copper grids. The grids were then blotted, washed on 5 drops of double-distilled water and negatively stained on 2 droplets of 2% uranyl acetate (pH 4.3) solution. Samples were imaged at a nominal magnification of 130000X using a Phillips CM10 electron microscope (Philips, Eindhoven, The Netherlands) operating at 80 kV. Electron micrographs were recorded on a 2000 by 2000 pixel charge-coupled device camera (Veleta, Olympus soft imaging solutions GmbH, Münster, Germany) mounted in the 35 mm port of the TEM, yielding a final pixel size of 0.38 nm on the specimen level. Reference-free alignment was performed on manually selected particles from raw micrographs using the EMAN image processing package.33 A total of 300 to 3000 particles of each imaged samples (monomers or dimers) were extracted from the micrographs, aligned, and classified by multivariate statistical analysis yielding class averages. The class averages with the best signal-to-noise ratio were presented in Figs. 1b, 3, 5b, and 6. When sample heterogeneity and domain flexibility prevented automated class averaging (Fig. 5a), images of selected particles from raw micrographs were low pass filtered to enhance the contrast and finally manually contoured to produce interpretable views.

Small angle X-ray scattering
SAXS data of mAb_B of dimer1 as well as dimer2 were collected at the PETRA III, P12 beamline (DESY synchrotron, Hamburg).34 Using a PILATUS 2M pixel detector at a sample-detector distance of 3.1 m and at an energy of 10 keV (λ= 1.24 Å), the range of momentum transfer 0.01 < s < 0.45 Å⁻¹ was covered (s = 4π sin(θ)/λ, where 2θ is the scattering angle). The initial stock solutions in D-PBS (Gibco Life Technologies) were measured at 24 mg/ml and 28 mg/ml, respectively, as well as concentration series hereof. For the final calculations, data measured at c₁ = 3 mg/ml (dimer1*) and c₂ = 3.5 mg/ml (dimer2) were used. The linearity of the Guinier plots and the overall parameters determined showed that there were no traces of aggregates/higher oligomeric species detected at these concentrations. The automated sample changer was employed to load the samples and pump the sample through the observation capillary during the exposure period to constantly remove the irradiated sample. For both dimers 5% (v/v) glycerol was added to the sample to minimize the radiation damage.25 20 successive exposures of 30 ms exposure time were collected and compared to detect and discard the radiation damage effects. The data were normalized to the intensity of the transmitted beam and radially averaged; the scattering of the buffer was subtracted and the difference curves were scaled for protein concentration. These primary data processing steps were performed using the automated data pipeline SASFLOW.36

SAXS analysis was performed using various programs of the ATSAS 2.6 package (http://www.embbl-hamburg.de/biosaxs/download.html).37 The forward scattering I(0) and the radius of gyration r_g were extracted from the Guinier approximation calculated with the AutoRG function within PRIMUS.38,39 These parameters were also computed from the entire scattering patterns using the indirect transform package GNOM,40 providing the pair distribution function, p(r), and the maximum size D_max of the particle as well. To evaluate the flexibility of the 2 dimer species, dimensionless Kratky plots were generated.41,42 The molecular weight (MW) of the solute was evaluated by comparison of the forward scattering with that from a reference solution of bovine serum albumin. The MW estimations were cross-validated using the particle excluded (Porod) volumes as previously described.37 The ab initio reconstructions were generated with the program DAMMIF.43 To check the stability of the solution 10 independent DAMMIF runs were performed for each dimer and were superimposed onto each other by SUPCOMB.44 Using DAMAVER common structural features were determined and a starting model was derived for a final run of DAMMIN.45 Models were calculated with and without imposing symmetry (P2). Model representations displayed in the figures were generated with Pymol Molecular Graphics System (Schrödinger, LLC).
CE-SDS analysis was performed on a Beckman Coulter Capillary Electrophoresis System Proteome Lab PA800 in the presence of SDS. All samples were labeled with 3-(2-furyl)-quinoxine-2-carboxaldehyde (Molecular Probes) or 5-carboxytetramethylrhodamine succinimidyl ester (Sigma Aldrich) for fluorescence detection. For reducing conditions, DTT was added and samples were incubated at 70°C for 10 minutes. All samples were injected to pre-conditioned capillary (40°C) with 5 kV for 20-40 seconds. The system was used under reverse polarity mode using a run voltage of 15 kV.

**Protein digestion with FabRICATOR®**

Purified fractions of monomer, dimer1* and dimer2 of mAb_B were digested with FabRICATOR® (Genovis AB) according to manufacturer’s instructions. mAb solutions were present at 5 mg/mL in D-PBS, pH 7.4 (Gibco Life Technologies). One unit FabRICATOR® was used per 1 µg protein. The solution was incubated for one hour at 37°C before it was applied to SEC using Superdex™ 200 10/300 GL column (GE Healthcare) that separated the crude protein mixture into 200 kDa dimeric F(ab')2-F (ab')2 fragments and residual heavy chain fragments. For both dimer fractions, monomeric F(ab')2 fragments were also present. D-PBS (Gibco Life Technologies) was used as mobile phase.

**Protein digestion with papain**

Purified fractions of monomer, dimer1* and dimer2 of mAb_B were digested with papain (Roche Diagnostics) in a ratio of 100:1 (w/w) in the presence of 1 mM L-cysteine (Sigma Life Science) using digestion buffer (100 mM Tris, 4 mM EDTA, pH 7.4). The solution was incubated for 2 hours at 37°C before the reaction was stopped by adding antipain (Sigma Life Science) in 2-fold (w/w) excess. After 30 minutes incubation at ambient temperature, buffer was exchanged with PD MiniTrap™ G-25 columns (GE Healthcare) to mobile phase (20 mM NaH2PO4, 150 mM NaCl, pH 8.0) according to manufacturer’s instructions. The eluate was loaded on a HiTrapp™ ProteinA HP column (GE Healthcare) using AKTA Explorer system (GE Healthcare). The flow through containing dimeric and monomeric Fab fragments was collected and applied to a preparative SEC column (HiLoad™ 16/600 Superdex™ 200 pg, GE Healthcare) that separated the crude protein mixture into 100 kDa dimeric Fab-Fab fragments and 50 kDa monomeric Fab. For preparative SEC, D-PBS (Gibco Life Technologies) was used as mobile phase.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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