Aggregation During Biopharmaceutical Production of Antibodies Induced by UV Radiation and Interface Exposure

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Abstract

In the manufacturing process of therapeutic monoclonal antibodies (mAbs) aggregate formation is a crucial problem. Proteins tend to aggregate due to several stress conditions during processing and storage. This may reduce their efficacy and can result in dangerous side effects such as immunogenicity.

Two aggregation-prone monoclonal antibodies (mAb1 and mAb2) were chosen for this study. Computer based analysis of their primary structure using the algorithm TANGO confirmed the presence of several aggregation prone regions (APRs) in mAb1 and mAb2.

Both, ultra violet (UV) light and interface exposure are stress factors with major relevance in the manufacturing process of mAbs. Experimental model systems in 96-well format were developed to analyze and characterize aggregation of mAb1 and mAb2 induced by UV light and interface exposure. The small scale models enabled parallel screening of multiple conditions with minimal sample consumption.

UV irradiation has proven to be an efficient approach for virus inactivation. However, a major problem of this method is photooxidation or physical degradation of antibodies, which can result in aggregate formation. MAb1 and mAb2 were exposed to UVC irradiation on a transilluminator. Spectral characteristics and radiation dose were similar to that of a UVC UVivatec® GMP Lab system (Sartorius Stedim Biotech). UV exposed mAbs resulted in distinct size exclusion chromatograms different from unstressed antibodies. Ag-
aggregate and fragment formation increased with exposure time. High molecular weight bands (>150 kDa), observed in reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), imply the formation of covalent, non-reducible photochemical crosslinks. Mechanisms responsible for this linkage as well as photooxidation, deamidation and other covalent modifications were characterized by mass spectrometry (MS). The detected covalent linkages were classified into intra- and intermolecular crosslinks based on the computationally modeled distances between the reactive atoms. UV induced conformational changes of the mAbs were analyzed by Fourier transform infrared (FTIR) spectroscopy and 8-Anilinonaphthalene-1-sulfonic acid (ANS) fluorescence spectroscopy. Alterations of the tertiary structure were detected in both mAbs whereas their secondary structure remained unaffected.

During the production process therapeutic proteins are exposed to numerous solid-liquid interfaces as well as to the air-liquid interface. In this study, aggregation kinetics of mAb2 exposed to glass, stainless steel and air interfaces were recorded. UV absorption at 340 nm and 280 nm were used to determine the turbidity and to calculate the aggregation index, the latter being a measure of the fraction of aggregates in the sample. Sigmoidal aggregation kinetics were obtained, which depended on the material and the interface area. Monomer and oligomer fractions were measured by size exclusion high performance liquid chromatography (SE-HPLC). The quick aggregation process prevented the detection of soluble aggregates. Insoluble aggregates were analyzed by fluorescence microscopy. For all materials aggregate size increased with exposure time and interface area. The steel interface resulted in the fastest aggregation kinetics. Aggregation rate as well as aggregate size were much higher compared to glass- and air-induced aggregation processes. Structural analysis of interface-stressed mAb2 revealed alterations of the secondary structure induced by steel and glass. The secondary structure of air-stressed mAb2 remained unchanged, though.

Three solvent additives, Tween 20, trehalose and trimethylamine oxide (TMAO)
were analyzed for their ability to inhibit interface-induced aggregation. Aggregation kinetics was recorded and the aggregate size, amount and structure were analyzed. Tween 20 completely prevented protein aggregation at the air interface whereas it was only moderately protective at glass and steel interfaces. Trehalose inhibited only glass-induced aggregation and to some extent aggregation at the steel interface. The methylamine TMAO showed no protective effect but in contrast increased aggregate formation.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>AAPS</td>
<td>American Association of Pharmaceutical Scientists</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AI</td>
<td>aggregation index</td>
</tr>
<tr>
<td>ANS</td>
<td>8-Anilinonaphthalene-1-sulfonic acid</td>
</tr>
<tr>
<td>APR</td>
<td>aggregation prone region</td>
</tr>
<tr>
<td>ATR</td>
<td>attenuated total reflection</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>Bis-ANS</td>
<td>4,4’-Dianilino-1,1’-binaphthyl-5,5’-disulfonic acid dipotassium salt</td>
</tr>
<tr>
<td>CDC</td>
<td>complement-dependent cytotoxicity</td>
</tr>
<tr>
<td>CDR</td>
<td>complementary-determining region</td>
</tr>
<tr>
<td>cGMP</td>
<td>current good manufacturing practice</td>
</tr>
<tr>
<td>CH</td>
<td>constant region of the heavy chain</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>C_L</td>
<td>constant region of the light chain</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micelle concentration</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>F_ab</td>
<td>fragment antigen binding</td>
</tr>
<tr>
<td>F_c</td>
<td>fragment crystallizable</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FTIR</td>
<td>fourier transform infrared</td>
</tr>
<tr>
<td>GMP</td>
<td>good manufacturing practice</td>
</tr>
<tr>
<td>HC</td>
<td>heavy chain</td>
</tr>
<tr>
<td>HMW</td>
<td>high molecular weight</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IC</td>
<td>internal conversion</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IND</td>
<td>Investigational New Drugs</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>LC</td>
<td>light chain</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>Ph. Eur.</td>
<td>European Pharmacopeia</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
</tr>
<tr>
<td>SE-HPLC</td>
<td>size exclusion high performance liquid chromatography</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>melting point</td>
</tr>
<tr>
<td>TMAO</td>
<td>trimethylamine oxide</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopeia</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet–visible</td>
</tr>
<tr>
<td>$V_H$</td>
<td>variable region of the heavy chain</td>
</tr>
<tr>
<td>$V_L$</td>
<td>variable region of the light chain</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Aggregation of therapeutic proteins

In the last three decades therapeutic proteins play an increasingly significant role in the pharmaceutical industry [1]. Protein drugs are rapidly developed for the treatment of a variety of human diseases like diabetes, immune disorders, infections or different forms of cancer [1, 2]. However, a major concern of proteins is their high propensity to aggregate [1, 3–5]. Protein aggregation may occur during any stage of the manufacturing process, like fermentation, purification, formulation and storage. Process conditions as well as storage conditions influence the aggregation mechanisms, the rate of aggregate formation and the aggregate species generated. Protein aggregates may cause adverse effects in the patient. They can impact drug efficacy due to reduced biological activity [6–9]. In addition, protein aggregates potentially stimulate immune responses or neutralize endogenous proteins with essential biological functions, which may become life-threatening.

To ensure the safety and efficacy of therapeutic proteins, aggregation must be reduced to an acceptable level. Therefore, full characterization of protein aggregates is necessary [3, 5]. All aggregate species have to be quantified and the origin, cause and mechanism of aggregate formation should be identified and understood [3–5]. For the analysis of the great diversity of aggregates the use of a variety of orthogonal analytical techniques is mandatory [3].
1.1.1 Different types of aggregates and their classification

In the pharmaceutical quality control characterization of protein aggregates is an important issue. For a long time protein aggregates were simply defined as multimeric species of higher molecular weight, such as oligomers. However, since several types of protein aggregates are known, researchers developed a more detailed classification scheme [5, 10–13]. Today, protein aggregates are most commonly classified based on the four categories (Table 1.1): (1) conformation, (2) linkage, (3) reversibility, and (4) size [5, 10, 13].

<table>
<thead>
<tr>
<th>Category</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>conformation</td>
<td>native</td>
</tr>
<tr>
<td></td>
<td>non-native</td>
</tr>
<tr>
<td>linkage</td>
<td>covalent, reducible</td>
</tr>
<tr>
<td></td>
<td>covalent, non-reducible</td>
</tr>
<tr>
<td></td>
<td>non-covalent</td>
</tr>
<tr>
<td>reversibility</td>
<td>reversible</td>
</tr>
<tr>
<td></td>
<td>non-reversible</td>
</tr>
<tr>
<td>size</td>
<td>soluble (&lt;100 nm)</td>
</tr>
<tr>
<td></td>
<td>subvisible (0.1-100 µm)</td>
</tr>
<tr>
<td></td>
<td>visible (&gt;50-100 µm)</td>
</tr>
</tbody>
</table>

1) Conformation
Protein aggregates can either show native or non-native structure [5, 10, 11, 13]. Native aggregates are assemblies of folded monomers. The high-order structure of the active proteins remains unaffected during the aggregation process [13].
Protein crystals are a special form of native aggregates [14].
The monomer structure of non-native aggregates however, is altered. One distinguishes misfolded, partially unfolded and unfolded aggregates [11]. Special forms of non-native aggregates are synucleins consisting of inherently disordered proteins and amyloids characterized by a cross-$\beta$ diffraction pattern [11].

2) Linkage
Aggregates can be formed through covalent crosslinking [11,13]. Reducible disulfide bonds are mainly responsible for this linkage. However, covalent crosslinks can also be non-reducible like thioethers or dityrosins [5,10,11,15]. Non-covalent aggregates interact through weak physical forces like hydrophobic, electrostatic, van der Waals interactions or hydrogen bonds [5,13]. The chemical linkage is indicative for the reversibility of protein aggregates [11].

3) Reversibility
Reversible protein aggregates dissociate into their monomers upon removal of the stress condition, which is responsible for aggregation and simultaneous treatment with a denaturant or reducing agent [11,13]. Self-association is a common mechanism in the formation of reversible aggregates. Reversible aggregates are mainly formed through non-covalent linkages [10,11,13] and mostly retain their native structure in the aggregated state [13]. They are assumed to exist in equilibrium with each other or with the native monomer [10,11,13]. Changes in the solution condition may result in a shift of equilibrium [10].
In comparison, irreversible aggregates retain their high-molecular weight structure when stress conditions are removed [11,13]. Dissociation of irreversible aggregates is kinetically inhibited [13].
4) Size

Small aggregates which are smaller than 100 nm and are able to pass a 0.22 µm filter are defined as soluble [10, 11, 13]. They are neither visible to the naked eye nor by an optical microscope [10,13]. Soluble aggregates are oligomers (e.g. dimers, trimers) composed of different numbers of monomeric units [5,11,13]. The maximal monomer number still allowing the aggregate to remain soluble depends on the protein itself and the solvent composition.

Particles in a size range of 0.1 to 100 µm are defined as subvisible aggregates [11, 13]. Large subvisible aggregates may be visible through an optical microscope and probably to a trained eye [13]. Solutions containing such aggregates in sufficient concentration may turn turbid.

Visible aggregates are larger than 50 to 100 µm and are visible to the naked eye [11,13].

Most of the recent literature classifies aggregates according to these four categories. However, in 2012 Narhi et al. and the Focus Group "Protein Aggregation and Biological Consequences" of the American Association of Pharmaceutical Scientists (AAPS) focused on introducing a standardized nomenclature to describe protein aggregates. They added an additional category, the morphology [11]. This characteristic can be helpful to differentiate between different species of protein aggregates as well as between protein aggregates and other particles introduced during the protein production process.

The aggregate morphology can be characterized by properties such as the aspect ratio and the surface roughness and by optical parameters like the refractive index and the transparency. Furthermore, aggregates can differ in structure, which can be regular or amorphous, fiber like or spherical.

The type of generated aggregates is influenced by both the kind of protein as well as by the solvent or stress conditions [5,11–13]. These so-called induction
factors (see chapter 1.1.3) define the mechanism of protein aggregation and consequently the aggregate characteristic.

1.1.2 Proteins can aggregate by different mechanisms

Aggregation of proteins varies depending on protein properties as well as on environmental conditions (e.g. buffer, container and temperature). A protein can follow one or a combination of several mechanisms to form aggregates. Four major aggregation mechanisms can be distinguished [4,12,16]: (1) Protein self-association, (2) Aggregation of conformational altered monomers, (3) Aggregation through chemical modification, (4) Surface-induced aggregation (Figure 1.1).

Figure 1.1: Overview of the most important aggregation mechanisms:
1) **Protein self-association**
Proteins can aggregate without any conformational changes directly from their native state [4]. Aggregation is induced through self-complementary surfaces on the protein [12]. In a single protein multiple patches can be involved in this process [4]. Electrostatic, hydrophobic as well as van der Waals interactions are responsible and trigger the formation of reversible oligomers (see chapter 1.1.1, 3) Reversibility). At high concentration this aggregates grow over time and finally may serve as precursors for irreversible aggregates [12].

2) **Aggregation of conformational altered monomers**
Conformational changes or partial unfolding of proteins may be a trigger for aggregate formation [12]. Usually, in a protein solution an equilibrium exists between the native protein and the altered monomer [4]. The native form is not likely to aggregate whereas intermediates and unfolded proteins show a strong affinity to each other [12].
External stresses like heat or pH-shift may induce conformational changes of the protein [4,12]. This favors protein aggregation due to increased flexibility and the exposure of hydrophobic patches [4]. The aggregate size increases with time and with increasing size the solubility of the aggregates decreases [4]. Conditions which stabilize the native monomer or the addition of solvent additives can inhibit aggregate formation [12].

3) **Aggregation through chemical modification**
Aggregation can also be promoted by chemically modified proteins [4,12]. Chemical degradation as well as crosslink formation result in a change of the physical properties of the protein [4]. Alteration of its hydrophobicity, electric charge and structure may increase the tendency for protein aggregation [4,16]. Chemical degradation processes can be a result of oxidation, deamidation, dimerization or proteolysis [4,12]. Furthermore, variants of the native monomer due to missing glycosylation or the formation of intermolec-
ular crosslinks like disulfide bonds or dityrosins may induce protein aggregation [4,12,16]. Usually, the aggregation propensity is increased in a modified protein [12]. However, in some cases a modification can improve the protein stability and therefore decreases the tendency for protein aggregation.

4) Surface-induced aggregation
All kinds of surfaces or interfaces are potential triggers for aggregate formation (see Chapter 1.1.3.2) [16–18]. The native protein interacts with these surfaces by hydrophobic or electrostatic interactions. This may result in a conformational change of the protein and induce protein aggregation [12,17,18]. The altered monomer can aggregate either directly at the surface or after being released and removing back in solution [12,18].

Aggregation kinetics
Protein aggregation reactions often follow a nucleation-growth mechanism. The nucleus, by definition, is the aggregation state of a protein with the highest free energy $\Delta G$ (Figure 1.2) [19,20]. Once this energy barrier has been overcome, aggregates will grow spontaneously. The reaction kinetics of the nucleation-dependent aggregation shows a sigmoidal course, which can be divided into two parts [4,12]: In the lag phase, also called nucleation step, nuclei of critical size ($r^*$) are formed. The duration of this initial step depends on solution and surface conditions [4]. In the so-called growth phase, which is autocatalytic, the nucleus rapidly grows by addition of monomers. The formation of visible particles is characteristic for this mechanism, small oligomers are rare [12].
Figure 1.2: Schematic representation of the free energy change $\Delta G$ of aggregate formation as a function of the aggregate size $r$ [20].

1.1.3 Induction factors of protein aggregation

Protein aggregation is influenced by a variety of factors [21]. They can be classified as internal and external factors and affect the aggregation rate, the aggregation mechanism as well as the type of aggregates formed [4,21]. Internal factors are defined by the protein structure and include protein glycosylation, charge distribution and hydrophobicity. Aggregation prone regions of monoclonal antibodies are discussed in chapter 1.2 in more detail. External factors are characterized by the protein environment. During the manufacturing process of biopharmaceuticals therapeutic proteins are exposed to a variety of stress conditions. Process steps including fermentation, purification, filling, shipment and storage potentially induce protein aggregation [4,5,21]. Furthermore, solution conditions such as pH, salt type and concentration, protein concentration and the presence of impurities play an important role in the aggregation process [5,11,21,22].

Proteins are amphoteric molecules [21]. Their folded state is defined by the
charge distribution of the amino acids, which depends on the pH. Therefore, proteins are stable only in a narrow pH range [21, 22]. The pH affects electrostatic interactions which play a key role in aggregate formation. Thus, buffer systems are usually used to set an optimal pH. However, salt ions can interact with charged amino acid side chains and also influence the protein stability. Salts trigger aggregate formation depending on the type and concentration [23]. Kosmotropes, for example, stabilize the native protein but simultaneously decrease the protein solubility. This effect called salting-out results in increased protein aggregation. Chaotropes, on the other hand, induce salting-in behavior. The native protein is destabilized whereas the protein solubility is increased. Protein concentration affects the aggregation process due to its involvement in macromolecular crowding effects [5, 21, 24, 25]. Furthermore, it defines the effective volume available for each molecule and therefore, the probability of bi-molecular collisions [5, 25]. Impurities including other proteins, cells or particles may act as nucleation source which induce protein aggregation, as well.

Apart from solution conditions, environmental factors like temperature and UV-light and processes such as freeze-thawing and agitation are possible induction factors [5, 11, 21]. Additionally, solid-liquid interfaces between the protein solution and materials of fermenters, tubes, pumps and others or the air-liquid interface are further induction factors. The temperature mainly affects protein stability. Proteins are only stable in a narrow temperature range [4, 21, 22]. An increasing as well as a decreasing temperature leads to protein destabilization, partial unfolding and in consequence protein aggregation [4, 21, 22]. Aggregate formation is accelerated at elevated temperatures by effects such as reduction of activation energy, enhancement of hydrophobic interactions and increasing protein diffusion and frequency of molecular collision [4]. Under appropriate environmental conditions very low temperatures below the freezing point are often used for long time storage of proteins. By the restricted flexibility at these low temperatures proteins can retain their stability and bioactivity [4, 5, 21].
However, the freeze-thawing process itself is also a stress factor. It may induce protein aggregation due to the ice-water surface formed or local environmental changes like pH change or concentration increase, respectively \[4,21,26\]. The external induction factors UV-light and interface interaction in combination with agitation were investigated in this study. Hence, they are discussed in more detail in the following section.

1.1.3.1 UV light

Light, especially UV-light, has been shown to be a major degradation factor for proteins \[5,21\]. Proteins are sensitive to light exposure, which probably induces photooxidations and finally may result in photolysis or crosslink formation. Photooxidation is a critical process which can occur during the production process of biopharmaceuticals \[27\]. Protein detection by UV light, long term storage, packaging and delivery processes are only a few examples where photooxidation of proteins may occur. Proteins are susceptible to direct photooxidation (Type I) due to the presence of intrinsic chromophores \[28\]. Tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), cystine and cysteine (Cys) residues as well as the peptide backbone are main targets of direct photooxidation \[27–30\]. Each chromophore absorbs UV light in a specific spectral region (Table 1.2) causing electrons to be transferred to the first excited singlet state. Tryptophan, which has the highest molar absorption coefficient, absorbs light in the near-UV region between 280-305 nm \[27,30,31\]. Despite of its low occurrence, Trp is the major point of photooxidation in proteins.
1.1. Aggregation of therapeutic proteins

Table 1.2: Intrinsic chromophores of proteins with their absorption in the near-UV region [27,30,31].

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Absorption wavelengths [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>280-305</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>260-280</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>240-270</td>
</tr>
<tr>
<td>Cystine</td>
<td>250-300</td>
</tr>
<tr>
<td>Peptide backbone</td>
<td>180-230</td>
</tr>
</tbody>
</table>

The excited state species can eject an electron from the chromophore (direct photoionization) or transfer their energy to other groups [27,28]. Energy transfer to oxygen can induce the formation of peroxy radicals or singlet oxygen (indirect photooxidation: Type II) [27,28,30]. Both react with the amino acids to form photoproducts [27,28]. Reaction of peroxy radicals with methionine for example was shown to form methionine sulfoxide. Reaction with cysteins results in the formation of sulfones and cysteine sulfonic and sulfinic acids and tryptophan-based peroxy radicals react to photoproducts like kynurenine and N-formylkynurenine (Figure 1.3). The tryptophan photoproducts kynurenine and N-formylkynurenine are critical because they serve as photosensitizers to visible light and lead to further protein damage. Energy is also likely to be transferred from tyrosine or phenylalanine to a nearby tryptophan [27] or from tryptophan to disulfide bridges which result in photolysis and eventually in the formation of thiolates or thyl radicals [30].
Chapter 1. Introduction

Figure 1.3: UV induced modification pathways of tryptophan [27]

Photooxidation can be accompanied by changes in the protein structure and altered physical and chemical properties. This may result in protein unfolding, aggregation through crosslink formation and/or fragmentation [28, 30]. The pathway of light-induced protein damage, the kind of photoproducts generated and possible structural alterations depend on the reaction conditions [27, 28, 30]. External factors like pH, ionic strength, temperature and protein concentration of the solution, protein characteristics like hydrophobicity, structure or nearby amino acids and the wavelength of the excited light affect the photooxidation process [27, 28, 30].

The only method to completely prevent photooxidation is to protect the protein from light during production and delivery [27, 29]. In addition to a well-designed packaging material, packaging in an inert atmosphere, as well as the addition of additives like methionine, which act as peroxide scavenger, may be possible methods to reduce photodamaging of proteins.
1.1.3.2 Interface exposure

Proteins come in contact with a variety of materials during the manufacturing process. The adsorption of proteins to an interaction surface is defined by the surface hydrophobicity and charge distribution as well as by the surface energy and morphology [18]. Hydrophobic surfaces are most critical. Proteins show a higher affinity to hydrophobic surfaces than to hydrophilic surfaces [17, 18]. Air, for example, is the most important hydrophobic surface in the production process of therapeutic proteins [17, 32, 33]. Solid materials like steel and glass are also often used in biopharmaceutical industry. Glass is a rather hydrophilic surface, whereas steel shows a more hydrophobic character [34].

In the initial stage of protein adsorption protein-interface interaction is the major driving force [17, 18]. The protein orientates in such a way that electrostatic attraction is maximal. With increasing surface coverage protein-protein interactions become increasingly dominant. Both, protein-protein interaction as well as protein-interface interaction influence conformational rearrangements [18]. If the native protein comes in contact with a surface, protein-interface interactions as well as entropic forces cause structural reorganization of the protein [18, 35]. The protein spreads at the surface and changes its secondary structure to the maximal favorable conformation. This stabilizes the protein at the surface but, on the other hand, affects the biological function of proteins and may induce protein aggregation [18, 32]. Protein aggregates can be formed either directly on the surface mediated by cooperative adsorption and strong protein-protein interactions or initial aggregation can be induced in solution. The protein clusters formed in solution subsequently stick to the surface where they spread and flatten [18].

The adsorption process of proteins at surfaces is influenced by the type of protein, the surface properties and external solution conditions [18]. Proteins are complex molecules which differ in size, structural stability and composition. Their complex structure and protein surface properties affect the interaction
with surfaces. Adsorption of proteins on surfaces is also affected by external factors [5, 17, 18]. The process is influenced by the protein concentration, the pH (Figure 1.4), the temperature and the ionic strength of the protein solution as well as by additives and mechanical stresses like agitation. The diffusivity of proteins in solution is accelerated at elevated temperatures and thus, the adsorption rate is increased [18]. Furthermore, adsorption is an endothermic, entropy-driven process [17, 18, 35]. Therefore, the amount of protein adsorbed at a surface is increased at higher temperatures. The solution pH determines the electrostatic state of proteins due to their amphoteric structure [18]. If protein and surface are oppositely charged, electrostatic attraction results in a high adsorption rate. However, the maximal amount of adsorbed proteins is reached at the isoelectric point where protein-protein repulsions are minimized [17, 18]. Electrostatic interactions between the protein and the surface as well as lateral interactions between proteins are lowered by salt ions [17, 18]. At a high ionic strength, protein adsorption to oppositely charged surfaces is reduced whereas adsorption to like-charged surfaces is enhanced [18]. Salts and organic solvents are often used as additives in protein solutions [17]. As already mentioned, salts reduce electrostatic interactions but enhance hydrophobic interactions. Organic solvents, on the other hand, enhance electrostatic interactions but reduce hydrophobic interactions.
1.1. Aggregation of therapeutic proteins

During the manufacturing process, proteins are often subjected to mechanical stress, like shaking, stirring or pumping. It was shown, that hydrodynamic shear alone does not or only rarely cause protein damage [5,36–38]. However, agitation enhances the interfacial damaging effect [5,36,39]. When proteins adsorb and unfold at a surface they can be mixed back into solution by agitation and the surface is free for the adsorption of further proteins. Thus, the interface is steadily renewed [33,36]. Furthermore, agitation accelerates the mass transfer of proteins by convection which increases the probability of protein-surface contacts.

Hydrophobic interaction is the major driving force for the adsorption of proteins to hydrophobic surfaces (Figure 1.5) [17]. The adsorption rate is influenced by the protein stability and the protein concentration. Proteins with low conformational stability are adsorbed more rapidly to hydrophobic surfaces than stable proteins. Labile proteins are more likely to unfold, which reduces the surface energy and increase the adsorption rate [17,35]. Low

Figure 1.4: Protein adsorption at the air interface depending on external factors: (A) low protein concentration, (B) pH 3, (C) pH–pI (based on [17]).
protein concentrations, on the other hand, reduce the adsorption rate. This results in an increased conformational and orientational change because proteins show a tendency to cover the interaction surface to reduce the surface energy (Figure 1.4) [17,18]. Adsorption at hydrophilic surfaces is driven by electrostatic attraction [17]. It is accompanied by less conformational changes but proteins adapt their orientation to maximize favorable surface-protein interactions [17,18,40].

\[
\begin{align*}
\text{Hydrophobic surface} & \quad \rightarrow \\
\text{Hydrophilic surface} & \quad \\
& 
\end{align*}
\]

Figure 1.5: Interface interaction of proteins at (A) hydrophobic and (B) hydrophilic surfaces.

The external induction factors discussed in this chapter and the internal factors stated in chapter 1.2 are only a selection of many more stress conditions which are known to induce protein aggregation. It is important to mention that these factors must not be regarded individually. Often, a combination of several stress conditions is responsible for the final aggregation process [21].
1.1.4 Solvent additives that prevent protein aggregation

1) Polyhydroxy compounds (sugars)
Polyhydroxy compounds such as sugars are stabilizing co-solvents (osmolytes), which stabilize the structure of native proteins without affecting their function [41,42]. Their protective effect increases with concentration [21,41,43,44] and is most effective at high concentrations between 0.3 M to 1 M [43,45,46]. In protein liquid formulations sucrose and trehalose are most commonly used [41,43,45]. Sugars show only limited or no specific binding to protein surfaces [21,41]. Their stabilizing effect is a result of preferential exclusion [41,45,46]. This mechanism is based on the fact that the interaction between sugars and proteins is thermodynamically less favorable than between water and proteins. Thus, sugars are preferentially excluded from the protein surface so that it is surrounded by a layer of water mainly without solvent additives (Figure 1.6).

Figure 1.6: Schematic overview of the mechanism of preferential exclusion. Sugar (black) in a aqueous solution (blue) is preferentially transferred through a imaginary membrane (green) and excluded from the protein (based on [41]).
As a result of preferential exclusion the chemical potential of the solution increases (Figure 1.7, left) [41,45,46]. The increase is proportional to the protein surface area as unfolding would increase the protein surface [41,45,46]. The solvent additive drives the protein into the folded state, thereby reducing the free energy of the system. Thus, in the presence of sugars the protein becomes stabilized. On the other hand, protein self-association results in a reduction of the overall protein surface area [41]. In this case, the addition of sugars would result in a decreased energy barrier between the native and the aggregated state. This theory suggests an enhanced aggregation in the presence of sugars (Figure 1.7, right) [47]. However, protein aggregation is generally induced by protein unfolding and thus, sugars mainly stabilize proteins by preferential exclusion. The higher melting point $T_m$ observed for proteins in the presence of sugars verified this assumption [41].

Figure 1.7: Energy diagrams of (A) protein unfolding and (B) protein aggregation from the native monomer state [41].

A further advantage of sugars is their positive effect in preventing oxidative damage [43]. In contrast, preferential exclusion of sugars may increase the
viscosity of protein solutions [45,48]. This has to be considered during formulation development.

2) Amino acids
Amino acids are a further group of commonly used solvent additives. Their stabilizing effect is not uniform but amino acids show rather different mechanisms [21,41,45]. Most of them stabilize proteins by preferential exclusion similar to sugars and other osmolytes [41,43,45]. However, direct binding, buffering capacity or antioxidant properties are further stabilizing mechanisms [45]. Histidine, glycine and arginine are amino acids commonly used as solvent additives (excipients) in drug formulations [41,45].

Histidine and glycine are both used as buffering agents to stabilize proteins [41,45]. Arginine, on the other hand, is not a protein-stabilizing additive. Its effect is not based on preferential exclusion or strong binding to proteins. It rather increases the solubility of proteins by being slightly chaotropic [21,41,49]. Thus, arginine is a highly effective aggregation suppressor [41,50]. The solubilization effect of arginine increases with concentration [41]. Concentrations above 0.1 M are most effective. Arginine shows weak affinity for most protein side chains and for the peptide bond [49]. This means, that arginine can reduce both, electrostatic and hydrophobic interactions. Its affinity for aromatic side chains is most pronounced [41,49]. The interaction is based on \( \pi-\pi \)- and cation-\( \pi \)-interactions and is therefore pH-dependent [41].

Arginine showed enhanced binding to the protein monomer due to its increased hydrophobic surface [41,51]. In solution arginine forms clusters through the alignment of its three methylene groups [41,51]. The increased hydrophobic surface of the arginine clusters enhances its interaction with hydrophobic patches of the protein surface. Arginine clusters mask the hydrophobic protein surface and suppress intermolecular interactions among aggregation-prone, unfolded proteins [41]. Consequently, the protein becomes solubilized [21,49]. At the same time, arginine is preferentially excluded from protein-protein com-
plexes and thus, destabilizes oligomers [21]. These two phenomena contribute to slowing down the protein aggregation process [21,41]. Furthermore, arginine positively affects high concentration formulations by reducing their viscosity [21,41,52].

3) Methylamines

Methylamines are naturally occurring osmolytes, which stabilize intracellular proteins [41,53]. Trimethylamine oxide (TMAO), for example, protects organisms from protein denaturation by environmental stresses [42,54,55]. It counteracts the destabilizing effect of urea [42].

The amphiphilic structure of TMAO (Figure 1.8) enables intermolecular interactions with both, water and protein molecules [42,54,55]. Therefore, enthalpic as well as entropic effects result in destabilization of the unfolded state and at the same time stabilize the native protein.

The three methyl groups of TMAO contribute to protein stabilization by the mechanism of preferential exclusion comparable to sugars (see chapter 1.1.4, 1)) [54,55]. The interaction of TMAO with the protein backbone is highly unfavorable whereas that between TMAO and apolar amino acid side chains is slightly favorable [42]. This strengthens backbone-backbone hydrogen bonds in proteins and results in chain compaction [54,55]. The oxygen atom serves as hydrogen bond acceptor and can interact with water molecules [54,55]. Thus,
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the enthalpic stabilization mechanism results from attenuated hydrogen bonds between water and protein molecules in the presence of TMAO [55]. This destabilizes especially the unfolded state due to its enhanced number of amide groups on the protein surface. Additionally, water-water hydrogen bonding is increased and an enhanced water structure is formed, which on the other hand, stabilizes the native protein [42].

4) Surfactants

Most proteins are surface-active molecules. Interface interaction may lead to activity loss, structural unfolding and aggregation [16,56,57]. To prevent or minimize surface adsorption and stabilize proteins against aggregation, surfactants are often added to biopharmaceutical drug formulations. Surfactants are amphipathic, surface-active molecules [16,58], which are effective even at very low concentrations (0.0003-0.3 % (w/v)) [41,46,58]. They can be divided into nonionic and ionic surfactants [21]. Currently, the nonionic polysorbate 20 (Tween 20) and polysorbate 80 (Tween 80) are most commonly used as solvent additives [16,44,46]. Polysorbates are biocompatible molecules which consist of a mixture of structurally-related fatty acid esters of polyoxyethylene sorbitan [16,58].

The amphipathic structure of surfactants defines their mode of action [16,58]. Surfactants show several mechanisms to prevent protein interface interaction. The driving force of all mechanisms is the hydrophobic effect and at high surfactant concentrations additionally van der Waal interactions [16,56]. Surfactants compete with proteins for surface adsorption [16,46,56,58]. They accumulate at the surface and prevent protein binding. The binding affinity of protein and surfactant to the surface depends on the surface properties, the surfactant properties and the protein. Especially, the surface hydrophobicity affects the protein adsorption process. Furthermore, the surfactant concentration plays a key role in this mechanism. Proteins are mainly stabi-
lized at surfactant concentrations just above the critical micelle concentration (CMC) [56]. Above the CMC all surfaces are saturated with a monolayer of surfactant molecules and micelles start to form (Figure 1.9). From this concentration protein adsorption at surfaces is minimized.

Direct binding of surfactants to proteins is a second mechanism [16,46,56,58]. The hydrophobic tail of surfactants binds to hydrophobic patches on the protein surface. The resulting surfactant-protein complexes show reduced surface affinity and protein-protein interactions are prevented. For this mechanism the molar binding stoichiometry between protein and surfactant defines the effective surfactant concentration rather than the CMC [16,56]. Additionally, binding of the surfactant to the native state increases the free energy of unfolding and stabilizes the native protein [46,56]. Surfactants may also act as chemical chaperons by binding transiently to partially folded proteins. This prevents intermolecular interaction and refolding is favored [56].

Figure 1.9: Surfactant adsorption to the air interface and to proteins at concentrations above the critical micelle concentration (CMC) (based on [16]).

On the one hand surfactants bring a lot of benefits in formulation of biopharmaceutical, but on the other hand they can cause serious problems. The ether linkages and unsaturated alkyl chains of polysorbates are prone to autooxidation [16,44,58]. Peroxides are formed, which might induce protein oxidation mainly at methionine and tryptophan residues. Furthermore, hy-
drolysis of the fatty acid ester bond as well as formation of reactive aldehydes can occur [16, 44, 58]. All these surfactant-induced damaging reactions are affected by environmental conditions like temperature, pH, light and the presence of metal ions or oxygen as well as by the surfactant concentration [58]. The degradation products of polysorbates destabilize or even damage proteins [21, 44, 56, 58]. Thus, polysorbates even increase aggregate formation and immunogenicity under unfavorable conditions [16, 44, 56, 58]. Due to these serious problems surfactant concentration should be kept to a minimum [56, 58].

1.1.5 Immunogenicity of protein aggregates

Protein aggregates, which are accumulated in therapeutic protein formulations, are potential inducers of an immune response in patients [59]. The immunogenicity of protein aggregates depends on a variety of factors [59–61]. Extrinsic factors include treatment related factors as the route and frequency of application, the immune status of the patient or concomitant medications and product related factors as the product origin, the presence of contaminants or the product formulation. The great diversity of proteins and generated protein aggregates influences the immune response as well. The immune response varies depending on different product related intrinsic factors. One factor is the aggregate size. High molecular weight aggregates $>$100 kDa seem to be more immunogenic than small aggregates [59, 62]. Especially multimerization was shown to play a major role in immunogenicity. Repetitive epitopes resemble the structure of microbial pathogens and are therefore highly immunogenic [59, 60]. Additionally, aggregates of proteins with a native-like conformation show a higher tendency for immune reactions [3, 59, 60]. Native-like aggregates trigger the formation of conformation-dependent antibodies, which may neutralize the monomeric product. Aggregates of denatured proteins, on the other hand, do not induce direct formation of neutralizing antibodies. The hydrophobicity as
well as the glycosylation pattern of the aggregates are further intrinsic factors which impact the immunogenicity. Considering the quantitative influence, it was shown that aggregates of relatively low abundance endogeneous proteins are more immunogenic than those formed from high abundance proteins [60].

Depending on their epitope structure aggregates induce different immunological mechanisms [59]. Neoepitopes, which are results of the aggregation process or cryptic epitopes, which are usually hidden in the native protein and become exposed by protein unfolding, induce the classical immune response. This fast and robust immune reaction is based on a T-cell dependent B-cell activation. Memory cell formation is induced and conformation-dependent antibodies with high affinity to the specific epitopes are formed. Conformation-dependent antibodies show no neutralizing effect due to their unspecificity to the native protein epitopes. However, the generation of neutralizing antibodies is facilitated by the presence of binding antibodies due to epitope spreading. During epitope spreading the epitope specificity is transferred to subdominant epitopes. Repetitive epitopes of protein aggregates induce another mechanism of immune response by breaking immune tolerance [3,59,60,62]. T-cell independent B-cell activation is characteristic for this process. B-cells are activated upon crosslinking of B-cell receptors by the repetitive epitopes. This results in a delayed formation of antidrug antibodies like neutralizing antibodies and a lack of memory cells [59]. Antidrug antibodies can crossreact with the monomeric product or its endogenous counterpart [59,60]. This may neutralize the efficacy of the product, alter its pharmacokinetics or induce immediate hypersensitivity such as anaphylaxis [59,63].

In summary, high order multimeric protein aggregates formed from native-like protein molecules which present repetitive epitopes are most immunogenic [59]. Thus, protein sequence analysis, the identification of epitopes and physico-chemical characterization to identify protein modifications are important tools to predict the immunogenicity of therapeutic proteins [61]. Additionally, an-
mal models can be used to induce the generation of binding and neutralizing antibodies, which offers a further opportunity to assess the protein immunogenicity [59,61].

### 1.1.6 Regulatory guidance - Aggregate specifications and limits

Biologics as biotechnology products or other products of complex natural source have to meet requirements to ensure its continued safety, purity and potency [63]. The requirements for biologics are regulated in “The Public Health Service Act” or “The Federal Food, Drug and Cosmetic Act”. According to these laws critical quality attributes have to be defined for each individual product and manufacturing process. Protein products are very challenging due to their inherent heterogeneity. Therefore, product monitoring and control is an essential part of lot release and stability studies. Lot-to-lot consistency, stability during distribution and storage as well as consistency in product quality, even after manufacturing changes, have to be proved. Protein aggregates can be formed at each stage throughout the manufacturing process, triggered by external and internal factors (see Chapter 1.1.3). They can potentially impact product yield, bioavailability, potency and immunogenicity. Thus, aggregation is defined as a critical quality attribute.

Aggregate assessment is an essential step of the regulatory process of therapeutic proteins [63]. The “Food and Drug Administration” and the “International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use” release several guidance documents to ensure product quality, safety and efficacy. Investigational New Drugs (INDs) have to conform to these guidelines to become approved and all manufacturing processes must comply
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with current good manufacturing practice (cGMP). Analytical methods for an accurate assessment of protein aggregates, for example, are listed in these documents. The methods need to be validated before their use. It must be demonstrated that the methods provide the required result and sensitively detect aggregates at each processing step. Methods for aggregate assessment have to be selected according to the nature and characteristics of the aggregates and the methods should not alter the product during sample preparation. Appropriate specifications and acceptance criteria for aggregates in therapeutics must be set and adapted during the product development process due to gained experience. However, up to now there are no specific acceptance criteria for protein aggregates officially defined [3, 62, 63]. In the United States Pharmacopeia (USP) and in the European Pharmacopeia (Ph. Eur.) only limits for visible and subvisible particulates are specified (≥10 μm: 6000 particles/container, ≥25 μm: 600 particles/container or ≥10 μm: 3000 particles/container, ≥25 μm: 300 particles/container depending on the analytical method used) [3, 64, 65]. Protein aggregates have to be monitored and characterized in each manufacturing step as part of product release and in stability studies [63]. Accelerated stability studies are performed to imitate long-term storage. In such studies the product is exposed to defined stresses and aggregation is monitored. Full characterization of all different aggregate species with only one analytical method is impossible. The use of orthogonal methods is necessary to detect the whole spectrum of aggregates. For monitoring aggregates >0.1 μm a variety of GMP-conform methods like size exclusion chromatography is provided. However, for aggregates between 2-10 μm only a limited number of GMP-methods is available.

Risk assessment of a therapeutic protein regarding protein aggregation is carried out based on the results received from the analytical methods. [63]. The products are assessed according to their inherent tendency to form aggregates during manufacturing and storage, the ability to capture and characterize all aggregate species and their potential to induce an immune response.
1.2 Monoclonal antibodies and their aggregation propensity

Antibodies or immunoglobulins (Ig) are recognition proteins which specifically bind an antigen [66, 67]. Each individual host possesses a great number of different antibodies. In humans they can be divided into five different classes (IgG, IgA, IgM, IgD, IgE) according to their biological activity. IgG antibodies, with their four subclasses are the most frequent antibodies in the blood. They are capable of entering tissues and can transfer the placenta to provide fetal protection. IgG antibodies coat antigens like bacteria or other target cells. This makes them more vulnerable for immune cells and speeds the attack and uptake by phagocytic cells. Furthermore, antigen-antibody complexes with IgG can attach to Fc receptors on B-cells and inhibit their activation. IgA antibodies, which possess two subclasses, are abundant in body fluids. In the serum, IgA exists as monomer whereas secretory IgA is dimeric. IgA antibodies protect the body from toxins and viruses which try to enter the body or cells. IgM is the largest antibody in humans. Once produced, the pentamer remains in the blood. Therefore, IgM is the antibody which initiates an immune response. The antigen-antibody complex of IgM activates the complement system and efficiently kills blood bacteria. The function of IgD is still unclear. The antibody, which is membrane-bound on the surface of B-cells, seems to regulate the immune response. In the blood IgE antibodies are found only in trace amounts. IgE triggers allergic reactions by binding to mast cells or basophils.

Despite of their numerous biological functions, the basic structure of immunoglobulins is similar for all antibody classes. The IgG structure is generally used as representative model for all antibody structures (Figure 1.10) [66,68]. IgG consists of four polypeptide chains, two identical light chains (LC – 23 kDa)
and two identical heavy chains (HC – 50 kDa), and has a total molecular weight of about 150 kDa. The heavy and the light chains are linked by disulfide bonds and other noncovalent interactions, which define the characteristic Y-shape of antibodies. The hinge region, where the arms and the stem are connected, assigns segmental flexibility to the antibody. The arms are responsible for the specific antigen binding. Therefore, these antigen-binding ends are also called F_{ab} fragments (fragment antigen binding). The stem, also called F_{c} fragment (fragment crystallizable), defines the biological properties of the antibody, like binding to cell-surface receptors or complement activation.

![Antigen-binding site](image)

Figure 1.10: Schematic structures of an immunoglobulin G molecule. yellow: disulfide bridge, green: carbohydrate.

Carboxyl-terminal ends of the polypeptide chains are highly conserved and are therefore called constant region. At the amino-terminal ends variable regions are located. However, the variable regions of heavy and light chain are not completely variable. Only the complementary-determining regions (CDRs), which compose about 25% of the variable region, are hypervariable. The heavy and the light chain possess three CDRs each and together they define
the specificity of the antigen-binding site. The regions between the CDRs, called framework residues, are highly conserved. The four polypeptide chains consist of several domains. The light chains possess one variable domain (V_L) and one constant region (C_L). The heavy chains also consist of one variable region (V_H) but three or four constant domains (C_H).

Antibodies are glycosylated to some extent [68]. The glycans affect the function of the immunoglobulin, depending on their location and specific structure. A highly conserved N-glycosylation site is located at asparagine 297 of the F_c part of IgGs and participate in the binding to FcγR. About 10-20% of all antibodies contain additional glycosylation sites which are located in the variable region. This glycosylation affects antigen binding.

1.2.1 Monoclonal antibodies

Antibodies of a single idiotype and with a predefined antigen specificity are called monoclonal antibodies (mAbs) [66]. Monoclonal antibodies play an important role in diagnostic and clinical applications. Today, 66 monoclonal antibodies are approved by the Food and Drug Administration (FDA) for the treatment of various human diseases [69]. The main application is the therapy of human malignancies [70]. Further clinical application fields of mAbs are for example the therapy of infectious diseases, transplantation and autoimmune diseases [70,71]. Diagnostic applications of mAbs include epitope specific immunoblotting, immunofluorescence and immunohistochemistry [70]. Almost all approved antibody drugs are IgGs because they possess desirable pharmacological characteristics [72]. The monomeric antibody shows a good solubility and stability. Furthermore, IgGs are highly specific and rarely form toxic metabolites. A further advantage is the binding of the IgG stem to F_c receptors [72–74]. Binding to FcγRs activates antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which are
important effector function for the destruction of pathogenic cells. Additionally, binding to FcRn contributes to the long half-life (21 day) of IgGs, which is a further reason for their use as therapeutic agent.

The production of monoclonal antibodies is based on the hybridoma technology developed by Milstein and Köhler in 1975 [75]. They produced mAbs using immortal B-cells which were fused with antibody forming B-cells from an immunized mouse [66]. This technology was adapted in the last years to reduce the high immunogenicity of mouse monoclonal antibodies in humans. Transgenic mice or recombinant antibody technologies, respectively, were developed to produce chimeric, humanized and fully human antibodies with reduced immunogenicity (Figure 1.11) [76, 77].

![Figure 1.11: Progression of antibody engineering from murine to fully human monoclonal antibodies. orange: mouse sequence, gray: human sequence, yellow: disulfide bridge, green: carbohydrate.](image-url)
1.2.2 Aggregation prone regions in monoclonal antibodies

Therapeutic proteins like monoclonal antibodies are exposed to several stresses during their production process (see chapter 1.1.3). This may impact the efficacy and quality of the product. Protein aggregation for example is a great concern due to its tendency to cause an immune response. The intrinsic stability of proteins and therefore their propensity to aggregate is coded in the protein’s sequence and molecular structure [78,79].

Nearly all proteins contain at least one aggregation prone region (APR) [80]. Thus, APRs are assumed to play an important role in protein function and folding. Furthermore, it was suggested, that protein folding and aggregation are driven by the same forces. In monoclonal antibodies 2-8 APRs per light chain/heavy chain pair were found [79]. They could be detected in all domains except of the C\textsubscript{H1} domain. In the variable region APRs are located primarily in the CDR loops or in the adjoining framework \(\beta\)-strands [78,79]. Thus, CDRs are suggested to promote self-association in the absence of an antigen. In the constant region APRs are highly conserved [79].

APRs are characteristic sequences of about 5-15 amino acid residues with an intrinsic aggregation propensity [78,80]. The sequences are rich in aromatic (Phe, Tyr, Trp) and hydrophobic, especially aliphatic (Ile, Val, Leu) \(\beta\)-branched residues [78,79]. Hydrophilic hydroxyl-containing Ser or Thr residues are also characteristic amino acids in APRs, whereas charged amino acids (Asp, Glu, Ly, Arg, His) are rare. The light chain CDR3 regions of monoclonal antibodies were shown to have an increased number of Gln and Asn residues [78,79]. Amino acids which are characteristic for APRs are also necessary for correct protein folding. In native proteins these amino acids are buried in the hydrophobic core [80]. However, APRs are conformationally unstable or flexible. They become exposed to the solvent due to unfolding or conformational alterations possibly induced by protein mutation or chemical modification like deamidation or oxidation caused by external stresses [78,79].
Today, therapeutic proteins are specially designed to minimize their aggregation propensity [78, 80]. Potential APRs are identified using computational tools like TANGO [78–83]. Based on this, the protein sequence can be adjusted in such a way that APRs are eliminated. The electrostatic properties of the protein are optimized to improve its solubility and native state stability [78]. For this purpose, nature-inspired strategies like the assembly of gatekeepers flanking APRs are useful tools [78, 80]. Gatekeepers are charged amino acid residues which disrupt hydrophobic regions and can be used to lower the aggregation propensity of proteins due to increased repulsive effects [80, 84].
1.3 Aim of the study

In the present study protein aggregation was investigated to understand the aggregation mechanisms induced by several stress factors typical for the manufacturing process of therapeutic proteins. The latter included the development of experimental setups as well as the selection and establishment of appropriate analytics.

Numerous studies have investigated aggregation of monoclonal antibodies. Aggregation depends on both, process related stress factors (external factors) as well as on the protein itself (internal factors). In the present study two common stress factors occurring in downstream processing, namely UV and interface exposure, were investigated. UVC light, for example, is used for virus inactivation in industry. Interface exposure is a common problem throughout the whole protein production process, from fermentation, through purification to filling and storage.

Experimental setups able to mimic those stress conditions at small scale were developed. The experiments were robust and lead to reproducible formation of antibody aggregates. Both, aggregation kinetics and structural properties of the generated aggregates were studied thoroughly. Analytical methods included various spectroscopic methods, as well as techniques to quantify aggregates belonging to various size classes. Covalent modifications and crosslinks induced by UV radiation were analyzed by mass spectrometry.

In a next step, different kinds of solvent additives were tested for their ability to reduce or prevent interface-induced aggregation. Their influence on aggregation kinetics, aggregate type and structure was analyzed.
2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

ANS Sigma-Aldrich
Bis-ANS Santa Cruz Biotechnology
Gel Filtration Standard Bio-Rad
PBS pH 7.2 Genaxxon bioscience GmbH
Protein Marker VI (10 - 245) prestained PanReac AppliChem
TMAO Sigma-Aldrich
Trehalose PanReac AppliChem
Tween® 20 Carl Roth

2.1.2 Antibodies

Two aggregation-prone monoclonal antibodies, mAb1 and mAb2, were studied. Protein A-purified mAb1 was kindly provided by Boehringer Ingelheim Pharma and was stored in phosphate buffered saline (PBS) pH 6.2 at 4°C at
a concentration of 50 mg/mL until use. mAb2 was produced in a CHO DG44 cell line [85,86]. Briefly, cells were cultivated in defined protein-free medium (SFM4CHO, Thermo Scientific) supplemented with 10 g/L glucose (Sigma-Aldrich) and 4 mmol/L L-Glutamine (Life Technologies). The antibody was purified by Protein A chromatography (MabSelectTM, GE Healthcare Bio-Sciences AB) and subsequently by mixed-mode chromatography (CaptoAdhere, GE Healthcare Bio-Sciences AB), filtered (0.2 µm) and stored in 50 mM phosphate buffer (Carl Roth) / 350 mM arginine (Merck Millipore), pH 6.2 at a concentration of 6.2 mg/mL. Both mAbs were diluted in 1x PBS to the appropriate concentration for each experiment.

2.1.3 Beads

For interface exposure investigation different surface materials were tested. Glass beads with a diameter of 2 mm (AR-Glas® (soda glass), Glaswarenfabrik Karl Hecht GmbH & Co KG) and stainless steel beads with a diameter of 1.4 mm (Type 316 stainless steel, Bio Spec Products Inc.) were used.

The hydrophobicity of the materials influences the aggregation process at interfaces. The contact angle is a measure for the hydrophobicity of solid materials and defines their wettability [34]. Hydrophilic materials show small contact angles. Thus, the liquid spreads over hydrophilic surfaces. On hydrophobic surfaces the liquid forms droplets. Hydrophobic materials are defined to have a contact angle $>90^\circ$. Materials tested in this study show different hydrophobicities. Soda glass has a contact angle of $<15^\circ$, steel a contact angle of 70-75°.

The surface area of the glass and steel beads were calculated from the number of beads $n$ and their diameters $d$:
\[ \text{surface area (beads)} = n \times \pi d^2 \] \hspace{1cm} (2.1)

Air interface area was approximated by assuming a hollow cylinder shape of the liquid during shaking with the air volume \( V \). The air-liquid interface therefore corresponds to the inner surface of the hollow cylinder. The height \( h \) of the microtiter plate (UV-Star® Microplate, Greiner Bio-One GmbH) after sealing with a CapMat (Greiner Bio-One GmbH) was set to 7 mm. The air interface area was calculated by:

\[ \text{interface area (air)} = 2 \times \sqrt{\pi \times V \times h} \] \hspace{1cm} (2.2)
2.2 Protein aggregation induction systems

2.2.1 UV irradiation

2.2.1.1 UVivatec® GMP Lab system

Virus inactivation is an essential step in downstream processing. One effective method to inactivate viruses is the use of UVC light at 254 nm [87–89]. This wavelength shows a high selectivity for deoxyribonucleic acid (DNA) versus protein absorption. However, some amino acids are still expected to absorb at this wavelength, potentially leading to photoreactions.

The UVC UVivatec® GMP Lab system (Sartorius Stedim Biotech) is a continuous-flow UVC reactor with a helical flow through a coiled channel around a mercury vapor lamp (254 nm) (Figure 2.1) [87–89]. It is claimed by the manufacturer to guarantee homogeneous sample irradiation and hence, effective virus inactivation (>4 log10) of small non-enveloped and larger enveloped viruses with minimal damage to the protein product.

Figure 2.1: (A) UVC UVivatec® GMP Lab system (Sartorius Stedim Biotech) and its (B) helical flow channel around the UV lamp [88].
2.2. Protein aggregation induction systems

Irradiation Procedure:

The UVivatec system was used to expose mAbs to UVC: The UVivatec system was first rinsed with 1x PBS. Then, 100 mL of mAb1 (1 mg/mL in 1x PBS) or mAb2 (0.1 mg/mL in 1x PBS) were pumped through the system at the minimal flow rate of 2 L/h and the maximal intensity of 8 W/m² (UV sensor: 20 mA). These settings correspond to the maximal achievable dose. Temperature control was set to 21 °C.

2.2.1.2 Experimental setup: Transilluminator

A model system at small scale was developed due to the large sample volume needed for the UVivatec system (Figure 2.2).

For small scale experiments 250 µL per well of 1mg/mL mAb1 or mAb2 were transferred into UV transparent microtiter plates (UV-Star® Microplate, Greiner Bio-One GmbH), placed onto a transilluminator (UVT-20 SM, Herolab GmbH Laborgeräte) and exposed to 254 nm light at maximal intensity for different time periods as indicated. Temperature of samples before and after irradiation was controlled.

Figure 2.2: Overview of the developed UV irradiation setup using a transilluminator.
2.2.2 Interface exposure

To investigate interface-induced protein aggregation an experiment in 96-well plates was developed (Figure 2.3). The effect of glass, steel and air on aggregate formation was studied.

Figure 2.3: Overview of the experimental setup for interface-induced aggregation.

MAb2 was transferred into 1x PBS using PD10 Desalting columns (GE Healthcare Life Sciences) and adjusted to a concentration of 0.5 mg/mL. Different numbers of glass or steel beads were placed into UV transparent microtiter plates (UV-Star® Microplate, Greiner Bio-One GmbH) (see Chapter 3.3.1, Table 3.4). The wells were filled up with antibody solution to a total volume of 280 µL so that no air space was left after sealing. For air-liquid interface
studies the sample volume in the wells was chosen to be less than 280 µL and no beads were added. A sample without beads and without air space (280 µL) was used as reference. For each sample a blank without antibody was added. The samples were prepared in triplicates, the blanks in duplicates. The 96-well plate was sealed with a CapMat (Greiner Bio-One GmbH) and shaken at 1200 rpm on an Orbit™ P2 Digital Shaker (Labnet International, Inc.) with 3 mm orbit size. Shaking was performed at room temperature for different time periods.

The experiment was repeated three times for each time point. The aggregation kinetics for each material was determined calculating the aggregation index (see chapter 2.3.5.1, equation 2.4). Aggregate characterization was performed using different analytical methods.

The same experiment was repeated by adding solvent additives. Trehalose (0.5 M), TMAO (0.5 M) or Tween 20 (0.5% (w/v)), respectively, were tested for their efficiency in inhibiting protein aggregation. The experiment was repeated two times for each time point.
2.3 Analytical methods

2.3.1 Total protein concentration determination

The total protein concentration of mAb2 after 32 h shaking was determined by bicinchoninic acid (BCA). The method is based on the reduction of Cu$^{2+}$ to Cu$^+$ in the presence of proteins in alkaline solutions [90]. The BCA specifically reacts with Cu$^+$ and forms a colorful complex with an absorption maximum of about 560 nm. The absorption of the samples at 560 nm was measured using the Infinite® M200 PRO NanoQuant (Tecan). For accurate determination of the protein concentration a calibration curve was recorded using unstressed mAb2 in 1x PBS at different concentrations between 0 and 1.0 mg/mL.

2.3.2 pH-measurement

In the interface project the pH of mAb2 (0.5 mg/mL in 1x PBS) was determined before and after 24 h shaking with 8 glass beads, 4 steel beads and 90 µL air. An Orion™ Versa Star Pro™ pH Benchtop Meter (Thermo Fisher Scientific Inc.) was used for the pH measurements. MAb2 shaken for 24 h without beads and air space served as control.

2.3.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique commonly used to characterize complex protein mixtures [91]. Sodium dodecyl sulfate (SDS), an anionic detergent, is used to unfold proteins by breaking hydrogen bonds and to give the protein a negative net
2.3. Analytical methods

charge [90,91]. Thus, the migration velocity of proteins in the polyacrylamide gel is directly proportional to the molecular mass. Reducing agents like dithiothreitol (DTT) are often added to break disulfide bonds and separate the protein into its subunits. To detect proteins Coomassie staining with a detection limit of about 0.1-1 µg is most commonly used [90].

Unstressed and UV-stressed antibodies were analyzed by SDS-PAGE on 6% Tris-Glycin gels (biostep GmbH) followed by Coomassie staining ( [92] modified). 6 µg antibodies/lane were loaded. The gel was run at constant voltage of 125 V. Bands were cut out for MS analysis.

UV-stressed and interface stressed mAb2 was analyzed by SDS-PAGE on 4-20% Tris-Glycin gels (Amersham ECL Gels, GE Healthcare Life Sciences) followed by Coomassie staining ( [92] modified). 3 µg antibodies/lane were loaded. The gel was run at constant voltage of 160 V.

For both gel types the Protein Marker VI (10 - 245) prestained (PanReac AppliChem) was used.

2.3.4 Size exclusion chromatography

Size exclusion chromatography (SEC) separates molecules according to their size [90,93–95]. The stationary phase media consists of a porous material of spherical particles [94]. The analytes dissolved in the mobile phase are eluted isocratically. They do not interact with the stationary phase but rather move through the matrix depending on their stokes radius [90,93,94]. The pore size and geometry of the matrix restrict the access of molecules. Large molecules are totally excluded from the pores and elute first. Smaller molecules can enter the pores and are retarded in the order of decreasing size.
Preparative SEC

Preparative SEC was carried out using an ÄKTApurifier 100 (GE Healthcare Europe GmbH). UV-exposed mAb2 was concentrated by ultrafiltration (Vivaspin 20, 50.000 MWCO PES, Sartorius AG) and separated (sample volume below 0.5 mL) on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Europe GmbH). 1x PBS was used as mobile phase. Protein was detected by absorption at 280 nm. The eluate was fractionated, and the fractions of each peak were pooled separately. After pooling, samples were concentrated again by ultrafiltration as above.

SE-HPLC

SE-HPLC was used to determine the molecular weight distribution of unstressed and stressed antibody samples. An Ultimate 3000 (Thermo Fisher Scientific) HPLC system controlled by Chromelone software (version 6.80, Thermo Fisher Scientific) was used for chromatography. Samples were filtered (0.2 µm) before separation by SEC. A MAbPac SEC-1G, 5 µm, 4 mm × 300 mm column with a pre-column (MAbPac SEC-1G, 5 µm, 4 mm × 50 mm) was used to analyze samples. 1x PBS was used as mobile phase and the flow rate was set to 0.2 mL/min. Protein was detected by absorption at 280 nm. Protein concentration was calculated from peak areas using an absorption coefficient of 1.4 L/(g·cm) for both antibodies.

2.3.5 Spectroscopy

2.3.5.1 UV-Vis spectroscopy

In UV-Vis spectroscopy the absorption of light in the ultraviolet range (100-400 nm) and in the visible range (400-700 nm) is measured [90]. Proteins exhibit several chromophoric groups like the peptide bond and aromatic side
chains, which interact with electromagnetic radiation at different wavelengths [90,96]. The peptide bond absorbs light in the far UV resulted from $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions (Figure 2.4). The absorption maxima of these transitions are at 190 nm for $\pi \rightarrow \pi^*$ transitions and 220 nm for $n \rightarrow \pi^*$ transitions. In aromatic side chains of proteins $\pi \rightarrow \pi^*$ transitions lead to absorption at $>230$ nm. Tryptophan shows the most intensive absorption. Its absorption maximum is at 280 nm. The absorption of tyrosine ($275$ nm) and phenylalanine ($260$ nm) is weaker.

![Molecular orbital diagram](image)

Figure 2.4: Molecular orbital diagram representing relative energies of electronic transitions (based on [97]).

**Concentration determination**

Tryptophan absorption enables concentration determination of proteins in solutions based on the law of Lambert-Beer [90,96]. This law states that the intensity of monochromatic light passes through a transparent sample drops exponentially with the path length. Thus, the absorption $A$ is defined as:

$$A = \log \frac{I}{I_0} = {\epsilon \ast c \ast d}$$  \hspace{1cm} (2.3)
where $I_0$ is the intensity of incident radiation and $I$ the intensity of radiation after passing the sample, $\epsilon$ is the molar absorption coefficient $[\text{L/(mol*cm)}]$, $c$ the concentration of the solute $[\text{mol/L}]$ and $d$ the path length of the sample [cm]. All parameters depend on the wavelength and thus, the absorption can be plotted as a function of the wavelength, in a so called absorption spectrum. The law of Lambert-Beer is valid for homogeneous solutions of one absorptive component at low concentrations, which show no light scattering and photo-reactions during the measurement. In this case, concentration determination is relatively simple and precise. Otherwise, correction of the measured absorbance is needed.

For concentration determination of mAb1 and mAb2 the NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific) was used. Absorption was measured at 280 nm and the concentration was determined using Lambert-Beer's law. The absorption coefficient was set to $\epsilon_{\text{percent}} = 1.4 \text{L/(g} \ast \text{cm)}$.

**Turbidity**

Aggregated protein solutions show a certain degree of turbidity as a result of light scattering [13]. Aggregate concentration and size affect the degree of light scattering and thus, the turbidity of the protein solution [13, 98]. Small particles ($R < \lambda/20$) lead to Rayleigh scattering, whereas large particles ($R > \lambda/20$) induce Mie scattering (Figure 2.5) [99]. Thus, light scattering of aggregated protein solutions is a combination of these light scattering phenomena. Rayleigh scattering is symmetric [100]. It is maximal in the forward and backward direction of incident light and shows a minimum at 90°. In Mie scattering, on the other hand, light scattering in the forward direction increases with particle size.
2.3. Analytical methods

Figure 2.5: Rayleigh and Mie scattering [101].

The turbidity of protein solutions is typically measured in a UV-Vis spectrometer at protein non-absorbing wavelengths of 320-800 nm [13]. Light scattering is most intensive at low wavelengths. Therefore, in the present study the turbidity of interface stressed mAb2 was measured at 340 nm and normalized by the absorption at 280 nm, which correlates with the protein concentration. The aggregation index (AI) was used as a measure of the fraction of aggregates in the antibody samples [102,103].

Sample preparation and analysis

150 µL interface stressed mAb2 was transferred to a new UV transparent microtiter plate (UV-Star® Microplate, Greiner Bio-One GmbH) and the absorption at 280 nm and 340 nm were measured on an Infinite® M200 PRO NanoQuant (Tecan). The AI was calculated using equation 2.4.

\[
AI = \frac{A_{340}}{A_{280} - A_{340}} \times 100
\]  
(2.4)

The AI was plotted as a function of time and fitted with the Gompertz function (equation 2.5) using Origin 2015G (OriginLab Corporation):
\[ y = ae^{-e^{-k(x-x_c)}} \]  

(2.5)

where \( a \) is the maximum AI, \( x_c \) the center of the curve and \( k \) the aggregation rate.

### 2.3.5.2 Fluorescence spectroscopy

Fluorescence occurs when an excited molecule returns to the electronic ground state by emission of a photon \([90, 96]\). The radiative energy of fluorescence is lower than the absorption energy and thus the emitted light is shifted to longer wavelengths (redshift). Fluorescence is a rather rare energy transition phenomenon, because of the flexibility of most molecules. In flexible molecules the vibrational levels of the electronic ground state and the electronic excited state overlap. Thus, non-radiative energy transition is predefined.

Fluorescence molecules possess rigid fluorescent groups (fluorophores) like aromatic rings or ring systems \([90, 96]\). It can be distinguished between intrinsic and extrinsic fluorophores. Intrinsic fluorophores are present directly in a molecule such as the aromatic amino acids tryptophan, tyrosine and phenylalanine in proteins. Tryptophan fluorescence is typically used to determine intrinsic protein fluorescence because it is selectively excitable at 295-305 nm with an emission at 300-350 nm. Extrinsic fluorophores have to be chemically introduced into a non-fluorescent molecule \([96]\). 8-Anilinonaphthalene-1-sulphonic acid (ANS) is a well-known extrinsic fluorophore, which can be used to determine the polarity of molecules or distinguish between different protein folding states.

**Recording of fluorescence spectra**

Tryptophan alteration and conformational changes due to UV irradiation were
analyzed by fluorescence spectroscopy. Experiments were performed both with mAb1 and mAb2 irradiated on the transilluminator for 0, 1, 5 and 15 min. 300 µL samples at a protein concentration of 0.1 mg/mL were measured in triplicate in microtiter plates (UV-Star® Microplate, Greiner Bio-One GmbH). All measurements were performed at a temperature of 25°C and the spectra were corrected for solvent fluorescence. Tryptophan emission spectra were recorded from 305 to 550 nm (increment: 5 nm) at 295 nm excitation wavelength on a SpektraMax M5® Microplate Reader (Molecular Devices).

Binding of the fluorescent probe ANS (Sigma Aldrich) to the antibody was used as a measure of its folding state, i.e. for conformational changes or aggregation induced by UVC exposure. The emission maximum of unbound ANS at 545 nm shifts to 470 nm when bound to misfolded proteins [104]. 0.1 mM ANS were added to the UV exposed antibody sample. Fluorescence emission spectra from 400 to 650 nm (increment: 5 nm) were recorded at 380 nm excitation wavelength.

### 2.3.5.3 Fourier transform infrared spectroscopy

Light of the infrared (IR) spectral region (0.76-1000 µm) excites mainly vibrational and rotational transitions of molecules depending on its frequency [90]. Light in the near infrared (0.76-3 µm) enables low energetic electronic transitions, in the mid infrared (3-30 µm) vibrational transitions dominate and in the far infrared region (30-1000 µm) rotational transitions are excited.

IR spectroscopy in the mid-infrared is used for protein structure investigations [90, 105, 106]. Absorption of IR light causes stretching and bending vibrations of functional groups of the same frequency [106, 107]. Basic prerequisite for vibrational transitions is a molecular dipole moment which changes upon vibration. Molecules with a dipole moment are therefore called infrared
active molecules. The dipole moment defines the vibrational frequency and the absorption probability of the functional group \([90,106]\). Furthermore, the vibrational frequency depends on the atom mass and the type of chemical bond \([107]\). Triple and double bonds absorb light of higher frequency than single bonds. Hydrogen bonding induces weakening of the bond strength. These properties are useful for structural investigations.

In proteins functional groups of the peptide backbone as well as some amino acid side chains are IR active groups, which absorb light at different frequencies \([90,105,107]\). The Amide I is used for secondary structure analysis due to C=O stretch vibrations of the peptide bond. Each secondary structure element (\(\alpha\)-helix, \(\beta\)-sheet, \(\beta\)-turn and random coil) has its individual hydrogen bonding pattern and thus a characteristic C=O vibration frequency (Table 2.1).
Table 2.1: Deconvoluted Amide I frequencies characteristic for proteins in water [105,108].

<table>
<thead>
<tr>
<th>Mean Frequencies</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1624 ±1.0</td>
<td>(antiparallel) β-sheet</td>
</tr>
<tr>
<td>1627 ±2.0</td>
<td>(antiparallel) β-sheet</td>
</tr>
<tr>
<td>1633 ±2.0</td>
<td>(parallel) β-sheet</td>
</tr>
<tr>
<td>1638 ±2.0</td>
<td>(parallel) β-sheet</td>
</tr>
<tr>
<td>1642 ±1.0</td>
<td>(parallel) β-sheet</td>
</tr>
<tr>
<td>1648 ±2.0</td>
<td>Random</td>
</tr>
<tr>
<td>1656 ±2.0</td>
<td>α-Helix</td>
</tr>
<tr>
<td>1663 ±3.0</td>
<td>3_{10} Helix</td>
</tr>
<tr>
<td>1667 ±1.0</td>
<td>β-Turn</td>
</tr>
<tr>
<td>1675 ±1.0</td>
<td>β-Turn</td>
</tr>
<tr>
<td>1680 ±2.0</td>
<td>β-Turn</td>
</tr>
<tr>
<td>1685 ±2.0</td>
<td>β-Turn</td>
</tr>
<tr>
<td>1691 ±2.0</td>
<td>(antiparallel) β-sheet</td>
</tr>
<tr>
<td>1696 ±2.0</td>
<td>(antiparallel) β-sheet</td>
</tr>
</tbody>
</table>

For investigation of biomolecules special technical equipment is necessary. High time resolution of Fourier transform infrared spectroscopy (FTIR) enables the investigation of protein structures, molecular mechanisms of protein reactions and protein folding [106]. An interferometer is the key element of an FTIR spectrometer (Figure 2.6). It is built up of a fixed and a movable mirror which enables simultaneous measurement of many wavelengths [90]. A light beam is directed onto a beam splitter and the two resulting beams are reflected at the two mirrors [106]. Depending on the optical path difference of the mirrors, the two beams undergo constructive and destructive interference when
recombined. The light intensity is measured with respect to the position of the movable mirror. The resulting interferogram $I(x)$ is a Fourier transform of the spectrum $I(\nu)$ [90,106]. The data can be reconverted back into a spectrum by Fourier transformation.

Figure 2.6: Overview of an interferometer installed in FTIR spectrometers (based on [106]).

For IR spectroscopy two different sampling techniques are commonly used. In transmission measurements the infrared light is directed through a cuvette containing the sample [90,106]. However, this technique is problematic for biological samples because water strongly absorbs in the mid-infrared region and overlaps with the Amide I band of proteins. Attenuated total reflection (ATR) was developed as alternative technique (Figure 2.7). The IR light is directed onto a crystal with an index of refraction larger than that of the
sample, which is placed on top of the crystal. The angle of incidence is chosen in such a way that total reflection occurs at the interface. Total reflection causes the formation of an evanescent wave at the interface. It is of the same frequency as the incoming light beam and penetrates into the sample with exponentially decreasing intensity. The sample absorbs light according to its structure and thus, the reflected light beam carries the information of the individual IR spectrum. The advantage of this technique is the low penetration depth of the evanescent wave into the sample. This enables measurement of even aqueous solutions.

![Diagram of ATR system](image)

Figure 2.7: Schematic representation of an ATR system (based on [106]).

**Recoding of FTIR spectra**

Protein secondary structure was determined by infrared spectroscopy. A BRUKER Vertex 70 FTIR spectrometer (Bruker Optik GmbH) equipped with an ATR unit (Silicon ConcentratIR2; Harrick Scientific Products, UQA-E-XXX) was used to record samples adjusted to 0.5 mg/mL in PBS. For signal recording a liquid nitrogen cooled mercury-cadmium-telluride detector (InfraRed Associates, Inc., D315/6) was used. Each spectrum was recorded within a spectral range of 1600 to 1700 cm\(^{-1}\) wavenumbers and represents the average of 1000 sample scans with a spectral resolution of 2 cm\(^{-1}\). The OPUS Spectroscopy Software (Bruker Optik GmbH) was used for atmospheric compensation and smoothing (13 point Savitzky Golay [109]).
2.3.5.4 Mass spectrometry

Mass spectrometry (MS) is a microanalytical technique to determine the molecular weight of ions in a high vacuum system [90,110]. The data can be used to characterize analytes due to their amount, elemental composition and molecular structure [110]. A mass spectrometer consists of three major components [90,111]: an ion source, a mass analyzer and a detector (Figure 2.8).

![Diagram of a mass spectrometer](image)

Figure 2.8: Schematic illustration representing the major components of a mass spectrometer [111].

Ionization of the analyte is a prerequisite for MS analysis [110]. For large, non-volatile and thermally labile compounds such as biomolecules, the two ion sources electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are commonly used today [90,110,112]. In ESI, which was used in the present study, the solubilized sample passes a high voltage capillary at atmospheric pressure and charged droplets are produced [112,113]. The ions are separated from the solvent before they enter the mass analyzer. Here, the ions are separated according to their mass-to-charge ratio (m/z) [110–112]. After passing the mass analyzer the ions are detected and a mass spectrum is generated, at which the abundance of an ion species of a defined m/z ratio is plotted as a function of m/z.

Complex sample mixtures with analytes of different structure or elemental
composition may generate ions of the same nominal m/z value [110]. Such samples have to be separated prior entering the ion source or tandem mass spectrometry (MS/MS) needs to be used [110,112]. Separation of similar compounds based on their structure can be achieved by specific chromatographic methods like reversed phase chromatography. Tandem MS instrumentations are used to isolate ions of desired m/z and subsequently, induce fragmentation of these ions. The fragment ions are analyzed in a second MS step. The high information content of MS/MS data enables structural analysis of compounds even at low concentrations as well as in complex mixtures [112].

Mass spectrometry can be used to identify posttranslational modifications of proteins with known amino acid sequence by comparing the theoretical molecular weight and the experimentally determined weight [90]. For protein identification or analysis of covalent modifications and crosslinks proteins are usually enzymatically degraded prior to MS analysis (bottom up sequencing) [90,113,114]. The generated MS or MS/MS data are compared to theoretical peptide masses recorded in a database for numerous proteins [114].

**Sample preparation and analysis**

Sample preparation and MS analysis were performed by Hohenheim University Service Centre: Proteins, which were cut-off from SDS gels, were digested in-gel using trypsin (Roche) according to Shevchenko et al. [115]. Nano-LC-ESI-MS/MS experiments were performed on an ACQUITY nano-UPLC system (Waters) coupled to a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific). Tryptic digests were concentrated and desalted on a pre-column (2 cm x 180 µm, Symmetry C18, 5 µm particle size, Waters) and separated on a 25 cm x 75 µm BEH 130 C18 reversed phase column (1.7 µm particle size, Waters). Protein was eluted in a 1-40 % acetonitrile gradient in 0.1 % formic acid within 90 min. The LTQ-Orbitrap was operated under the control of XCalibur 2.1.0 software. Survey spectra (m/z = 250-1800) were recorded in the Orbitrap detector at a resolution of 60,000 at m/z = 400. Data
dependent tandem mass spectra were generated for the seven most abundant peptide precursors in the linear ion trap. For all measurements using the Orbitrap detector, internal calibration was performed using lock-mass ions from ambient air as described in Olsen et al. [116].

**Data analysis**

Mascot 2.3.02 (Matrix Science) was used as search engine for protein identification. Spectra were searched against Hohenheim University Service Centre’s internal sequence database in FASTA-format. Search parameters specified trypsin as cleaving enzyme allowing three missed cleavages, a 5 ppm mass tolerance for peptide precursors and 0.6 Da tolerance for fragment ions. Carbamidomethylation of cysteine residues was set as fixed modification. Methionine oxidation was allowed as variable modification. Scaffold 4.4.3 (Proteome Software Inc.) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability by the Peptide Prophet algorithm. Crosslinks were identified using the MassMatrix Database Search Engine (MassMatrix version 2.4.2, Center for Proteomics & Bioinformatics Case Western Reserve University) [117–119]. Search parameters were set as for Mascot but with a 10 ppm mass tolerance for peptide precursors and oxidation and deamidation of several amino acids as additional variable modifications.

### 2.3.6 Fluorescence microscopy

The fully automated cell imager NyONE (SynenTec) is commonly used for screening, detection and classification of cells [120]. It combines fast non-invasive brightfield imaging and fluorescence imaging with three different fluorescence light sources. Apart from cell imaging the high resolution enables detection of unsoluble protein aggregates labeled with a fluorescence dye.
Sample preparation and analysis
MAb2 stressed in the interface project was diluted to 25 µg/mL in Dulbecco’s PBS (Biochrom) and labeled with 10 µM 4,4’-Dianilino-1,1’-binaphthyl-5,5’-disulfonic acid dipotassium salt (Bis-ANS, Santa Cruz Biotechnolgy). Bis-ANS is a bifunctional fluorophore which binds to proteins via hydrophobic interaction [121]. Its absorption maximum is at 395 nm and its emission maximum at 500 nm. The samples were labeled in black UVStar 96-well pates (Greiner Bio-One GmbH). The plates were centrifuged at 200 g for 1 min before analyzing by fluorescence microscopy on a NyONE cell imager (SynenTec). Imaging was done using an objective lens Olympus 20x (NA 0.5, Resolution ~550 nm ppx). Aggregates with a detected area of 0-500 µm² were counted and classified into ten sections of 50 µm² each.

2.3.7 Computer based models

2.3.7.1 Analysis of APRs using the TANGO software

TANGO is a statistical mechanics algorithm to predict cross-β aggregation in peptides and denatured proteins [82]. It calculates the partition function of the protein phase-space which encompasses different structural states. Each protein segment can populate the random coil state, β-turn, β-sheet and α-helix conformations as well as α-helical and β-aggregates according to a Boltzmann distribution. The frequency of population of each state is based on statistical and empirical considerations and is defined by its energy. For an accurate prediction of aggregating sequences TANGO takes different energy terms into account like the hydrophobicity, solvation energetics, electrostatic interactions and hydrogen bonding. Furthermore, the algorithm considers protein stability and physico-chemical parameters like pH, protein concentration and ionic strength supplemented by the assumption that the core region of β-aggregates
is fully buried.

TANGO determines the propensity for \(\beta\)-aggregate conformation for each residue of the protein. Potential aggregation segments are defined by the TANGO guideline to consist of at least five consecutive residues with a TANGO score above 5%.

**Data analysis**

The aggregation prone regions of mAb1 and mAb2 in 1x PBS as well as potential aggregation segments were computed using TANGO. Parameters were set based on the experimental conditions in the study: protein concentration: 0.5 mg/mL, pH: 7.2, ionic strength: 0.1627 M, temperature: 25°C.

**2.3.7.2 Distance calculation**

To estimate the minimum distance between two atoms of reacting residues models of conformational ensembles of mAb1 and mAb2 were computationally generated. This was performed by Dr. Daniel Seeliger (Boehringer Ingelheim Pharma). In a first step an ensemble of 100 different conformations of the Fab fragments was generated with the distance constraint-based method tconcord [122]. This method gives a reasonable estimate of the protein flexibility, in particular the large global protein motions. For each of the individual conformations of this ensemble all side-chain rotamers for the residues of interest were computed using the Dunbrack backbone-dependent rotamer library [123]. Hence, the conformational model accounts for large collective backbone flexibility as well as local flexibility on the level of bond rotations. Subsequently, the pairwise distances for all rotameric states were calculated and the minimum distance was used as criterion to classify the MS detected linkages into intra- and intermolecular crosslinks. For classification an 8 Å cutoff according to Clementi et al. [124] was used.
3 Results and Discussion

3.1 Antibody sequence characterization

In this study two monoclonal antibodies, mAb1 and mAb2, were used to analyze stress-induced protein aggregation. MAb2 is known to be an antibody with high aggregation propensity. Bickel et al. identified a reversible aggregation mechanism inducible at low pH and high ionic strength [102]. Aggregation was accompanied by changes in tertiary structure whereas the secondary structure remained unaffected. The study of Bickel et al. was limited to the stresses pH and ionic strength. Further stresses had to be tested to get a precise assessment of the aggregation propensity of mAb2. UV light and interface exposure were chosen in this study due to their relevance in the manufacturing process of mAbs. For comparative studies a second antibody (mAb1) was introduced for UV experiments. No stability studies has been published for mAb1, yet.

The primary structure of proteins significantly contributes to their stability. Computer based analysis using TANGO was done to get an initial assessment of the aggregation propensity of mAb1 and mA2. Regions which tend to β-aggregation were identified (Figure 3.1). The aggregation prone regions with a TANGO score above 5% for at least five consecutive residues are highlighted in the primary sequences (Figure 3.2).

For both antibody sequences eight APRs were detected in the heavy chain
and five in the light chain. A slight shift between the APR positions of mAb1 and mAb2 was observable due to their different sequence lengths (Figure 3.1). However, most of the APR sequences were identical in both mAbs with similar scores (Figure 3.2).

![Figure 3.1: TANGO score for β-aggregation of mAb1 (red) and mAb2 (black) plotted as a function of the primary sequences of (A) heavy chain and (B) light chain.](image)

APRs in the variable region of mAbs are located in proximity to CDRs [78, 79]. Unfortunately, detailed structural information of both mAbs used in this study were missing. Literature research revealed a patent of a monoclonal antibody which almost fitted to the primary structure of mAb2 [125]. Thus, the CDRs from the patent were transferred to the primary structure of mAb2 and were compared with the position of the identified APRs. Two of the APRs detected in the heavy chain of mAb2 flanked the CDRs or even overlapped with them. In the light chain three of the APRs overlapped with the CDRs.
3.1. Antibody sequence characterization

**mAb1-HeavyChain**
EVQLVESGGG LVKPGGSLRL SCAASGFTFS SYDMSWVRQA PGKGLEWVST
ISSGSGSYTVY LDISKGRTFV SRDNKASNLQ LQMNLSRAED TAVYYCARIQG
LDYWGRFELY TVSSASTKGPS SVFPLAPSSK STSGTAAALG CLVQDDYPFPV
VTWNSWSGAL TSGVHTFPAPV LQSSGLYLSL SVTVPSSSSL GTQTYICNVN
HKPSNTKVDK KEPEKSCDFTK HTCPCPAPAE LLGPGPSVLFL PPKPDITLMI
SRPTEVTCCV YDVSHEDEPV KENWYVQGVE VHNAKTPKRE EYQNYSTRYVV
SVLTVLHIDWD LNQKEYKCKV SNKALPAPIE KTISAKAQGQ REPQYVTIPLP
SRDELTKNQV SLTCLVKGFLY PSNIAWEVES NGQPENNYFT TPPVLDSDGS
FFLYSKLTVD KSRWQQQGNNF VSCVMHEALH NYHTQKSLSL SPGK

**mAb1-LightChain**
EIVLTQSPAT LSLSGERAT LSASASSSIN YTVYQQKPGQR LLIYLT
NLASGVAPAR FGSGSGETDF TLTISSLEPE DFEAVYVLQGW VSNFTFLGGS
TKVEIKRTVA APSVFIFPPS DEQLKGTAS VSCLNENFYPP REAKVQVKVDD
NALQNSNSQG SSYEQQDSKDD TYSLSSTLTTL SKADYEKHKV YACEVTHIQGL

**mAb2-HeavyChain**
QVQLVQSGAE VKPGASVKVY SCKTSRYTFT EYTIHVRQQA PQRLEWIGG
INPSNGPSSY NQKFGKSTIY TVDSTASTAY MELSLLRSED TAVYCARER
IAQYDVGEIH A MDYRQQCTSLY TVSASTKGP SVFPLAPSSK STSGTAAALG
CLVQDDYPFPV VTWNSWSGAL TSGVHTFPAPV LQSSGLYLSL SVTVPSSSSL GTQTYICNVN
GTQNYICNVN HKPSNTKVDK KEPEKSCDFTK HTCPCPAPAE LLGPGPSVLFL PPKPDITLMI
PPPKDITLMI SRPTEVTCCV YDVSHEDEPV KENWYVQGVE VHNAKTPKRE EYQNYSTRYVV
EQYNSTVRIVY SVLTVLHIDWD LNQKEYKCKV SNKALPAPIE KTISAKAQGQ REPQYVTIPLP
REPQYVTIPLP SREEMKTNQV SLTCVLKGFY PSNIAWEVES NGQPENNYFT TPPVLDSDGS
TPPVLDSDOG FFLYSKLTVD KSRWQQQGNNV VSCVMHEALH NYHTQKSLSL SPGK

**mAb2-LightChain**
DIVMTQSPDS LAVSLGERAT IINCKSSQSLYY SRNQKNDVLA WYQQKPGQPP
KEFILWASTR ESGPVPDFSG SGFGTDEFTLT ISSLQAEVDA VYQCYQQFSS
PLTFQQGTKV EIKRTVAAAPS VFIPPSDEQK LKSQGATSAVC LNLNFPREAE
KVQWKVDNALE QSNSQESVET EQSDKSTSTSY LSSTTLTSKA DYEKHKVYAC
EVTHQGILSP VTQSNFRGEC

Figure 3.2: Primary sequences of mAb1 and mAb2 with their aggregation prone regions identified by TANGO (gray). CDRs transferred from the literature [125] to mAb2 are highlighted in red.
3.1.1 Antibody sequence characterization: Discussion

The computer based model TANGO not only calculated the aggregation propensity but also identified aggregation prone regions in mAb1 and mAb2. At first glance, the results of both mAbs were identical. Both showed the same number of aggregation prone regions located in similar regions of the primary sequence and exhibited similar amino acids. The APRs amount detected is consistent with previous studies on commercial mAbs [79]. They identified 2-8 APRs per light and heavy chain. The similarity can be explained by the highly conserved structure of IgGs. The constant region is mainly identical in all antibodies and even the variable region contains conserved parts [66,68]. IgGs primarily differ in the CDR regions. Based on the CDRs of the patented mAb, which were transferred to mAb2, a large number of the APRs detected in this study were located in close proximity to or even overlapped with the CDRs. In previous studies it was shown, that APRs are often located in CDR loops or in adjoining framework $\beta$-strands [54,55]. Wang et al. identified an accumulation of Gln and Asn in the CDR3 region of the light chain [79]. In the commercial mAb cetuximab (Erbitux) the sequence YCQQNNN and in efalizumab (Reptiva) the sequence YCQQHNE were identified as APRs in the CDR3 of the light chain. The detected APRs of mAb1 and mAb2 showed no accumulation of Gln and Asn. However, in the light chain of mAb2 the fourth APR (VAVYYC) adjoined the CDR3 region is accompanied by two flanking Gln.

Having an overall look at the APRs identified in mAb1 and mAb2 the sequences mainly contain the hydrophobic amino acids Val and Leu or the aromatic Tyr. To some extent further hydrophobic and aromatic amino acids were detected, too. The identified APRs also contained several hydrophilic, hydroxyl containing amino acids like Thr and Ser. Charged amino acids, on the other hand, were not found. These results correspond to the APR characteristics defined by Wang et al. and Beerten et al. (see chapter 1.2.2) [79,80]. In the present study, additionally some Cys and Ala were detected in the APRs.
3.1. Antibody sequence characterization

Based on the information of tools like TANGO, proteins with low aggregation propensity can be designed. With specific antibody engineering the tendency for aggregation and thus, the immunogenicity of therapeutic antibodies can be reduced [126,127]. The enrichment of so-called gatekeeper residues were used to prevent aggregation due to protein unfolding [80,84]. Gatekeepers flank APRs and disrupt hydrophobic patches which are responsible for aggregate formation. Charged amino acids (Arg, Lys, Asp, Glu) for example, were introduced for their repulsive effect [80]. Furthermore, Pro and Gly which were incompatible with the $\beta$-structure were used as gatekeepers [128,129]. Most of the APRs identified in mAb1 and mAb2 were actually flanked by amino acids known as gatekeepers.

To conclude, the results confirm the experimentally found tendency of mAb1 to form aggregates. However, the similar results of mAb1 and mAb2 made it difficult to state which antibody has the higher aggregation propensity. Experimental studies had to be done for both mAbs to provide more information.
Chapter 3. Results and Discussion

3.2 Aggregation induced by exposure to UVC radiation

3.2.1 Development of a scale-down experiment reflecting UVC exposure during viral inactivation

To investigate the extent of conformational and covalent damage in monoclonal antibodies induced by UVC exposure, mAb1 solutions were initially passed through the commercially available UVivatec virus inactivation system. However, as the latter is designed for pilot to large scale manufacturing, experiments consumed too much antibody for extended investigations. Hence, the UV irradiation step was scaled down by exposing mAb samples in UV-transparent 96-well plates placed on a UV transilluminator. The transilluminator has similar spectral characteristics as the UVivatec due to its mercury lamp and a similar filter set. Downscaling enabled an extended screening and the inclusion of a second antibody (mAb2) in the studies.

The formation of cross-linked species both in the UVivatec system and in the scale-down experiment was analyzed by SDS-PAGE to achieve comparable radiation dosages. Different passage numbers in the UVivatec system were compared to different exposure times on the transilluminator (Figure 3.3). Analysis of the UVivatec stressed, non-reduced samples (lanes U6-U8) on 6% Tris-glycin gels showed the appearance of an additional band >245 kDa not observable in the unstressed antibody (lane U6 vs. lane U5). UV light appears to induce intermolecular crosslinks which result in dimer and trimer formation.

In the reduced, UVivatec stressed samples (lanes U2-4), three additional bands at about 80 kDa, 125 kDa and 150 kDa were observed. These bands indicate the formation of non-reducible, intra- or intermolecular crosslinks between antibody chains. The crosslinked bands could represent HC-LC, (HC)2-LC and (HC)2-(LC)2 combinations, but this assumption was not tested further. Mab1
irradiated on the transilluminator for different times was analyzed in lanes T1-T8. UV exposure on the transilluminator for 5 min (lanes T4 and T8) resulted in a similar band pattern compared to the one from a single passage through the UVivatec system (lanes U2 and U6).

![Figure 3.3: SDS-PAGE Coomassie stained 6% Tris-glycin gels of mAb1 exposed to UVC light by the UVivatec system (U) or the transilluminator (T). Reduced (U1-4, T1-4) and non-reduced samples (U5-8, T5-8) were analyzed. U1,U5: unstressed mAb1, U2,U6: one run, U3,U7: two runs, U4,U8: three runs on the UVivatec system; T1,T5: unstressed mAb1, T2,T6: 1 min, T3,T7: 2 min, T4,T8: 5 min UV exposure by the transilluminator; M: marker.]

### 3.2.2 Kinetics of aggregate and fragment formation

The kinetics of UV induced aggregate and fragment formation was followed by SE-HPLC. Chromatograms of mAb1 and mAb2 before and after irradiation on the transilluminator (Figure 3.4 (A) and (B)) showed that UV exposure led to additional peaks. Non-reducing SDS-PAGE confirmed that peaks eluting before the monomer were aggregates while those eluting after the monomer were fragments (Figure 3.5). Both aggregate and fragment content increased with exposure time.
Figure 3.4: (A) and (B) SE chromatograms representing the kinetics of aggregate (dimer, trimer) and fragment formation induced by UV irradiation using an UV transilluminator (254 nm) of (A) mAb1 and (B) mAb2. Color code: black: 0 min, red: 1 min, blue: 2 min, green: 5 min, orange: 15 min, magenta: 30 min. (C) and (D): Antibody concentration derived from peak areas as a function of time for (C) mAb1 and (D) mAb2. Color code: black: total chromatogram, red: trimers, blue: dimers, green: monomers, orange: fragments. Error bars represent standard deviations of triplicates.
3.2. Aggregation induced by exposure to UVC radiation

Figure 3.5: SDS-PAGE Coomassie stained 4-20% Tris-glycin gel of mAb2 exposed to UVC light by the UVivatec system after SEC fractionation. Non-reduced (1-4) and reduced (6-10) samples were analyzed. 1&7: unstressed mAb, 2&8: aggregates, 3&9: monomer, 4&10: fragments, 5: marker.

Total soluble protein as well as monomer, oligomer and fragment concentration were calculated from peak areas of the SE-HPLC chromatograms (Figure 3.4 (C) and (D)). For both antibodies the total fraction of soluble protein remained constant. The monomer concentration decreased with extended exposure time, while aggregates increased. Dimer and trimer formation could be observed for both antibodies. In the first five minutes of UV exposure both, dimers and trimers, were formed. With longer exposure time the dimer fraction slightly decreased whereas the trimer fraction continued to increase. Fragments showed a slight, but continuous increase with exposure time.

The temperature increase due to irradiation was controlled during the kinetics experiment to exclude any temperature influence on protein aggregation or modification (Figure 3.6). After five minutes exposure the temperature was
increased by about 3°C (21 to 24°C). Thirty minutes exposure resulted in a temperature increase of about 10°C (20 to 30°C).

Figure 3.6: Temperature of mAb2 as a plot of UV exposure time. Error bars represent standard deviations of triplicates.

3.2.3 UVC irradiation results in alterations of the antibody structure

Secondary structure
To characterize secondary structure alterations caused by UV exposure, Uvivatec stressed mAb2 was separated by preparative size exclusion chromatography into fractions of dimers, monomers and fragments. FTIR analysis of the Amide I band (1600-1700 cm⁻¹) of these fractions showed that dimers did not differ from monomers in secondary structure (Figure 3.7). The absorption maximum at 1640 cm⁻¹ indicated mainly β-sheet structure, which is characteristic for the native IgG fold [105,106,130]. In contrast, the fragment fraction
3.2. Aggregation induced by exposure to UVC radiation

contained protein with a significantly different secondary structure. The spectrum showed increased absorption between 1700 and 1650 cm$^{-1}$ and decreased absorption between 1640 and 1600 cm$^{-1}$ whereas the maximum at 1640 cm$^{-1}$ was shifted to slightly higher wavenumbers. Absorption at wavenumbers between 1660 and 1700 cm$^{-1}$ is characteristic for beta-turns [105,106,130].

**Figure 3.7:** (A) FTIR spectra of unstressed and UV-irradiated mAb2 before and after SEC fractionation. Spectra are normalized with respect to the maxima. (B) Second derivative spectra of (A). black: unstressed, red: UV stressed (UVivatec) as well as blue: dimers, green: monomers and orange: fragments separated by preparative SEC.

**Tertiary structure**

ANS fluorescence was used to reveal changes in mAb tertiary structure. ANS binds to hydrophobic patches of the antibody, which results in a shift of the emission maximum from 545 nm to 470 nm. Fluorescence intensity of bound ANS increased with UV exposure time of both mAb1 and mAb2 when added after exposure (Figure 3.8). This is an indication that UV exposure led to a partial denaturation and exposure of additional hydrophobic residues for both antibodies.
Figure 3.8: Relative ANS fluorescence at 470 nm in the presence of mAb1 (open circles) and mAb2 (filled circles) as a function of UV exposure time. Fluorescence at exposure time zero was set to 1. Error bars represent standard deviations of four replicates. Lines represent linear fits with relative fluorescence at $t = 0$ fixed to 1.

### 3.2.4 Modifications and crosslinks resulting from UV exposure

Monoclonal antibodies exposed to UV light on the transilluminator were separated by SDS-PAGE. Protein bands labeled in Figure 3.9 were isolated from the gels, digested by trypsin and subjected to Nano-LC-ESI-MS/MS. Modifications as well as crosslinks were identified by database search.
3.2. Aggregation induced by exposure to UVC radiation

Figure 3.9: SDS-PAGE Coomassie stained 6% Tris-glycin gels of (A) non-reduced and (B) reduced samples of mAb2 (1-4) and mAb1 (5-8) exposed to UVC light on the transilluminator were analyzed. Samples were loaded the same on both gels: 1&5: unstressed, 2&6: 1 min, 3&7: 2 min, 4&8: 5 min UV exposure by the transilluminator; M: marker. I-XI: numbered bands isolated from the gel for MS analysis.

UV induced modifications

The most common modifications for mAb1 and mAb2 exposed to UVC light on the transilluminator are listed in Table 3.1. Oxidation occurred most frequently. Even monomer bands of the UV stressed sample (II&VI), seemingly indistinguishable by SDS-PAGE from the non-irradiated control, were heavily modified. Some of those modifications could also be identified in the mAb2 sample stressed by the UVivatec system (Table 3.2). Tryptophan and methionine seemed to be main targets for oxidation. Especially kynurenine was a major product of tryptophan photooxidation.
Table 3.1: UV induced modifications (gray) of mAb1 and mAb2 irradiated by the transilluminator. I-XI: numbered bands isolated from SDS gel (Figure 3.9) for MS analysis. HC: heavy chain, LC: light chain. Amino acids (AA) are listed in one letter code. gray: modified AA

<table>
<thead>
<tr>
<th>Gel band</th>
<th>Chain</th>
<th>AA number</th>
<th>AA sequence</th>
<th>Modification [ΔDa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI</td>
<td>HC</td>
<td>99-106</td>
<td>(R)QGLDYWGR(G)</td>
<td>Dioxidation (+32)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>366-389</td>
<td>(K)GFYPSDIAVEWESNGQPENNYK(T)</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>HC</td>
<td>77-87</td>
<td>(K)NSLYLQ_MNSLR(A)</td>
<td>Dioxidation (+32)</td>
</tr>
<tr>
<td>X</td>
<td>HC</td>
<td>77-87</td>
<td>(K)NSLYLQ_MNSLR(A)</td>
<td>Dioxidation (+32)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>99-106</td>
<td>(R)QGLDYWGR(G)</td>
<td>Dioxidation (+32)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>246-252</td>
<td>(K)DTLMISR(T)</td>
<td>Dioxidation (+32)</td>
</tr>
<tr>
<td>XI</td>
<td>HC</td>
<td>99-106</td>
<td>(R)QGLDYWGR(G)</td>
<td>Dioxidation (+32)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>246-252</td>
<td>(K)DTLMISR(T)</td>
<td>Dioxidation (+32)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>423-436</td>
<td>(C)SV_MHEALHNHYTQK(S)</td>
<td>Oxidation (+16)</td>
</tr>
<tr>
<td>II</td>
<td>HC</td>
<td>27-38</td>
<td>(R)YTFTEYTIHWVR(Q)</td>
<td>Dioxidation (+32)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>101-128</td>
<td>(R)IAYGYDEGHAMDYwGQGTLVTVSSASTK(G)</td>
<td>Oxidation (+16) Dioxidation (+32)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>282-295</td>
<td>(K)FN_WYVDGVEVHNAK(T)</td>
<td>W → Kynurenine (+4)</td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>352-367</td>
<td>(R)EPQVYTLPPS_R_EEMTK(N)</td>
<td>Oxidation (+16)</td>
</tr>
<tr>
<td></td>
<td>LC</td>
<td>31-51</td>
<td>(K)NYLA_WYQQKPGQPPK(L)</td>
<td>Dioxidation (+32)</td>
</tr>
<tr>
<td></td>
<td>LC</td>
<td>31-51</td>
<td>(K)NYLA_WYQQKPGQPPK(L)</td>
<td>W → Kynurenine (+4)</td>
</tr>
</tbody>
</table>
3.2. Aggregation induced by exposure to UVC radiation

Table 3.2: UV induced modifications of mAb2 irradiated by the UVivatec system. HC: heavy chain, LC: light chain. Amino acids (AA) are listed in one letter code. gray: modified AA

<table>
<thead>
<tr>
<th>Chain</th>
<th>AA number</th>
<th>AA sequence</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>27-38</td>
<td>(R)YTFTEYTH WVR(Q)</td>
<td>Oxidation (+16)</td>
</tr>
<tr>
<td>HC</td>
<td>256-262</td>
<td>DTL MISR</td>
<td>Oxidation (+16)</td>
</tr>
<tr>
<td>HC</td>
<td>282-295</td>
<td>(K)FN W YVDGVEVHNAK(T)</td>
<td>Oxidation (+16)</td>
</tr>
<tr>
<td>LC</td>
<td>31-51</td>
<td>(K)NYLA W YQQKPGQPPK(L)</td>
<td>W → Kynurenine (+4)</td>
</tr>
</tbody>
</table>

Fluorescence spectroscopy was carried out to follow the kinetics of photooxidation in tryptophan side chains (Figure 3.10) [131]. An excitation wavelength of 295 nm was chosen to selectively excite tryptophan fluorescence. With extended UV exposure times the tryptophan emission maximum at 330 nm decreased, supporting the above finding of tryptophan modification.

![Fluorescence spectroscopy graph](image)

Figure 3.10: Intrinsic tryptophan fluorescence at 330 nm of mAb1 (open circles) and mAb2 (filled circles) as a function of UV exposure time. Fluorescence at exposure time zero was set to 1. Data were fitted to \[ y = y_0 + A \cdot e^{-kt} \]. Error bars represent standard deviations of five experiments.
UV induced crosslinks

A number of crosslinks between amino acid side chains were only found in the samples exposed to UVC light on the transilluminator. Most of them occurred between cysteine and serine or cysteine and threonine. In addition, histidine was found to form crosslinks with other amino acids such as cysteine or lysine. Crosslinks induced by UVC light could be classified into three categories (Table 3.3): (1) crosslinks between amino acids close in the protein sequence, (2) crosslinks between amino acids distant in sequence but close in the predicted tertiary structure and (3) crosslinks between amino acids distant both in primary and tertiary structure. Crosslinks of class 1 and 2 could have been formed by intramolecular reactions. Class 3 crosslinks most likely represent the covalent intermolecular bonds causing aggregate formation. The classification was performed based on the estimation of the minimum distance between the reacting atoms and a 8 Å threshold distance for atom interaction [124].
3.2. Aggregation induced by exposure to UVC radiation

Table 3.3: UV induced crosslinks of mAb1 and mAb2 irradiated by the transilluminator. I-XI: numbered bands isolated from SDS gel (Figure 3.9) for MS analysis. Code for amino acids: HC: heavy chain or LC: light chain - amino acids in one letter code with its position in the primary sequence. Grayed crosslinks are presented in the model structures (Figure 3.12)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gel band</th>
<th>Amino acid 1</th>
<th>Amino acid 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb1</td>
<td>VI</td>
<td>HC-K219</td>
<td>HC-H221</td>
</tr>
<tr>
<td>mAb1</td>
<td>VI</td>
<td>HC-C258</td>
<td></td>
</tr>
<tr>
<td>mAb2</td>
<td>VIII</td>
<td>HC-T373</td>
<td>HC-C374</td>
</tr>
<tr>
<td>mAb1</td>
<td>X</td>
<td>HC-C364</td>
<td>HC-T390</td>
</tr>
<tr>
<td>mAb1</td>
<td>VI</td>
<td>HC-H422</td>
<td>HC-H426</td>
</tr>
<tr>
<td>mAb2</td>
<td>VIII &amp; IX</td>
<td>HC-T24</td>
<td>HC-C96</td>
</tr>
<tr>
<td>mAb2</td>
<td>II</td>
<td>LC-K151</td>
<td>LC-H204</td>
</tr>
<tr>
<td>mAb2</td>
<td>II</td>
<td>LC-K155</td>
<td>LC-H195</td>
</tr>
<tr>
<td>mAb1</td>
<td>XI</td>
<td>HC-T136</td>
<td>HC-C141</td>
</tr>
<tr>
<td>mAb1</td>
<td>VI</td>
<td>HC-C217</td>
<td>HC-T222</td>
</tr>
<tr>
<td>mAb1</td>
<td>VI</td>
<td>HC-C258</td>
<td>HC-H265</td>
</tr>
<tr>
<td>mAb1</td>
<td>XI</td>
<td>HC-C318</td>
<td>HC-S3437</td>
</tr>
<tr>
<td>mAb1</td>
<td>X</td>
<td>HC-S361</td>
<td>HC-C364</td>
</tr>
<tr>
<td>mAb1</td>
<td>VII &amp; XI</td>
<td>LC-C193</td>
<td>LC-H197</td>
</tr>
<tr>
<td>mAb2</td>
<td>VIII IX</td>
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<td>HC-C96</td>
</tr>
<tr>
<td>mAb2</td>
<td>II</td>
<td>HC-T146</td>
<td>HC-C151</td>
</tr>
<tr>
<td>mAb2</td>
<td>VII</td>
<td>HC-S371</td>
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<td>LC-T20</td>
<td>HC-C374</td>
</tr>
<tr>
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<td>III &amp; VIII</td>
<td>HC-T373</td>
<td>HC-C374</td>
</tr>
<tr>
<td>mAb2</td>
<td>II</td>
<td>LC-H195</td>
<td>LC-C200</td>
</tr>
</tbody>
</table>
Examples of UV induced modifications (Figure 3.11) and crosslinks (Figure 3.12) of each of the three categories are labeled in model structures of mAb1 and mAb2.

Figure 3.11: PyMol model of mAb1 (left) and mAb2 (right). UV induced modifications are shown as red spheres.

Figure 3.12: PyMol model of mAb1 (left) and mAb2 (right). Amino acids involved in covalent crosslinking are shown as spheres. One example of each category was highlighted in red: category 1, orange: category 2, pink: category 3.
3.2.5 Aggregation induced by exposure to UVC radiation: Discussion

Virus inactivation by UV exposure at 254 nm has been reported to cause minimal damage in proteins [87–89]. In this study covalent and non-covalent modifications in two monoclonal antibodies as a result of UVC exposure were investigated. Radiation dose in the scale-down experiment was adjusted to result in similar damage as the maximal recommended dose of the UVivatec system as assessed by SDS-PAGE. This dose was subsequently chosen to expose mAbs before analysis. It is well aware that reducing the dose might still effectively inactivate viruses while reducing damage to the mAb product. However, as the aim of this work was to qualitatively identify modifications that may occur as a consequence of UV exposure, experiments were carried out at the maximal recommended dose.

Aggregation kinetics upon UV exposure seems to be somewhat dependent of the specific antibody type. For both mAbs tested, the monomer fraction decreased and aggregates increased with exposure time. Aggregates remained soluble but grew in size over time, while the total chromatogram area remained constant. This indicates that mAb1 and mAb2 stay soluble in the oligomeric state. In contrast, Mason et al. recently reported a decrease in soluble protein and a concomitant increase in insoluble aggregates for an IgG1 exposed to 302 nm UV light at different pH values [30].

An effect of temperature increase due to UV exposure on protein aggregation and modification could be excluded. Irradiation up to 30 minutes resulted only in a slight temperature increase from room temperature to maximally 30 °C. After five minutes exposure the temperature was below 25 °C. At this temperature mAbs stored in phosphate buffer have been shown to be stable for about four days [132].

No significant alteration of mAb2 secondary structure following UV exposure in the UVivatec system was detected by FTIR. Both, UV stressed monomers
Chapter 3. Results and Discussion

and dimers showed similar spectra as the unstressed mAb2. The latter is also consistent with Mason et al.’s findings [30]. They observed no structural alterations of an IgG1 in sodium phosphate buffer at pH 8 after exposure to 302 nm UV light. The spectrum of the fragments however, differed significantly from that of the intact mAb2. The additional peaks in the second derivative spectrum at 1665, 1670 and 1681 cm$^{-1}$ are characteristic for $\beta$-turn structure [105,106,130], while part of the native $\beta$-sheet structure was lost. Together, these findings indicate at least partial unfolding of the generated fragments. Cross-$\beta$ structure, which is characteristic of fibrillary aggregates, should result in a distinct band at 1620 cm$^{-1}$ [108,133,134], but was not detected in any of the fractions analyzed.

Although protein secondary structure was not significantly altered by UV irradiation in mAb monomers and dimers, ANS fluorescence indicated an increase in hydrophobicity with exposure time for both antibodies. The latter could result from interaction of ANS with the misfolded fragments, but also with full-length antibody molecules destabilized by photochemical modifications. Hydrophobic interactions play a key role in aggregation of proteins including mAbs [135,136]. Fink outlined that partially unfolded protein states tend to aggregate due to their extended hydrophobic surfaces [136]. Modifications induced by UV irradiation may therefore destabilize the tertiary structure and promote aggregation.

Amino acid modifications may lead to alteration of the protein surface properties which can, but don’t have to be accompanied by changes in secondary structure. Depending on the type of modification, the number of basic, acidic and hydrophobic side chains on the protein surface can change [89,137]. Mass spectrometry was used to analyze photochemical modifications in individual side chains. While aggregation analysis is a common method to test protein colloidal stability under various stress conditions, MS can also detect modifications in monomeric mAbs indistinguishable in solubility from the native protein. Indeed, it was found that one minute of UV exposure already resulted
3.2. Aggregation induced by exposure to UVC radiation

in significant amino acid modification and crosslinks. Positions of tryptophan (Trp) and methionine (Met) oxidation were spread over the antibody molecule. Several reviews [27,28,138] emphasize these amino acids as main targets for (photo-) oxidation and photodegradation. Oxidation of Met and Trp in the IgG heavy chain region 101-128 of an IgG was detected by Perdivara et al. [139]. Karanuakaran-Datt showed that Met is susceptible to oxygenation and forms sulfoxide (+16) or sulfone (+32) in the presence of oxygen [140]. This reaction is photoactivatable through absorption of the sulfur atom. The same is true for Trp, which has the highest absorption coefficient at 254 nm of all amino acids [141] and therefore undergoes extensive photooxidation [28]. A common product of Trp photoreactions is kynurenine, which was detected in both antibodies. Under ambient light, kynurenine is an even more effective photosensitizer than Trp, as it will absorb light in the visible range and hence cause additional damage [27,28]. Perdivara et al. identified kynurenine in a peptide homologous to LC 31-51 of mAb2 [139]. We followed kinetics of Trp modification by fluorescence spectroscopy. Trp fluorescence decreased with UV exposure time. However, fluorescence of kynurenine could not be detected, indicating that most Trp residues rather underwent photodegradation to other products or that kynurenine was short-lived and therefore did not reach a detectable concentration.

The results allow deducing plausible aggregation mechanisms initiated by photochemical reactions. Philo and Wang [4,12] suggest four molecular pathways leading to aggregates: (1) protein self-association of unmodified molecules, (2) aggregation of conformational altered monomers, (3) aggregation through chemical modification and (4) surface-induced aggregation. Photoinduced aggregates as investigated here certainly belong to mechanism (3). However, other mechanisms may also impact the aggregation process to some extent. For instance, side chain modifications can lead to conformational changes of proteins followed by aggregation according to mechanism (2). Direct photocrosslinking was monitored by the appearance of higher molecular weight
species on reducing SDS-PAGE. The bands were cut out from gels and subjected to MS. Many of the identified crosslinks were oxidized thioethers (sulfoxides) between cysteine and threonine or serine, respectively. Tous et al. first identified non-reducible thioether crosslinks between cysteines in the heavy and light chain of monoclonal antibodies [15]. Later, Mozziconacci et al. showed that UV exposure lead to thioether formation between cysteines [142]. Crosslinks between histidine and lysine as well as between histidine and cysteine were also detected by mass spectrometry. Crosslinking reactions can only occur between neighboring atoms. However, defining a threshold distance for atoms in contact is not trivial due to the inherent flexibility of protein molecules and possible errors of the model structure. An 8 Å cutoff was used in accordance with the contact potential approach of Clementi et al. [124]. Distances were not directly taken from the static model, but instead, the minimal distance was first determined from rotamer libraries. Starting from these minimal distances between crosslinked atoms, the crosslinks were classified into three categories: In category 1 and 2 distance is below the 8 Å cutoff and therefore, intramolecular crosslinks are possible. Categories 1 and 2 differ in the amino acid distance in the primary sequence: In category 1, they are separated by zero or one amino acid, whereas in category 2 they are further apart on sequence level. In category 3 the distance of the crosslinked atoms is above the interaction limit, i.e. larger than 8Å. Those crosslinks are assumed to be intermolecular. However, crosslinks of category 3 were also detected in the UV stressed monomer gel bands of mAb1 and mAb2. This does not fit to the interpretation at first glance. But the hinge region of an antibody enables high molecule flexibility [143] and thus, intramolecular crosslinks of atoms in larger distance may be possible.

The results show that exposure to UVC light resulted in aggregate formation. Depending on the antibody type, aggregates grew in size but remained soluble. Further characterization by MS indicated that (a) even monomeric mAb was heavily modified as a consequence of various photoreactions and (b) amino acid
modifications and crosslinking promoted aggregation. Both, chemical modifications as well as aggregate formation are highly undesirable in therapeutic proteins as they will reduce efficacy and may cause immunogenicity of the drug. Methods used to detect aggregates only cannot identify most of the covalent alterations identified here. It can be concluded that MS analysis should be included in quality control of biopharmaceuticals if the production process contains a UV irradiation step.
3.3 Aggregation induced by exposure to interfaces

3.3.1 Aggregation kinetics initiated by interface interaction

Exposure of proteins including mAbs to interfaces has been reported to result in aggregate formation during the production process [32, 39, 144]. To investigate the aggregation kinetics of the monoclonal antibody mAb2 at the air-liquid interface and at various solid-liquid interfaces a model experiment in 96-well plates was developed. Samples were shaken to control convective mass transfer and to accelerate aggregate formation. After different time points the amount of aggregates was determined by turbidity measurement and the generated aggregates were characterized according to their size and structure.

In a first step, the experimental setup was validated. MAb aggregation should occur specifically through interaction with the tested surfaces. Spontaneous aggregation induced by shear forces resulting from shaking or by contact with the plastic material forming the microtiter plate was excluded in a negative control. MAb2 was shaken for 32 h in a sealed plate without entrapped air and in the absence of other materials. The control, however, showed no increase of the AI over up to 32 h (Figure 3.13: gray).

The experimental setup was used to test the effect of air, glass and steel interfaces on mAb2 aggregation. Interface induced aggregation kinetics was recorded. The AI of the shaken samples increased with time. All kinetics showed a sigmoidal time course depending on the contact material and its surface area (Figure 3.13). Aggregation of mAb2 was induced by each of the tested materials. However, aggregation kinetics depended on shaking, as samples left without shaking did not change in AI over several hours (data not shown).
3.3. Aggregation induced by exposure to interfaces

Figure 3.13: Aggregation kinetics of mAb2 at the (A) air interface, (B) glass interface and (C) steel interface of different surface areas according to Table 3.4. Gray: reference, black: ID 1, red: ID 2, blue: ID 3, green: ID 4. Error bars represent standard deviations of triplicates.

Different numbers of beads or different air volumes, respectively, were chosen to investigate the influence of the surface area on antibody aggregation (Table 3.4). The surface area of the glass and steel beads were calculated from the number of beads and their diameters. Air interface area was approximated by assuming a hollow cylinder shape of the liquid during shaking. The air-liquid interface therefore corresponds to the inner surface of the hollow cylinder.

Table 3.4: Analyzed materials and surface areas. Corresponding surface areas are highlighted in gray.

<table>
<thead>
<tr>
<th>ID</th>
<th>air</th>
<th>glass</th>
<th>stainless steel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>volume [µL]</td>
<td>surface area [mm²]</td>
<td>number of beads</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>51.37</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>60</td>
<td>72.65</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>88.98</td>
<td>16</td>
</tr>
</tbody>
</table>
To directly compare the kinetics of the different materials, the aggregation rate as well as the maximal aggregation index were plotted as a function of the surface area (Figure 3.14).

Figure 3.14: (A) Aggregation rate and (B) maximal aggregation index of the aggregation kinetics of mAb2 shaken in the presence of different materials. Air (blue), glass (green) and steel (red). Error bars represent standard deviations of triplicates.

The aggregation rate as well as the maximal AI increased with the surface area for all materials. Steel induced by far the highest aggregation rate as well as the highest maximal AI. The aggregation rate linearly increased with the surface area. The highest maximal AI of about 170 was already reached with a surface area of 12 mm$^2$. Based on the aggregation kinetics, glass and air interfaces showed a similar aggregation behavior depending on their surface areas. Their aggregation rate as well as their maximal AI were on the same scale but lower than those of steel. At the air interface the aggregation rate seemed to reach its maximum at a surface area of about 70 mm$^2$ whereas the maximal AI steadily increased. For glass the aggregation rate increased for surface areas up to 50 mm$^2$, decreased at 100 mm$^2$ before it increased again. At 100 mm$^2$ glass showed its highest maximal AI. This decreased again for enhanced surfaces.
3.3. Aggregate induced by exposure to interfaces

3.3.2 Aggregate size and structure depend on the interaction material

The AI was a useful parameter to determine the aggregation kinetics of mAb2. It was shown that air, glass and steel surfaces induce mAb2 aggregation to different extents. However, this parameter did not provide any structural information of the aggregates formed. For further insight into the size distribution of the aggregates size exclusion chromatography and fluorescence microscopy were performed. Conformational analysis, especially analysis of the protein secondary structure, was done using FTIR spectroscopy.

3.3.2.1 Size distribution of the generated aggregates

Soluble aggregates
SE-HPLC was used to determine the size distributions of soluble aggregates formed during interface interaction. Samples of all data points of the aggregation kinetics were analyzed. As a typical result, the chromatograms of mAb2 at the inflection points of the aggregation kinetics incubated with the maximal air space or the maximal number of beads, respectively, are plotted in Figure 3.15 (A).

MAb2 incubated with air and glass only showed a single peak at a retention time of 9.4 min. This peak corresponds to the monomer peak of mAb2, which could be confirmed by the overlay with the chromatograms of the unstressed antibody and the already characterized UV stressed sample (see Chapter 3.2, Figure 3.4). No peaks eluted at lower retention times as in the UV-stressed sample. Thus, soluble multimers were not detectable in the interface interaction experiment. For the samples incubated with steel beads additionally to this monomer peak a second peak at a retention time of 11.5 min was observable. The same peak was detected in the blank which was shaken without
mAb2 but with steel beads (Figure 3.15 (B)). This indicates that the peak is not a result of the antibody. Further experiments were done to identify the origin of the second peak (see chapter 3.3.4). For the blanks of air and glass no additional peak eluted.

To summarize, no peak at retention times smaller than that of the monomer was observable for all interface stressed samples. All samples showed only one peak of protein origin. The peak was identified to correspond to the mAb2 monomer. Looking at the monomer peak in more detail, it becomes obvious that its peak maximum as well as the peak area got reduced through the incubation with different materials.

Figure 3.15: (A) SE chromatograms of mAb2 at the inflection points of the aggregation kinetics after shaking with the maximal number of beads or air space (Table 3.4 - ID4). Blue: 89 mm² air - 8 h, green: 201 mm² glass - 8 h, red: 49 mm² steel - 2 h shaking. The unstressed mAb2 (black) and mAb2 after 5 min UV exposure with the transilluminator (gray) representing the formation of soluble aggregates (dimer, trimer) were plotted as controls. (B) SE chromatograms of the blanks (without mAb2) shaken for 16 h with 73 mm² air (blue), 201 mm² glass (green) or 25 mm² steel (red).

To estimate whether the AI was a suitable measure for the aggregate content, the relationship between the monomer peak area and the aggregation index was tested. The aggregation index of interface stressed mAb2 was plotted as
a function of the appropriate aggregated protein fraction calculated from the
decreasing monomer peak areas (Figure 3.16). Interestingly, the data points
of all samples could be fitted with the linear equation 3.1 regardless of the
interaction material and its surface area. The coefficient of determination of
the linear fit was calculated to be $R^2 = 0.96617$.

$$y = 187.77x + 1.80$$ \hspace{1cm} (3.1)

Figure 3.16: Aggregation indexes of mAb2 shaken with air (blue), glass (green)
and steel (red) for different time periods plotted as a function of the aggregated protein fraction determined from the SE chromatograms. The black line represents a linear fit taking all materials into account ($y = 187.77x + 1.80$, $R^2 = 0.96617$).
Visible aggregates
The cell imager NyONE cannot only be used for cell visualization but also to detect fluorescent particles >1 µm² (personal communication: Albert Paul, University of Applied Sciences Biberach). This property was used to detect insoluble, visible aggregates formed during interface interaction. Antibody aggregates were labeled with the fluorescent dye Bis-ANS, which binds to hydrophobic surfaces of partially denatured and aggregated proteins. All mAb2 samples of the interface kinetics were analyzed using fluorescence microscopy.

For all materials the number and size of visible aggregates depended on the shaking time and the interface area (Figure 3.17 - 3.19). The reference which was incubated without air or beads (0 mm²) showed a negligible concentration of aggregates up to 16 h. After 32 h the number of visible aggregates was slightly increased.

In the samples incubated with air the aggregate number significantly increased with incubation time (Figure 3.17). With increasing surface area the total number of aggregates increased up to 73 µm² of interface area and remained nearly constant for larger surfaces. The particle size was mainly between 0 and 100 µm² and slightly shifted to larger aggregates with increasing incubation time and interface area. In comparison to the samples incubated with glass and steel, the total number of visible aggregates as well as the aggregate size was much smaller.

Interaction with glass interfaces led to the formation of a significant number of aggregates with a size up to 400 µm² (Figure 3.18). The particle size as well as the total number of aggregates increased with incubation time and surface area. However, after 32 h of shaking the aggregates in the samples incubated with 101 µm² glass were obviously larger in size than the aggregates in the samples incubated with 201 µm² glass.

Steel resulted in the formation of relatively large aggregates compared to samples incubated with air or glass, respectively (Figure 3.19). For small surfaces
3.3. Aggregation induced by exposure to interfaces

(6-12 µm²) the total number of aggregates and their size increased with incubation time. Large steel surfaces (25-49 µm²) lead to the formation of larger particles right from the beginning. Furthermore, the aggregate size increased up to 8 h incubation. However, the total number of aggregates was much smaller than for surfaces up to 12 µm². For samples incubated with 25 µm² steel the total number of aggregates increased up to 16 h before it slightly decreased. The largest surface of 49 µm² resulted in an increasing aggregate number only up to 4 h. Afterwards, the number of aggregates decreased before it stayed nearly constant from 16 h.
Figure 3.17: (A) NyONE images representing mAb2 kinetics of insoluble, visible aggregate formation after shaking with different air volumes. The size bar was set to 100 µm. (B) Size distribution kinetics of insoluble aggregate formation plotted as aggregate concentration [aggregates/ml] depending on shaking time and air volume. Aggregates were automatically counted by the NyONE cell imager. Error bars represent standard deviations of three replicates. black: 0-50 µm², red: 50-100 µm², blue: 100-150 µm², magenta: 150-200 µm², green: 200-250 µm², yellow: 250-300 µm², orange: 300-350 µm², violet: 350-400 µm², cyan: 400-450 µm², olive: 450-500 µm².
3.3. Aggregation induced by exposure to interfaces

Figure 3.18: (A) NyONE images representing mAb2 kinetics of insoluble, visible aggregate formation after shaking with different glass surface areas. The size bar was set to 100 µm. (B) Size distribution kinetics of insoluble aggregate formation plotted as aggregate concentration [aggregates/ml] depending on shaking time and glass surface area. Aggregates were automatically counted by the NyONE cell imager. Error bars represent standard deviations of three replicates. Color code see Figure 3.17
Figure 3.19: (A) NyONE images representing mAb2 kinetics of insoluble, visible aggregate formation after shaking with different steel surface areas. The size bar was set to 100 µm. (B) Size distribution kinetics of insoluble aggregate formation plotted as aggregate concentration [aggregates/ml] depending on shaking time and steel surface area. Aggregates were automatically counted by the NyONE cell imager. Error bars represent standard deviations of three replicates. Color code see Figure 3.17
3.3.2.2 Structural analysis of the aggregates

The secondary structure of mAb2 incubated for 32 h with the maximal air, glass or steel interface was determined by FTIR spectroscopy. The Amide I region of the peptide bond (1600-1700 cm\(^{-1}\)), which is characteristic for protein secondary structure elements, was analyzed in more detail.

![FTIR spectra](image)

Figure 3.20: (A) FTIR spectra and (B) FTIR spectra normalized with respect to the maximum absorption representing the effect of different materials on the protein secondary structure of mAb2 after shaking for 32 h with the maximal number of beads or air space (Table 3.4 - ID4). Black: unstressed mAb2; blue: mAb2 shaken with 89 mm\(^2\) air, green: mAb2 shaken with 201 mm\(^2\) glass, red: mAb2 shaken with 49 mm\(^2\) steel.

The absolute absorption of the interface stressed antibodies was decreased compared to the unstressed sample. An extreme reduction was observed for the samples incubated with glass and steel. A signal loss due to background effects of the blank was excluded. Furthermore, a BCA-assay rebutted the presumption of a protein loss due to interface adsorption (data not shown). The unstressed mAb2 showed a peak maximum at 1640 cm\(^{-1}\), which is characteristic for native β-sheet structure. This peak was also observable for mAb2 stressed with the different materials. The FTIR spectrum of air-stressed mAb2
was nearly identical to the spectrum of the unstressed mAb2. However, an additional maximum at lower wavenumbers appeared for the samples incubated with glass or steel, respectively. For mAb2 stressed with glass the maximum of the second peak was at 1615 cm$^{-1}$, whereas the maximum was at 1620 cm$^{-1}$ for mAb2 incubated with steel beads. A peak in this region is characteristic for cross-$\beta$ structure [108,133,134].

3.3.3 Inhibition of interface induced aggregation by solvent additives

The experimental setup was shown to be suitable to test the effect of solvent additives on interface induced aggregation kinetics. Three common additives known to influence protein aggregation were selected: the polyol trehalose, the detergent Tween 20 and the methylamine TMAO. A further reason for the choice of these solvent additives was their different mechanisms in preventing protein aggregation (see chapter 1.1.4). The solvent additives were tested in mAb2 samples incubated with the maximal surface area of the materials air, glass and steel (Table 3.4, ID 4).

The effect of these solvent additives on mAb2 aggregation depended critically on the type of interface (Figure 3.21). They affected the maximal degree of aggregation as well as the aggregation rate.
3.3. Aggregation induced by exposure to interfaces

Figure 3.21: Aggregation kinetics of mAb2 shaken with (A) the maximal air volume or the maximal number of (B) glass or (C) steel beads without solvent additives (gray) and with the addition of solvent additives. (D) Aggregation rate and (E) maximal aggregation index of the aggregation kinetics (A)-(C) of mAb2 shaken with different solvent additives. red: Tween 20, blue: trehalose or green: TMAO. Error bars represent standard deviations of duplicates.

Tween 20 completely prevented aggregation at the air interface, whereas it acted only moderately protective at steel and glass interfaces. At the glass interface the aggregation rate of mAb2 was reduced. Furthermore, the maximal aggregation index was slightly decreased compared to the reference incubated without solvent additives. Tween 20 showed a similar effect at the steel interface. The aggregation rate as well as the maximal AI were slightly decreased. Trehalose, on the other hand, slowed down aggregation at steel and glass interfaces, but not at the air interface. Nevertheless, trehalose did not reduce the aggregation maximum at steel interfaces. At the glass interface aggregation had not yet reached its maximum after 32 h. At the end of the record the aggregation index of the samples incubated with trehalose was lower than that of the reference. The aggregation kinetics at the air interface was identical to
the reference up to 16 h. Afterwards, the kinetics slowed down and showed a slightly smaller aggregation index after 32 h.

TMAO did not inhibit antibody aggregation independent of the material. It even caused adverse aggregation kinetics at air and glass interfaces. At the air interface TMAO shifted the entire curve to higher aggregation indexes. Aggregation was accelerated and the maximal AI was increased. TMAO did not influence aggregation at the glass interface up to 10 h. However, for longer incubation times the aggregation index was higher compared to the reference. At the steel interface TMAO showed no effect on antibody aggregation. The aggregation kinetics of mAb2 incubated with TMAO was almost congruent with the reference. The aggregation rate minimally increased whereas the maximal AI remained unchanged.

3.3.3.1 Influence of solvent additives on aggregate size and structure

Size distribution of the soluble aggregates

Size exclusion chromatography was performed to investigate the soluble protein fraction of mAb2 shaken for 32 h with air, glass or steel and the addition of different solvent additives (Figure 3.22). Some solvent additives showed absorption at 280 nm. Thus, the chromatograms of the blanks incubated without antibody were subtracted from the sample chromatograms.

In control experiments without shaking, without interface material but with the addition of solvent additives it was confirmed that the solvent additives do not induce aggregate formation of mAb2 autonomously (Figure 3.22 (A)). After 32 h incubation without shaking the chromatogram were almost identical. All chromatograms showed only the monomer peak and no further peaks appeared. Only Tween 20 shifted the monomer peak to a slightly higher retention time with a slightly increased absorbance compared to the reference without
3.3. Aggregation induced by exposure to interfaces

solvent additives. This phenomenon was also observed for mAb2 incubated
with Tween 20 at glass and air interfaces.

Figure 3.22: SE chromatograms representing the effect of different solvent ad-
ditives on (A) the unstressed mAb2 and mAb2 shaken with (B) air, (C) glass and (D) steel for 32 h. Black: mAb2 without solvent
additives, red: Tween 20; blue: trehalose; green: TMAO.

The negligible direct effect of the solvent additives on unstressed mAb2 enabled
the investigation of their influence specifically on interface induced aggrega-
tion (Figure 3.22). The addition of solvent additives to mAb2 during interface
exposure did not induce the formation of soluble aggregates at all material
interfaces. As for mAb2 without solvent additives only the monomer peak was
visible in the chromatograms. At the air interface the area of the monomer
peak did not alter using Tween 20 compared to the control experiment. MAb2
shaken at the air interface without solvent additives, with trehalose or TMAO,
respectively, resulted in a significant monomer loss. Compared to the sam-
ple stressed without solvent additives the monomer peak of the sample with trehalose is slightly increased whereas the sample with TMAO is slightly decreased. The chromatograms of mAb2 incubated with glass showed all smaller monomer peaks than those of the control experiment. The absorption maximum of the sample incubated with trehalose was a little higher than that without solvent additives. TMAO lead to the highest reduction of the monomer peak whereas Tween 20 showed only a moderate decrease. In the samples incubated with steel, no peak was visible at all independent of the presence and kind of solvent additives.

At first glance, the SEC results of mAb2 incubated for 32 h are consistent with the appropriate aggregation indexes plotted in the aggregation kinetics (Figure 3.21). Samples with a high aggregation index showed a small monomer peak and therefore a larger fraction of unsoluble aggregates. To test, if the calculated aggregation indexes and peak areas fit to the linear correlation seen for mAb2 incubated without solvent additives, the results were overlaid with Figure 3.16 (Figure 3.23). The data points of the samples incubated with solvent additives did not exactly match but are in proximity to the existing fit. Most of them showed slightly smaller aggregation indexes for the same fraction of aggregated protein compared to the samples incubated without solvent additives.
Figure 3.23: Aggregation indexes of mAb2 shaken for 32 h with air, glass or steel and with the addition of Tween 20 (red), trehalose (blue) or TMAO (green) plotted as a function of the aggregated protein fraction determined from the SE chromatograms. For comparison Figure 3.16 is plotted in gray.
Size distribution of the visible aggregates

The optical imager NyONE was used to analyze mAb2 after 32 h shaking with different interfaces and solvent additives to detect the formation of visible aggregates >1 µm$^2$ (Figure 3.24). Tween 20 was not possible to analyze using NyONE due to high background fluorescence.

![NyONE images representing the effect of trehalose and TMAO on the formation of visible aggregates of mAb2 after shaking with air, glass and steel for 32 h. As control mAb2 was shaken without solvent additives (PBS). The size bar was set to 100 µm.](image)

In samples incubated at the air interface with trehalose more aggregates were formed than without solvent additives. However, trehalose resulted in the formation of smaller aggregates. TMAO, on the other hand, increased the aggregate size but decreased the total number of aggregates. The aggregate size due to glass interaction was significantly reduced by trehalose. The number of aggregates however was slightly increased. The addition of TMAO did not result in an alteration of visible aggregates. Addition of solvent additives to samples incubated with steel did not show a strong effect on the formation of visible aggregates. Trehalose led to the formation of more aggregates than without solvent additives, whereas the aggregates were smaller in size. The number and size of aggregates formed with TMAO was similar to those
without solvent additives. However, the aggregates seemed to be more loosely packed.

**Structural analysis of the aggregates**

The effect of the tested solvent additives, trehalose, Tween 20 and TMAO on the secondary structure of mAb2 was analyzed by FTIR spectroscopy (Figure 3.25). The Amide I band (1600-1700 cm\(^{-1}\)) of the samples incubated with different solvent additives and interfaces were compared with the spectrum of interface stressed mAb2 shaken without solvent additives and with that of the unstressed mAb2. As already shown, the unstressed mAb2 showed a peak maximum at about 1640 cm\(^{-1}\), which is characteristic for native β-sheet structure. Air interface interaction did not result in a shift of the Amide I maximum even after the addition of solvent additives. Solvent additives only resulted in an intensity loss. Glass interface interaction was shown to result in a second peak maximum at about 1615 cm\(^{-1}\). This additional peak was not observable for mAb2 incubated with the different solvent additives. The IR absorption of the samples incubated with glass but without solvent additives was small compared to the unstressed antibody. A similar signal loss was observed with Tween 20. Samples incubated with trehalose and TMAO showed only a slight absorption decrease. For samples stressed with steel, absorption was reduced independent of the presence and kind of solvent additives. Steel interface interaction resulted in a second peak maximum at about 1620 cm\(^{-1}\) compared to the unstressed sample. This peak was still observable in the samples incubated with solvent additives, whereas the peak with a maximum at 1640 cm\(^{-1}\) was nearly completely reduced. However, due to the low absorption signal, it was difficult to determine the exact peak position and to define potential further peaks.
Figure 3.25: (A, C, E) FTIR spectra and (B, D, F) FTIR spectra normalized with respect to the maximum representing the effect of Tween 20 (red), trehalose (blue) and TMAO (green) on the protein secondary structure of mAb2 after shaking for 32 h incubated with (A, B) air, (C, D) glass or (E, F) steel.
3.3.4 Abrasion characterization

The sample medium may become altered during interface interaction. At the air-liquid interface, for example, oxidation reactions can occur. Shaking increases the antibody transfer to the air-liquid interface and therefore may result in enhanced oxidation reactions. Oxidations in turn can influence medium conditions like the pH. Furthermore, shaking may result in abrasions of the different materials. In this study, glass, steel but also polymer abrasions from the microtiter plate may trigger aggregation on their own or activate chemical reactions which may alter again the medium conditions. Hence, steel and glass abrasions as well as pH changes of the interface-stressed mAb2 were analyzed.

As already shown, the SEC chromatograms of mAb2 incubated with steel beads showed a second peak at higher retention times compared to the monomer peak (Figure 3.15). This second peak was found not to result from the antibody, because it was detected in the chromatogram of the blank, too. This assumption was confirmed by SDS-PAGE (Figure 3.26 (A)). Here, no band was visible smaller than the light chain of 25 kDa. To estimate the size of the unknown particles the chromatogram was overlaid with a protein size standard (Figure 3.26 (B)). The particles seemed to be of about 10 kDa. These results in combination with the gray coloring of the steel stressed samples indicate that the second peak of the size exclusion chromatogram was a result of steel abrasion.
The effect of steel abrasion on mAb2 aggregation was investigated by replacing the steel beads by different amounts of steel abrasion in the interface experiment. Steel abrasions were collected from the steel blank. The aggregation index of mAb2 incubated with steel abrasions increased with incubation time and depended on the abrasion amount. After 6 h, the addition of 75 µL abrasion solution resulted in an AI of about 23. A comparable aggregation was induced by 1 steel bead (6 mm²). The addition of 140 µL abrasion solution resulted in an AI of about 87 after 6 h, which is comparable with an interface interaction with 2-4 steel beads (12-25 mm²).

MAb2 samples incubated with glass were analyzed for abrasions using FTIR spectroscopy. Silicon dioxide absorbs light at about 1050-1100 cm⁻¹ due to Si-O stretching vibrations [145,146]. In some of the tested samples a significant peak in this region was observed (Figure 3.27). MAb2 shaken with glass beads
for 32 hour as well as the appropriate blank showed no absorption peak in this characteristic region. However, after centrifugation of 100 µL of this mAb2 sample at maximal speed for 5 min and resuspending the pellet in 30 µL 1x PBS a significant peak with a maximum at about 1050 cm\(^{-1}\) was visible. For the sample incubated with Tween 20 a similar peak was observable, too. The samples incubated with trehalose and TMAO did not absorb light in this region.

![FTIR spectra](image)

**Figure 3.27:** FTIR spectra of samples incubated with glass beads in the spectral region where silicon dioxide absorbs light. Black: 1x PBS, dark gray: mAb2, light gray: mAb2-pellet, red: mAb2 with Tween 20; blue: mAb2 with trehalose, green: mAb2 with TMAO.

To analyze changes of the pH due to the different surface materials, the pH of mAb2 and the blanks was measured before and after 24 h shaking with different materials. The mAb2 control as well as the blank control incubated without air and without any beads showed no pH change after 24 h. The glass interface resulted in an pH increase from 7.2 to 8.4 in the blank and from 7.2 to 7.9 in the mAb2 sample. Changes of the pH due to steel and air interfaces were negligible in both, in mAb2 as well as in the blank.
Figure 3.28: pH change of mAb2 (shaded) and of the blanks (white) after 24h shaking at air, glass and steel interfaces. pH 7.2 was set to 0.
3.3.5 Aggregation induced by exposure to interfaces: Discussion

Proteins are exposed to different materials during the manufacturing process. Some of these materials have already been shown to induce protein aggregation. Bee et al., for example, detected significant aggregation of a monoclonal antibody at the air-liquid interface and at stainless steel microparticles [32]. Glass, on the other hand, resulted in mAb adsorption but not in aggregation. Further studies revealed protein aggregation at hydrophobic interfaces like Teflon and polypropylene [147], silicone oil syringe lubricant [148] as well as stainless steel particles shed from a filler pump [149]. However, investigations of interface interaction studies are rare. To get statistically relevant results and to explain the effect of the materials on protein aggregation more studies are necessary.

In the present study, surface materials which are relevant in pharmaceutical industry were analyzed according to their probability to induce aggregation of a monoclonal antibody. Air interface is the most important hydrophobic surface [17]. Most of the production steps from fermentation through purification to filling are carried out under air conditions. Another important material commonly used in protein production is glass. Glass vials are not only used as closure container during production but also in sample formulation or storage. In the pharmaceutical industry production plants are often made of stainless steel. Steel 316 and 316L are usually used due to their favorable properties like corrosion resistance and good fabrication properties [150]. Air, glass and steel were used in the study to investigate interface-induced protein aggregation of mAb2. Material dependent aggregation kinetics were determined and the generated aggregates were characterized according to their amount, size and structure. In a second step three different solvent additives, Tween 20, trehalose and TMAO were tested for their ability to inhibit interface induced protein aggregation.
First, the validity of the experimental model system was confirmed by demonstrating that mAb2 aggregation was specifically induced by the tested materials air, glass and steel. Materials of the 96-well plate and of the lid as well as the shaking process were shown not to induce aggregation for up to 32 h. However, shaking was a prerequisite for interface induced aggregation. The tested interfaces air, glass and steel did not induce aggregation without shaking up to 32 h. The fact that shaking without interface exposure did not induce aggregation, but on the other hand, shaking was necessary to induce aggregation at interfaces indicated that shaking accelerated the aggregation process. Shaking increases the mass transfer of mAb2 from and to interfaces by convection. Furthermore, it can replace unfolded mAb2 adsorbed at the surfaces with native mAb2 [33]. The continuous renewal of the interaction interface may have resulted in faster mAb2 denaturation and therefore in accelerated aggregation.

Aggregation was induced by all tested interface materials. All aggregation kinetics showed a sigmoidal time course. However, the aggregation rate as well as the kind of the generated aggregates were specific for air, glass and steel and depended on the surface area and the material. The aggregation index was used as parameter to determine the individual aggregation kinetics. Based on the turbidity of a sample the aggregated fraction could be estimated depending on the protein concentration. Air induced only moderate mAb2 aggregation. The aggregation rate as well as the maximal aggregation index increased nearly linearly with the surface area. Kiese et al. showed a similar effect of air on protein aggregation, but with nearly identical aggregation kinetics for the two tested air volumes [39]. In the present study, glass showed similar aggregation kinetics as air. The aggregation rate and the maximal aggregation index were in the same range as for comparable air interface areas. However, for glass surfaces no linear dependency between the aggregation kinetics and the surface area was detected. At large glass interfaces the total amount of generated aggregates was decreased whereas the aggregation rate was increased. Proba-
bly, this effect can be explained by the pH increase which was only significant in samples incubated with glass. Steel resulted in a much faster and higher aggregate formation compared to glass and air interfaces. The aggregation rate steadily increased with the surface area whereas the maximal aggregation index was reached already almost at a surface area of about 10 mm$^2$.

The generated aggregates were characterized according to their size, amount and structure. Interestingly, no soluble aggregates were identified for all tested materials. Size exclusion chromatography revealed only a monomer loss of the interface stressed mAb2. This indicates that interface interaction result in only short-lived soluble aggregates, which immediately grow to high molecular weight (HMW) aggregates. The monomer loss, or rather the fraction of aggregated mAb2 calculated from the monomer peak, was shown to linearly correlate with the aggregation index. Thus, the aggregation index could be directly used to calculate the amount of aggregated mAb2. The correlation was even independent of the tested materials. At an aggregation index of about >150 mAb2 was completely aggregated. No monomer peak was left. In this study, complete aggregation was detected for mAb2 stressed with steel. A correlation as revealed here might be useful for industrial applications. The aggregation index, which can be determined simply by turbidity measurements, might replace the more time-consuming and complex size exclusion chromatography. However, such a correlation will have to be confirmed for each protein individually. Probably, it only exists for aggregation processes at which no soluble aggregates are formed and only a monomer loss occurred. Indeed, other interface studies of monoclonal antibodies revealed the formation of soluble aggregates. Bee et al. for example detected soluble aggregates in steel stressed mAb solutions [32] and Kiese et al. observed them in mAb samples shaken with an air space [39]. This might be a result of slower agitation in these studies compared to the present. Thus, the aggregation rate in the studies of Kiese and Bee was reduced and soluble aggregates were detectable.

In the present study, the monomer loss in combination with the lack of solu-
Chapter 3. Results and Discussion

Toxic aggregates clearly indicate the formation of visible aggregates induced by air, glass and steel. Bee et al. detected a monomer loss in antibody samples which were incubated with glass and stainless steel microparticles as well [32]. The formation of air-induced visible aggregates was consistent with Mahler et al. and Kiese et al. [39,144]. They showed that shaking with an air space induced the formation of visible aggregates and that the number and size of antibody aggregates increased with incubation time. A similar dependency was observed in the present study where visible aggregates were analyzed by fluorescence microscopy. For all tested materials the aggregate size increased with incubation time and interface area. The only exception was again glass at the largest interface area. The aggregate size was reduced compared to the size in samples induced with smaller interfaces. This might be again explained by the pH increase detected in glass samples. Air showed by far the smallest aggregates, steel the largest. The number of visible aggregates steadily increased in the samples incubated with air and glass. For steel, the number of aggregates decreased for larger interaction surfaces and incubation times. As already mentioned, complete aggregation of mAb2 stressed with steel >12 mm² was detected by size exclusion chromatography. Consequently, for larger interfaces the number of aggregates decreased with increasing aggregate size.

Total or at least partial unfolding is often a prerequisite for protein aggregation [12,22,41]. Aggregation induced by interface interaction is accompanied by adsorption to surfaces followed by structural alterations [18]. In this study, changes of the protein secondary structure were analyzed by FTIR spectroscopy. Unstressed mAb2 showed a single peak maximum at about 1640 cm⁻¹. Such a spectrum is characteristic for native antibodies with mainly \( \beta \)-sheet structure. Exposure of mAb2 to air did not induce structural rearrangement of the antibody which was consistent with Kiese et al. [39]. Glass and steel, on the other hand, resulted in alterations of the protein secondary structure. A second peak appeared in the Amide I band between 1615 and 1620 cm⁻¹. This region is characteristic for cross-\( \beta \) structure, which is present
in amyloid fibrils [108,133]. A peak in this region was not detected by Bee et al. who analyzed structural changes of mAbs after glass exposure and Tyagi et al. who studied steel stressed antibodies [32,149]. However, it must be considered that protein unfolding depends on various factors, like the protein itself, the interface area as well as on environmental conditions like temperature, pH and ionic strength. Thus, the inconsistency between the results is not surprising. Unexpectedly, the IR absorption of glass- and steel-stressed mAb2 was much smaller than the absorption of the unstressed mAb2 and of mAb2 incubated with air. A protein loss due to interface adsorption was excluded as well as any background effects due to the buffer system. Up to now the phenomenon of signal loss cannot be explained. In literature no information has been found, yet. However, one explanations might be that the monomeric antibody adsorbed stronger to the ATR crystal of the FTIR spectrometer than the aggregated antibody.

Interaction of mAb2 with interfaces was shown to induce protein aggregation. Thus, the aggregation process of this study can obviously be assigned to mechanism 4, surface-induced aggregation, as classified by Philo, Lee and Wang (see Chapter 1.1.2) [4,12,16]. Considering all results of this study, steel had the most critical effect on mAb2 aggregation. The aggregation rate was much higher than for air and glass interfaces. Larger aggregates were formed and the aggregates lost their native structure during the aggregation process. Having a look on the properties of the solid materials, a relationship between hydrophobicity and aggregation could be identified. Glass is a highly hydrophilic material [34]. Its contact angle with water is of about 15°. Steel, on the other hand, shows a rather high contact angle of about 70-75° and is therefore much more hydrophobic than glass. The major driving force for protein adsorption to surfaces depends on the surface hydrophobicity [17]. Hydrophobic interactions are mainly responsible for the adsorption of proteins to hydrophobic surfaces. Thus, at steel surfaces hydrophobic interactions dom-
inate and mAb2 aggregation was favored. Adsorption to hydrophilic surfaces is rather driven by electrostatic attraction. Under the experimental conditions at neutral pH the mAbs are positively charged due to their isoelectric point between 8.8 and 9.1. This resulted in electrostatic repulsion at hydrophilic surfaces of same charge and on the other hand, electrostatic attraction at oppositely charged surfaces. Furthermore, electrostatic interactions depend on the ionic strength [17]. A charged shielding double layer is formed at charged surfaces and reduces electrostatic interaction between proteins and oppositely charged surfaces. Soda lime glass used in this study is a hydrophilic, negatively charged material which shows a special mechanism in preventing protein adsorption. In aqueous solutions soda-lime glass interacts with water molecules. Na\(^+\) and Ca\(^{2+}\) are replaced with water ions like H\(^+\) and H\(_3\)O\(^+\) [151,152]. Additionally, a water film is formed which prevents protein-surface interactions. The hydrophobicity of the interaction material is a good explanation for protein aggregation at solid surfaces. However, this explanation could not be transferred to air-liquid induced aggregation. Air is assumed to have a contact angle of nearly 180° [153]. Thus, it is much more hydrophobic than steel. At the air interface, however, aggregate formation was lowest. This indicates that protein aggregation not only depends on the surface hydrophobicity but that other factors have to influence the aggregation process. The presence of abrasions in the samples stressed at solid interfaces indicated high shear forces during the experiment. This raises the question, if not shear additionally contributed to the aggregate formation of mAb2. In previous studies a direct effect of shear on protein aggregation was already excluded [5,36–38]. Shear alone did not affect protein aggregation but rather the synergistic effect of shear and gas-liquid or solid-liquid interface interaction. This would be consistent with the results of this study and might be explained by the study of Bee et al. who calculated a tension force perpendicular to the air-liquid interface of about 140 pN [38]. Thus, a protein which is adsorbed to the air-liquid interface experiences this force as well. The force is in the range of 20-150 pN which is
required to mechanically unfold proteins observed by atomic force microscopy. Unfortunately, comparable data are not known for solid-liquid interfaces, yet. Based on the results of this study the forces at solid-liquid interfaces are probably higher than at the air-liquid interface.

As already mentioned, shaking affected interface induced aggregation of mAb2. Increased mass transfer by convection accompanied by continuous renewal of the contact surface accelerated the aggregation process. Samples shaken for some hours contained abrasions of different materials. These abrasions might also indirectly affect the aggregation process. MAb2 shaken with steel showed a second peak in the size exclusion chromatogram, which was shown to not be of protein origin. The gray color of the pellet was an indication for abrasions of the steel beads. AAS measurements confirmed this assumption by identifying iron in the steel-stressed samples after removing the beads (data not shown, experiment qualitatively performed by Dr. Barbara Bottenbruch, University of Applied Sciences Biberach). This is consistent with Hedberg et al. who detected increased release of stainless steel components induced by protein adsorption [154]. In the present study, glass abrasions were detected in mAb2 samples incubated with glass. FTIR measurements revealed silicon traces in the concentrated mAb2 sample shaken with glass for 32h. The fact that in the unconcentrated sample no silicon dioxide was detectable cannot be explained, yet. Further investigations are necessary. Shaking with beads may also resulted in abrasions of the 96-well plate or of the lid. However, this was not tested, yet.

Abrasions in protein samples are critical because they can either directly induce protein aggregation like shown for steel or they react with medium components like salts and induce alterations of the medium. Changes of the pH, for example, were detected in the samples incubated with glass. This was probably a result of Na$^+$ and Ca$^{2+}$ substitution with H$_3$O$^+$. Thus, silicon oxide abrasions might induce basification of the samples which consequently affects the aggre-
In the second part of the interface project several solvent additives were tested for their ability to inhibit or even prevent interface induced mAb2 aggregation. The three solvent additives, Tween 20, trehalose and TMAO were chosen due to their positive effects already mentioned in literature and their different protein stabilizing mechanisms (see chapter 1.1.4). The tested solvent additives affected interface-induced mAb2 aggregation to different extents depending on the interface material (Table 3.5). Not only the aggregation kinetics but also the size as well as the structure of the aggregates were influenced by the solvent additives. However, it was confirmed that none of the solvent additives induced aggregate formation by their own.

As interface-stressed mAb2 without solvent additives, mAb2 with solvent additives did not result in the formation of soluble aggregation. However, the monomer loss indicated the formation of visible aggregates in this samples as well. Due to the similar results with solvent additives, the linear correlation between the aggregation index and the fraction of aggregated mAb2 was reexamined. The solvent additives only minimally influenced the linear correlation. The deviations from the linear fit determined from mAb2 incubated without solvent additives were a bit higher for the samples incubated with solvent additives.

Tween 20 is a surfactant which competes with proteins for surface adsorption [16, 46, 56, 58]. It was tested at a concentration greatly exceeding the critical micelle concentration. At this concentration Tween 20 completely prevented mAb2 aggregation at the air interface. The same result was revealed by Kiese et al. and Chou et al. [39, 56]. A possible explanation might be the reduction of the surface tension at the air-liquid interface in the presence of Tween 20 [58]. As stated in the first part, surface tension is a major aspect in
Table 3.5: Result overview of the effect of solvent additives on different protein aggregation properties.
++: strong increase, +: increase, 0: no effect, -: decrease, --: strong decrease

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aggregate formation. At the glass and steel interface Tween 20 also inhibited aggregation but did not completely prevent it. Tween 20 can cause desorption of mAbs from solid surfaces as investigated by Couston et al. [155]. Desorption was dependent on the surfactant affinity to solid surfaces. However, the exact mechanism of surfactants at solid-liquid interfaces is not well characterized, yet. Kerwin et al. suggested the formation of well ordered monolayers as well as a hemi-micellar structure of the surfactant at hydrophobic solid surfaces [58]. In the present study, visible aggregate detection by fluorescence microscopy was not possible for mAb2 samples with Tween 20 due to its hydrophobic property. The fluorescence dye Bis-ANS bound to the detergent micelles which resulted in high background fluorescence. Tween 20 prevented structural alterations induced by glass. However, cross-β formation at the steel interface remained unaffected by Tween 20. Probably, Tween 20 showed a higher affinity for the more hydrophilic glass surface than for steel. The increased silicon abrasion in samples incubated with glass and Tween 20 strengthen this assumption. In this study, a Tween 20 concentration far above the CMC was chosen. However, in drug formulations the addition of solvent additives has to be minimized due to their adverse effects. Tween 20 can induce autooxidation which may result in protein destabilization or even protein damage [16, 21, 44, 56, 58]. Thus, in further studies the effect of reduced Tween 20 amounts should be investigated. Trehalose, an osmolyte, stabilizes proteins by preferential exclusion [41, 42, 45, 46]. It was most efficient in inhibiting protein aggregation induced at the glass interface. The aggregation rate was significantly reduced. However, at the end of the record the fraction of aggregated mAb2 was in the same range as without the addition of any solvent additives. The visible aggregates were smaller than without trehalose, but the amount of aggregates was slightly increased. Alterations of the protein secondary structure induced at the glass interface were prevented by trehalose. At the steel interface trehalose showed only a moderate effect. It resulted in a reduced aggregation rate whereas the final aggregate amount remained constant. As for mAb2 without solvent additives
the complete antibody was aggregated. The visible aggregates were slightly smaller than without solvent additives, but the number of aggregates was increased. Assuming a linear relationship between the peak areas of the FTIR spectrum and the fraction of secondary structure elements, trehalose reduced the fraction of $\beta$-aggregated mAb2 in the steel stressed sample. Trehalose showed only a negligible effect on air induced aggregation. The aggregate size was slightly decreased whereas the amount was slightly increased. In summary, the results indicate that trehalose was mainly effective at solid interfaces, especially at hydrophilic ones. Previous studies of Docoslis et al. and Wendorf et al. were consistent with this results [156,157]. They stated that sugars bind to charged hydrophilic surfaces by electrostatic interactions and prevent protein-surface interaction. Contrary, they showed that sugars are excluded from the air–water interface leading to an increased surface tension.

TMAO is a further osmolyte. Its specific amphiphilic structure comprising one oxygen atom and three methyl groups defines its stabilizing mechanism. TMAO did not inhibit protein aggregation at all tested materials. The aggregation kinetics at the air and glass interface became even worse due to TMAO. Both, the aggregation rate as well as the fraction of aggregated mAb2 were increased. The size of visible aggregates was slightly increased at the air interface. Glass interface interaction resulted in similar aggregates than without solvent additives. Furthermore, TMAO prevented $\beta$-cross formation of glass stressed mAb2 like the other solvent additives. Aggregates incubated at the air interface retained their secondary structure when incubated with TMAO although the overall aggregation process was accelerated. Steel induced aggregation was not influenced by TMAO. Amount and size of visible aggregates were similar than without solvent additives. Additionally, secondary structure alterations of steel stressed mAb2 were not prevented by TMAO. Athawale et al. explained a comparable effect of TMAO by its amphiphilic structure [158]. Hydrophobic and hydrophilic regions of TMAO balance the mechanisms of preferential exclusion and interaction with functional groups. The interaction
of TMAO with air and glass seems to minimally shift this equilibrium to enhanced protein-protein interaction. The prevention of structural alterations induced at the glass interface might be a result of increased protein-water interaction induced by TMAO. Protein-water interaction stabilizes the protein’s native state [55].

To sum up, the solvent additives showed different effects on mAb2 aggregation depending on the interface material (Table 3.5). None of the solvent additives resulted in reduced aggregation accompanied by improved aggregate characteristics at all tested interfaces. At the air interface Tween 20 was the most effective solvent additive. It completely prevented mAb2 aggregation. For glass, Tween 20 as well as trehalose showed mainly positive effects. An explanation for the increased number of aggregates might be the reduction of the aggregate size. Preventing mAb2 aggregation induced by steel was only possible to some extent with the tested solvent additives. They were all inefficient in inhibiting cross-β formation induced by steel.

In practice, proteins come in contact with a variety of different materials during the manufacturing process. To inhibit interface-induced protein aggregation the use of solvent additives must be tested for each protein product individually. As shown here, often a single solvent additive would not be enough to completely prevent interface induced protein aggregation. Rather, a combination of solvent additives has to be used. However, any effect of each single solvent additive and their combinations on protein aggregation as well as on immunogenicity has to be strongly controlled.

In conclusion, experimental studies like the present could help to assess the risk of protein aggregation during downstream processing and to identify solvent additives to reduce this risk. The study revealed that interfaces can have tremendous consequences for therapeutic proteins and therefore for the patient. Of course, extreme experimental conditions were selected to accelerate protein aggregation. However, already a small amount of aggregates and dena-
3.3. Aggregation induced by exposure to interfaces

tured proteins might result in a high immunogenicity of a protein drug. Thus, interface effects have to be considered during process development of protein production. Materials have to be selected according to the results of studies such as the present, which for example, indicated that hydrophobic, solid surfaces are most critical for therapeutic proteins.
4 Conclusion

Proteins are molecules which tend to aggregate (reviewed in [4, 5, 159]). During the manufacturing process therapeutic proteins are exposed to a variety of stresses that may induce aggregate formation. This problem becomes more and more obvious to regulatory agencies. To guarantee high quality of drugs, risk assessment of the most important stress factors is necessary. The guidelines demand frequent analysis of the aggregation state of proteins in each production step as well as stability studies to minimize aggregate formation. However, up to now, the USP and the Ph. Eur. only set limits for the concentration of visible and subvisible particles \(>10 \mu\text{m}\). Soluble aggregates as well as subvisible aggregates \(<10 \mu\text{m}\) have not been considered, yet. However, these aggregates might also induce an immune response. Protein aggregates with a minimum of 20 repetitive epitopes were assumed to be most immunogenic, although they are commonly smaller than 10\(\mu\text{m}\) [59]. Furthermore, these small aggregates can serve as nuclei for further aggregate growth, for example, during storage. Thus, already the detection of small aggregates seems to be important.

In the present study, the two stress factors UV light and interface interaction, which are both highly relevant in downstream processing, were investigated according to their effect on monoclonal antibodies. Both antibodies studied were predicted by computer based algorithms to contain aggregation-prone regions. However, the complexity of the environmental influences on proteins during their production process makes experimental studies indispensable. To
investigate protein aggregation induced by either of the two stress conditions, experimental setups were developed, which enabled the determination of aggregation kinetics. This was accompanied by the establishment of appropriate analytical methods for an accurate aggregate characterization.

In the first part of this work, UV light at 254 nm was shown to induce the formation of soluble aggregates. The aggregate size as well as the amount of aggregates increased with exposure time. The secondary structure of the monomeric antibody and the aggregates remained unaffected. However, amino acid modifications were detected not only in the aggregates but also in the antibody monomers. It was concluded that UV induced amino acid modifications are responsible for the formation of intra- and intermolecular crosslinks and thus, for aggregate formation.

Amino acid modifications as well as possible alteration were not detectable with commonly used methods like SEC or SDS-PAGE, but only by mass spectrometry. This problem has to be considered when protein samples are exposed to UV light during the manufacturing process. Virus inactivation by UVC irradiation, for example, should be accompanied by mass spectrometry to identify alterations even in the protein monomer.

In the second part of the study, the effect of interface interaction on protein aggregation was investigated. Interface contact resulted in rapid formation of HMW aggregates whereas soluble aggregates could not be detected. Aggregation kinetics as well as the final aggregate concentration and size distribution depended on the interface material and its surface area. Aggregates were characterized according to their size and structure. Interestingly, only solid materials affected the antibody secondary structure, while contact to air did not result in measurable structural changes. Furthermore, solvent additives were identified which are able to prevent or at least reduce interface induced antibody aggregation. The effect of the additives on the aggregation rate was
specific for the interface materials. Air, glass and steel are three of the most common interfaces proteins get exposed to in the manufacturing process. Here, the kinetics and extent of aggregate formation depending on these interfaces were investigated in detail. To reduce air-induced protein aggregation, the production pipeline should be sealed off from air whenever possible. This is problematic during the whole production process but especially during upstream processing. Today, the majority of recombinant therapeutic proteins is produced in chinese hamster ovary cells (CHO) [160]. Gassing with oxygen is essential for eukaryotic cells like CHO [161]. Thus, already during the fermentation process air-liquid interface interaction is unavoidable for secreted proteins. The present study showed that the addition of detergents like Tween 20 completely prevent air-induced antibody aggregation. Such detergents, already used as formulation excipients, might also be useful in downstream processing. In upstream processing, however, cell toxicity of additives has to be considered and screening for protein stabilizers has to include their effect on cell culture. Stainless steel was shown to result in the strongest mAb aggregation compared to glass and air. In industry, production plants for biopharmaceuticals may include stainless steel reactor vessels, pumping systems or tubings. Considering the results of this study, single use equipment might be a good alternative to avoid contact with steel surfaces.

This study provides methods to quantitatively assess the effect of interface contact on antibody aggregation. The experimental setup might be useful to optimize production processes with respect to surface materials and solvent additives to minimize protein aggregation.
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Biberach, December 2016

Martina Merg
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Thesis: „Aggregation During Biopharmaceutical Production of Antibodies Induced by UV Radiation and Interface Exposure“
Supervisor: Prof. Dr. Hans Kiefer, Institute of Applied Biotechnology, Biberach University

10/2011 – 09/2013
M.Sc. Biochemistry, Ulm University
Degree: Master of Science
Thesis: „Distance dependence of FRET measured on mononucleosomes by single molecule experiments“ – Ulm University, Institute of Biophysics, Prof. Dr. Jens Michaelis

03/2008 – 08/2011
B.Sc. Pharamceutical Biotechnology, Biberach University
Degree: Bachelor of Science
Thesis: „Untersuchung der Glykosylierung von Antikörpern mittels Lektinaffinitätschromatographie“ – Boehringer Ingelheim, BP Quality, Quality Germany, Quality Control

2007
Abitur, Wieland Gymnasium, Biberach

Internship

Boehringer Ingelheim Pharma GmbH und Co. KG, Biberach
Department: BP Quality, Quality Germany, Quality Control

AAI Pharma GmbH & Co. KG, Neu-Ulm
Department: Mass Spectrometry

Biberach, December 10, 2016
Publications

Oral presentation

Olubukayo Oyetayo, Fabian Bickel, Martina Merg, Eva Herold, Oscar Mendez Lucio, Andreas Bender and Hans Kiefer
QSAR Analysis of Additive Effects on the Aggregation of Monoclonal Antibodies in Downstream Processing.
PENG Europe, Lisbon, Portugal (November 2016)

Martina Merg, Eva Herold and Hans Kiefer
Aggregation and Modification of Monoclonal Antibodies Induced by UV-C Irradiation.

Poster

Olubukayo Oyetayo, Fabian Bickel, Martina Merg, Eva Herold, Oscar Mendez Lucio, Andreas Bender and Hans Kiefer
Experimental Investigation and Analysis of Solvent Additive Effects on Monoclonal Antibody Aggregation.
MiBio 2016, Cambridge, UK (November 2016)

Olubukayo Oyetayo, Fabian Bickel, Martina Merg, Oscar Mendez Lucio, Andreas Bender and Hans Kiefer
QSAR Analysis of Additive Effects on the Aggregation of Monoclonal Antibodies.
Recovery of biological products XVII, Southampton, Bermuda (June 2016)

Martina Merg, Eva Herold and Hans Kiefer
Aggregation and Modification of Monoclonal Antibodies Induced by UV-C Irradiation.
11th annual BioProcess International European Summit (BPI), Düsseldorf Neuss, Germany (April 2015)

Martina Merg, Julia Fitz and Hans Kiefer
Protein Aggregation Induced by Interface Exposure.
11th annual BioProcess International European Summit (BPI), Düsseldorf Neuss, Germany (April 2015)

Olubukayo Oyetayo, Fabian Bickel, Martina Merg and Hans Kiefer
Experimental Investigation, Modeling and Prevention of Aggregate Formation in Downstream Processing.
Recovery of biological products XVI, Rostock, Germany (July 2014)

Martina Merg, Eva Herold and Hans Kiefer
Aggregation and Modification of Monoclonal Antibodies Induced by UV-C Irradiation.
34th International Symposia on the Separation of Proteins, Peptides and Polynucleotides (ISPPP), Würzburg, Germany (April 2014)

Biberach, December 10, 2016