It is never too late for a cocktail - Development and analytical characterization of fixed-dose antibody combinations

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Abstract

Monoclonal antibodies (mAbs) have been immensely successful as biological drugs. However, the treatment of some diseases requires combinations of antibodies that bind to different pharmacological targets. An elegant approach to delivering the therapeutic potential of antibody combinations is to develop drug products based on fixed-dose combinations (FDCs) of co-formulated mAbs. Since the first FDA approval of two co-formulated mAbs in 2020, the interest in antibody FDCs is increasing. However, there are different strategies to develop co-formulated antibodies and unique challenges related to their analytical characterization. In this review, we summarize the recent progress on antibody FDCs with a focus on important considerations during drug development and the analytical toolbox for co-formulated mAbs.

Keywords

Antibody drugs

IgG antibodies

Monoclonal antibodies

Protein formulations

Abbreviations

AC-SINS - affinity-capture self-interaction nanoparticle spectroscopy
AUC – analytical ultracentrifugation
BoNT - botulinum neurotoxin
CA – co-administration
CD – circular dichroism
CF – co-formulation
CIC – cross-interaction chromatography
cIEF - capillary isoelectric focusing
COVID-19 - coronavirus disease 2019
DLS – dynamic light scattering
DP – drug product
DSC – differential scanning calorimetry
DSP – downstream processing
ELISA - enzyme-linked immunosorbent assay
EMA – European medicines agency
EUA – emergency use authorization
FDA - U.S. Food and Drug Administration
FDC – fixed-dose combination
FTIR - Fourier transform infrared spectroscopy
HIC – hydrophobic interaction chromatography
HP-SEC - high-performance size-exclusion chromatography
HPLC - high-performance liquid chromatography
IEX - ion-exchange chromatography
IgG – immunoglobulin G
ITC – isothermal titration calorimetry
mAb – monoclonal antibody
MS – mass spectrometry
NMR - nuclear magnetic resonance spectroscopy
pAb – polyclonal antibody
RP – reversed-phase chromatography
SPR – surface plasmon resonance
USP – upstream processing

1. Introduction

The importance of therapeutic monoclonal antibodies (mAbs) has increased immensely over the past three decades.[1,2] The success of mAbs can be explained by their high specificities, long half-life, and good storage stability.[3] The time to develop a novel mAb as a drug product (DP) ready for clinical trials could be shortened to less than a year.[4,5]

Despite the huge success of therapeutic mAbs, there are drawbacks of having a single highly specific drug that targets only one epitope. For example, if the epitope undergoes mutations, a high-affinity mAb that was promising at first might become ineffective.[6] The issue of targeting single epitopes became even more apparent during the COVID-19 pandemic. For example, an emergency use authorization (EUA) of bamlanivimab as monotherapy was revoked due to fear of escape viral variants.[7] The resistance to individual mAbs is also an obstacle to developing antibody therapies against cancer.[8] There are different mechanisms by which cancerous cells can escape from a single therapeutic mAb like mutations in the targeted epitope[9,10] or induction of alternative growth signalling pathways.[11–13]

The development of mAbs has allowed the wide commercialization of well-defined and highly specific drugs that fit into the concept of a “magic bullet”.[14] The use of mAb monotherapies however does not perfectly resemble the natural immune response. The human immune system is fighting diseases by generating a variety of monoclonal antibody entities against different epitopes on the same antigen or against different antigens related to the same disease.[15,16] In other words, using a combination of antibodies could offer a more effective way of therapeutic intervention because multiple epitopes can be addressed with a single DP. However, early approaches to developing antibody mixtures were focusing on polyclonal antibodies (pAbs) showing cross-reactivity and high variability during manufacturing.[17,18] The characterization, development, and analysis of a mixture of dozens of antibodies remain challenging.

Fortunately, there is a way of combining the advantages of mAbs and pAbs by co-formulating mixtures of well-defined mAbs. Such fixed-dose combinations (FDCs) of therapeutic mAbs, casually called antibody cocktails, become increasingly popular but bring new challenges to their pharmaceutical development and analysis.[19–21] For example, a sound scientific explanation of the reason to combine two or more mAbs into FDCs will be required. This rationale should consider not only aspects related to the biological activity (non-overlapping epitopes, affinity, avidity, effector functions) but

also safety concerns, for example, related to the immunogenicity of the individual mAbs compared to their mixture. The analytical teams will also be more challenged with the development of antibody FDCs compared to single mAb products. Due to the structural similarities of mAbs from the same class, control strategies based on more and novel analytical techniques become feasible in the context of antibody FDCs. Finally, the development and validation of processes to produce the drug substance (DS) and the DP will be more complicated for FDCs compared to an individual mAb. Despite these challenges, several antibody FDCs have found their way into the market recently, and many others are in development.

Here, we summarize the recent progress on antibody cocktails and discuss basic aspects of their pharmaceutical development and analytical characterization.

2. At the interface between monoclonal and polyclonal antibodies

Antibody FDCs combine features of both mAbs and pAbs (Table 1). Multiple epitopes can be targeted with a mixture of mAbs to improve the pharmacological effect in comparison to individual mAbs.[22–24] At the same time, the development and manufacturing of a well-defined mAb combination can avoid many of the drawbacks related to pAbs like cross-reactivity or batch-to-batch variability. Antibody FDCs therefore offer a great opportunity to develop a well-defined DP with multiple desired specificities.[19] The antibody FDC can be composed of (i) already approved mAbs, (ii) new mAbs without previous approval, or (iii) a combination of previously approved and new mAbs.[25]

3. Fixed-dose antibody combinations compared to bispecific antibodies

Another approach to combining specificities for two different pharmacological targets is to generate bispecific antibodies.[26] There are some key differences between mAb FDC products and the bispecific antibodies. First, the different specificities will be combined in the same molecule in bispecific antibodies allowing the generation of therapeutics that link two different targets. For example, a bispecific T-cell engager antibody can have one specificity for the cluster of differentiation 3 (CD3) receptor and a second specificity for a cancer antigen.[27] However, if target linking is not the aim of the pharmacological mechanism, an antibody FDC might offer advantages like the flexibility to prepare different dose ratios of the combined mAbs or to have higher binding avidity of each active substance (for example, each immunoglobulin G (IgG) in

an FDC will have two binding sites for the same target). Second, the bispecific antibodies are engineered molecules that contain different variable domains. While various types of bispecific and multispecific antibodies can be engineered, the outstanding challenge is to obtain molecules with favorable physicochemical properties.[26] In contrast, the conventional mAbs in FDCs have a structure that resembles the naturally occurring human antibodies with two identical target-binding sites. There is substantial knowledge about how to select and develop conventional antibodies with desired physicochemical behaviour.[28–31] Third, a bispecific antibody will be produced as one DS, while the mAbs in FDCs could be produced as different DSs. The advantage here is that a DS used for antibody FDCs could also be used in other DPs. On the downside, the processes and analytics will have to be developed for each separate DS.

Overall, both bispecific antibodies and FDCs of mAbs have their place as drugs. The decision on which path to take should be taken after carefully considering the advantages and disadvantages of these two drug formats.

4. Manufacturing approaches

There are two general approaches to manufacturing antibody FDCs. The *first approach* is to express and purify each mAb as a separate DS. The mAbs can then be co-formulated before fill-and-finish in one primary package. Producing each mAb separately is attractive for several reasons. The manufacturing and analytical methods for a DS comprising a single mAb are well-established, allowing for good control during upstream (USP) and downstream processing (DSP).[32,33] Having the different mAbs separately allows for precise mixing to achieve the desired fixed-dose ratio. Furthermore, a DS comprising one mAb could be used for multiple DPs. In fact, it is possible that some mAbs that were initially developed as monotherapies will be included in antibody cocktails in future.[34] The major disadvantage of the first manufacturing approach is that the processes and methods have to be developed for each mAb in the final DP, making this strategy feasible for products containing only a few mAbs. For example, NTM-1634 (Ology Bioservices) is a fixed-dose antibody combination containing four mAbs that were expressed and purified individually.[35] The four mAbs were combined in equimolar amounts to obtain the final DP.[35] Each of the four mAbs targets a different epitope on serotypes C and D of the botulinum neurotoxin (BoNT). NTM-1634 is therefore a well-defined alternative to the polyclonal BoNT

antitoxin.

The *second approach* to manufacturing antibody cocktails is to co-express and simultaneously purify all mAbs as a mixture.[21,36] This approach could be susceptible to more manufacturing difficulties related to the purification and analytical characterization of the drug substance, but it becomes feasible when the antibody mixture contains many mAbs. This is the case of rozrolimupab (Symphogen), an antibody cocktail containing 25 unique mAbs produced by co-expression from a mixture of 25 CHO cell lines each expressing one of the antibodies.[37,38] Rozrolimupab was developed as a defined alternative to plasma-derived anti-rhesus D immunoglobulins for the treatment of primary immune thrombocytopenia.

5. Co-formulation versus co-administration

Each antibody FDC comprises two or more co-formulated mAbs.[39,40] The co-formulation is a feasible approach when the fixed-dose ratio between the mAbs in the cocktail will not change over the course of the clinical therapy. Ideally, the fixed-dose ratio will also be the same for different patient groups. Furthermore, it is important that the co-formulated antibodies are compatible, that is, combining them in the same solution does not lead to detrimental effects on antibody solubility and stability.[41,42] If these prerequisites are met, the co-formulation offers several benefits like reduced risk of dosing errors, easier handling, more convenient administration, and better patient compliance. For example, the FDC of trastuzumab and pertuzumab was developed with the benefit that the combination can be administered subcutaneously at home, and by thus, reducing the costs and burden of infusions of multiple singular DPs in a hospital setting.[43]

Besides the co-formulation, there are also other approaches to deliver a synergistic combination of antibodies to patients. For example, each mAb from a therapeutic combination can be formulated in an individual dosage form, and the individual mAbs can be mixed together prior to administration.[21] The co-administration of antibodies has to be distinguished from the sequential administration that is used in combination therapies.[21] In the sequential administration, the first mAb is administered alone, followed by the second mAb, followed by the third mAb, etc., while in co-administration the mAbs are combined directly before administration; for example, by mixing them in the same infusion bag. The co-administration approach saves time and reduces the number of injections/infusions. However, mixing mAb products should only be done

when recommended by the manufacturers based on available in-use stability data and compatibility studies. The post-production handling of mAbs should be performed with great care as deviations from the manufacturers' recommendations could compromise the quality of the proteins.[44,45] A benefit of the co-administration approach (compared to co-formulation) is dosing and formulation flexibility since every mAb will be provided in a separate, tailor-made dosage form. In this case, it is important that the antibodies and formulations that will be mixed right before administration are compatible.

6. Marketed and clinically tested antibody FDCs and other antibody combinations

Several antibody FDCs have already received marketing authorization (Table 2). For example, *Phesgo*® is a fixed-dose combination of trastuzumab and pertuzumab for treating patients with HER2-positive breast cancer.[46] A third protein, vorhyaluronidase alfa, is also added to the product to facilitate the subcutaneous administration. The final DP is a solution for injection. Each mL of Phesgo® contains 60 mg pertuzumab and 60 mg trastutzumab or 80 mg pertuzumab and 40 mg trastuzumab. The formulation contains histidine buffer (pH 5.5 ± 0.3), trehalose, sucrose, L-methionine and polysorbate 20. Noteworthy, trastuzumab and pertuzumab were first established as a combination that is administered sequentially.[47] Later, a seminal paper by Genentech demonstrated that trastuzumab and pertuzumab were stable when mixed in the same infusion bag.[48] These findings paved the way for the co-administration of the two antibodies in a hospital setting and later for the development of the FDC product with the view to enable subcutaneous administration at home.[43,49]

Another approved FDC product, *Inmazeb*®, contains three mAbs (atoltivimab, maftivimab, and odesivimab) for the treatment of infections caused by *Zaire ebolavirus*.[50–52] Each of the three mAbs has a concentration of 16.7 mg/mL. Inmazeb® is a solution that is diluted with either 0.9 % sodium chloride or 5 % dextrose solution and administered as a single intravenous infusion. The formulation of Inmazeb® contains histidine buffer (pH 6), sucrose and polysorbate 80.

A third antibody FDC, *Opdualag*™, contains nivolumab and relatlimab-rmbw for the treatment of unresectable or metastatic melanoma.[53] The DP is a solution for injection containing 12 mg/mL nivolumab and 4 mg/mL relatlimab. The formulation contains histidine buffer (pH 5.8), sucrose, polysorbate 80 and pentetic acid. Opdualag™ is

administered intravenously undiluted or diluted in 0.9 % sodium chloride or 5 % dextrose solution.

Furthermore, there are several recent EUAs of antibody FDCs to treat patients with COVID-19 (Table 2). *Ronapreve*™ is a cocktail composed of two mAbs, casirivimab and imdevimab, supplied as a fixed-dose co-formulated product or in individual vials intended for co-administration.[54] The excipients in the individually formulated and co-formulated casirivimab and imdevimab are the same – histidine buffer (pH 6), sucrose and polysorbate 80. Another antibody combination used for the treatment of COVID-19 contains *bamlanivimab and etesevimab*. However, the two antibodies are formulated separately and are mixed in the same infusion bag right before administration.[55] In this case, the formulations of individually-packaged bamlanivimab and etesevimab are slightly different. Bamlanivimab is formulated with histidine buffer (pH 6 ± 0.5), sucrose, sodium chloride and polysorbate 80. Etesevimab is formulated only in histidine buffer (pH 6 ± 0.5), sucrose, and polysorbate 80. The concentration of each mAb is 35 mg/mL. An antibody combination that is based on a different formulation approach is *Evusheld*™.[56] Evusheld™ is used under EUA for prophylaxis of COVID-19 and contains two co-packaged antibodies (tixagevimab and cilgavimab). In contrast to the previous examples, the two antibodies in Evusheld™ are administered sequentially via intramuscular injections. Interestingly, the formulations of tixagevimab and cilgavimab are the same and contain histidine buffer (pH 6), sucrose and polysorbate 80. Each of the two mAbs is formulated at a concentration of 100 mg/mL. Overall, the listed antibody combination products with market approval or under EUA reveal different drug development strategies.

Besides these products, there are also several antibody FDCs that entered clinical trials (Table 2). Currently, the leading indications for antibody cocktails are the treatment of infections and cancer. Considering the large number of clinical trials exploring synergistic mAb combinations and the recent approvals of the first FDC antibody products, it seems that the full therapeutic potential of antibody FDCs is yet to be revealed.[19–21,57]

7. Analytical toolbox for antibody mixtures

Antibody mixtures will have to be analyzed in two cases – (i) during the development and quality control of co-formulated antibodies, and (ii) for in-use stability studies of antibodies mixed in the same infusion bag for co-administration. The analytical methods used for antibody mixtures must be carefully selected according to the development stage and the target product profile. Orthogonal analytical techniques will be required to demonstrate the compatibility and stability of the mAbs (Table 3). A good approach would be to analyze the stability of the individual antibodies and compare them to the stability in the co-formulation.[42] It is important to understand the limitations and opportunities of applying analytical methods to antibody mixtures. For example, it is possible that due to overlapping peaks or a poor resolution of the methods, some degradation products of a mAb cannot be detected in the mixture.[69] Therefore, a critical risk assessment is needed with a focus on the suitability of the selected analytical toolbox to detect degradation products that can compromise safety and quality.

7.1. Analyzing potency

Good knowledge of the binding modes to the pharmacological targets is essential during the development of antibody cocktails. Typically, each mAb in a cocktail will target either a unique antigen or a unique epitope. Analyzing the binding of each mAb and looking for mAbs with synergistic binding will require a larger analytical effort in comparison to single mAb products. At first, *in vitro* assays can be applied to look for synergy in the antigen-binding modes of mAbs. *Enzyme-linked immunosorbent assay (ELISA)*-based competition-binding assays were used to find antibodies with non-overlapping epitopes and synergistic potency.[70] Specific ELISA can also be applied to analyze the potency of each antibody in the FDC.[69] Other methods that were used to simultaneously analyze antibodies with non-competing binding are *surface plasmon resonance (SPR)* [71] and *biolayer interferometry (BLI)*.[72] Ultimately, the potency and synergistic effects of mAb combinations have to be confirmed in cell culture experiments and animal models.[70]

7.2. Structure and conformational stability

In addition to the target binding, it is important to analyze the structure of the antibodies in the mixture. *Circular dichroism (CD)* can be used to assess the protein secondary and tertiary structure, as well as the thermal stability of co-formulated proteins. [73,74] For

example, a mixture of two compatible mAbs exhibited a far-UV and near-UV CD spectra identical to the calculated spectrum from measurements with the individual mAbs.[42] Such measurements could provide information that the structure of the individual mAbs does not change (e.g., due to cross-interactions) when the proteins are mixed in the same solution. However, a limitation of CD is that the measurements are performed at low protein concentrations (e.g., 0.1-1 mg/mL). Orthogonal techniques like *Fourier transform infrared spectroscopy (FTIR)* can be used to analyze the protein structure at high protein concentrations (≥100 mg/mL).[75] FTIR has already been used for antibody mixtures to measure spectra in the region of the Amide I band providing information about the secondary protein structure.[42] The calculated FTIR signal from the measurements of the individual mAbs was superimposable to the experimental data for the co-formulated mAbs. Despite the examples where CD and FTIR were applied to study the structure of antibody mixtures, it is important to note that the secondary structure of IgGs is very similar.[42] Therefore, anticipated changes in the CD and FTIR spectra might not be assigned to one of the components in the antibody mixture. Moreover, subtle structural changes in antibodies might not be evident from the CD spectra.[76] Therefore, methods with higher resolution and sensitivity than CD and FTIR could find their way into the analytical toolbox for antibody FDCs.

Differential scanning calorimetry (DSC) is the standard method to study the conformational stability and higher-order structure of therapeutic proteins.[77] DSC analysis can be performed on individual mAbs or antibody mixtures. For example, a co-formulation of nine mAbs was characterized by DSC. [69] The authors showed that the thermal stability and higher-order structure of the individual mAbs is not affected in the cocktail by comparing the DSC curve of the mixture to the sum of the DSC spectra of the single mAbs. DSC was also used in other studies on antibody co-formulations.[42,78] A major disadvantage of DSC however is the low throughput. Therefore, other techniques that provide information about the conformational stability of antibodies in co-formulations can be used during early-stage development. As an orthogonal approach to DSC, *nanoDSF* (based on intrinsic protein fluorescence) can be used to analyze the thermal unfolding profiles and apparent melting temperatures of antibody mixtures.[41] The nanoDSF measurements can be performed quickly by consuming only 10 µL of the sample. However, the method will detect only structural changes and unfolding events associated with a change in the intrinsic fluorescence

of the protein.

7.3. Colloidal stability and cross-interactions

The colloidal stability of mAbs is determined by weak protein-protein interactions in solution.[79] However, potential cross-interactions between different antibodies in the same solution add another level of complexity to the development of FDCs. It is challenging to predict mAb interactions in co-formulation, and the risk for cross-interactions has to be evaluated during DP development. *Dynamic light scattering (DLS)* can be used to obtain the apparent hydrodynamic radius (R_h) of the proteins. The resolution of DLS is not sufficient to differentiate between the monomers of two different mAbs.[80] However, a larger R_h for the protein mixture compared to the individual proteins will give the first indication for cross-interaction and oligomer formation.[42] Furthermore, DLS can be used to measure the mutual diffusion coefficient at different protein concentrations to determine the diffusion interaction parameter (k_D) of individual antibodies and their mixtures.[41] Studies on the concentration dependence of the mutual diffusion coefficient can provide information on cross-interactions between co-formulated mAbs.[81] Another useful application of DLS is to identify the aggregation onset temperatures (T_{on}) of the co-formulated antibodies and compared the values obtained for the individual mAbs.[41] Orthogonal techniques to look for cross-interactions between antibodies are also available. For example, *static light scattering (SLS)* can be applied to study cross-interactions between mAbs.[81,82] *Affinity-capture self-interaction nanoparticle spectroscopy (AC-SINS)* was also employed to screen for compatible antibody co-formulations by using miniature protein amounts.[83] However, there is still little experience with the robustness of AC-SINS to detect cross-interactions of antibodies, and more studies will be needed to validate the AC-SINS approach via comparisons to other techniques. If sufficient amounts of the mAbs are available, *isothermal titration calorimetry (ITC)* can provide further insights into potential cross-interactions. ITC is able to detect a wide range of strong and weak interactions with K_D values from the nM to the mM range.[84] The label-free detection of interactions directly in the formulation without immobilization is very useful for the analysis of protein mixtures and ITC has been already applied to show the absence of cross-interactions between co-formulated mAbs.[42] The disadvantage of ITC is that the required sample amount is significantly more compared to DLS or AC-SINS. Therefore,

ITC is more feasible during the later stages of FDC development when more material from each of the antibodies is available.

Analytical ultracentrifugation (AUC) can also be used to study cross-interactions in protein mixtures.[85] For example, AUC was used to analyze the self- and cross-interactions of a fluorescently-labelled mAb.[86] The labelled tracer mAb-1 was combined with non-labeled mAb-1 or mAb-2 in concentrations of up to 20 mg/mL to study differences in the sedimentation velocity of mAb-1 caused by either self- or cross-interactions.[86] Besides the fluorescence-based approach, AUC was also applied to study cross-interactions between unlabeled mAbs.[42] The main drawback of AUC is the low throughput. For example, a sedimentation velocity experiment will take about one day and typically only up to 7 samples can be measured. Further technique that can be used to assess interactions between different mAbs is *cross-interaction chromatography (CIC)*.[87] In this case, one mAb can be immobilized on the column and other different mAbs can be run through the column to assess potential cross-interactions that lead to longer retention times.[87] Some of the limitations of the CIC approach are related to the immobilization. For example, if an antibody is immobilized in a certain orientation, this could occlude cross-interaction sites that will be accessible when the protein is in solution. More advanced techniques like *nuclear magnetic resonance (NMR)* can also be applied to study cross-interactions between antibodies. For example, NMR spectroscopy was used to study the individual labelled antibodies in protein mixtures by ^{19}F NMR.[88,89] The application of different ^{19}F-labels on two mAbs allowed the investigation of protein interaction and aggregation for both mAbs in the mixture.[90] However, a drawback of the approach is that the antibodies for ^{19}F NMR are labelled. The labelling moiety could potentially influence the physicochemical properties of the antibodies and the cross-interactions.

The risk of detrimental cross-interactions between co-formulated mAbs might depend on the protein concentration.[41,81] Antibodies for subcutaneous administration have to be formulated at high protein concentrations (e.g., >100 g/L),[91] where short-ranged hydrophobic interactions become relevant and can outweigh long-ranged electrostatic interactions.[92] It has been proposed that cross-interactions in protein mixtures at high concentrations can be detected by deviations of the measured viscosity of the protein mixture from the calculated *viscosity* that is predicted by the extended Mooney equation.[93,94] For binary mixtures of structurally similar proteins such as mAbs, the Arrhenius mixture model can be applied as well to predict the viscosity of the mixtures

based on the viscosities of the single protein solutions.[81,93] Deviations from this mathematical relationship can indicate additionally arising cross-interactions in the binary mixture compared to the individual protein self-interactions. For example, Woldeyes *et al.* detected attractive cross-interactions in a binary mixture of different mAbs by viscosity measurements,[81] where the measured viscosity of the binary mixture exceeded the prediction of the Arrhenius mixture model. Interestingly, the approach to measure the viscosity of binary antibody mixtures could predict the viscosity of a bispecific antibody derived from the two mAbs in the mixture.[81] While the viscosity measurements are very valuable to obtain information about the compatibility of the antibodies at high concentrations, such studies are feasible during the later stages of development because large amounts of material are required.

7.4. Analyzing aggregates in mAb mixtures

Cross interactions between proteins could lead to the formation of aggregates under stress conditions and during storage. Several studies have investigated the co-aggregation of proteins, for example, mixtures of ovalbumin and lysozyme,[95] or ovotransferrin and lysozyme.[96] *High-performance size-exclusion chromatography (HP-SEC)* is the workhorse method used to detect aggregates and fragments in antibody formulations.[97] HP-SEC has also been applied to analyze the aggregates and fragments in co-formulated mAbs.[42,78,98] One limitation of HP-SEC applied to antibody mixtures is that the method cannot separate proteins with similar hydrodynamic radii (e.g., IgG antibodies) or provide information on whether homo- or hetero- aggregates are present in the sample.[98–100] However, HP-SEC can be coupled with other techniques to provide more detailed information. For example, aggregates obtained from forced degradation studies on a mixture of two mAbs were collected by HP-SEC and analyzed by SPR to demonstrate that only one of the antibodies is present in the mixture.[42] Important to note, the SPR approach to detect a specific antibody in aggregates relies on the assumption that certain epitopes of the native protein are also accessible in the aggregates.

Besides HP-SEC, there are other approaches to understanding the composition of the aggregates in antibody mixtures. For example, a recently published patent application presents an approach for the quantification of hetero-dimers in mAb co-formulations by immunoprecipitation and subsequent liquid chromatography-assisted mass spectrometry.[101] In some cases, the co-aggregation may cause the formation of

morphologically distinct structures compared to the pure protein aggregates, that could be detected by transmission electron microscopy.[102] Larger aggregates (e.g., in the micrometer range) in antibody co-formulations can be analyzed with flow imaging microscopy.[98]

7.5. Analysis of charge variants

The mAbs in a mixture can be separated based on different isoelectric points and charge. In such cases, *ion-exchange chromatography (IEX-HPLC)* is a valuable technique. Several studies successfully separated mixtures of mAbs by strong cation exchange chromatography or weak cation exchange chromatography.[42,100,103] Either salt gradients or pH gradients can be used to elute the antibodies bound to the column. For example, precise adjustments of a method employing a pH gradient were used to achieve different elution times of mAbs with different isoelectric points.[104] The good separation of the mAbs could be sufficient to enable charge variant characterization of the individual antibodies.[42,105]

Further, *capillary isoelectric focusing (cIEF)* is an alternative method to IEX to separate protein mixtures based on their IEPs and has been applied to detect chemical changes in the individual proteins in co-formulations.[42,100] cIEF is commonly applied in proteomics research due to the very high resolution and has been reported to baseline separate components with a difference in their respective IEP of only 0.01.[106]

A limitation in both IEX and cIEF is that certain degradation products from one antibody could overlap with the main peaks or degradation products from another antibody. Therefore, a sound method development combined with stress studies on the individual antibodies and their mixture is essential.

7.6. Chemical changes

Reversed-phase high-performance liquid chromatography (RP-HPLC) is commonly used to detect chemical changes in proteins. RP-HPLC can achieve a good separation of co-formulated mAbs.[98] The coupling of RP-HPLC to *mass spectrometry (MS)* is a well-established strategy to identify chemical changes in specific parts of the protein. For example, Perez-Robles *et al.* demonstrated the simultaneous identification and quantification of up to four different co-formulated mAbs by RP-HPLC coupled to MS.[107] It is also possible to use peptide mapping and RP-HPLC-MS to identify the exact position of chemical changes occurring in co-formulated mAbs.[42] In another

example, Cao *et al.* developed a peptide mapping method to identify and quantify deamidation in the complementarity-determining regions of one specific mAb in a co-formulation.[105] The sensitivity was sufficient to allow the quantification of site-specific deamidation of a low concentrated mAb in presence of a second mAb at a higher concentration.

7.7. Quantification of individual mAbs in a mixture

The quantitative analysis of each antibody in a cocktail will rely on separation techniques. A recent study compared SEC, cIEF, RP-HPLC, WCX-HPLC and *hydrophobic interaction chromatography (HIC)* with the goal to obtain good separation between the peaks of three co-formulated mAbs.[100] The mAbs had similar IEPs which further complicated the separation. Interestingly, the HIC method performed best. The authors were able to precisely measure the content of each of the three mAbs with acceptable precision, accuracy, and linearity. However, the separation of the antibodies in HIC will depend on the physicochemical properties of each protein.[29] Therefore, the HIC approach to separate and quantify individual mAbs in FDCs will not be universal.

7.8. Application of in silico approaches to antibody FDCs

The development of antibody FDCs can be supported by computational methods. The simplest way to use a computation approach for antibody FDCs is to calculate the charges of the mAbs to predict potential electrostatic cross-interactions.

For example, one of the first studies on the compatibility of trastuzumab and pertuzumab used homology models of the antibodies to calculate the theoretical net charge of the proteins at different pH.[48] Based on the very similar charge of trastuzumab and pertuzumab, the authors anticipated that no significant electrostatic interactions should occur between the two antibodies.

We have also used in silico approaches in the context of cross interactions in binary antibody mixtures.[41] In this work, we calculated molecular descriptors like surface charge, hydrophobic surface area, aggregation scores to select antibodies with diverse properties in the variable domains. Interestingly, several biophysical assays did not detect cross-interactions between antibodies with very different properties evident from the in-silico approach.

In future, we expect that more advanced computational approaches employing coarse-grained models or all-atom molecular dynamics simulations will be used to understand and predict the sites of interactions between different mAbs in an FDC.[108,109]

Summary and Outlook

The field of therapeutic antibody combinations is evolving rapidly. Several other reviews have previously addressed general aspects of co-formulating biologics,[25,40] the pharmacological benefits of combining mAbs,[20,118] or production aspects for antibody cocktails.[19,25] However, we have witnessed the approval of the first four antibody FDCs in the past two years, as well as exciting publications demonstrating the pharmacological benefits of using antibody cocktails, for example, against infectious agents.[23,119,120] These recent developments fuelled the interest in antibody FDCs but also raised many questions related to the strategies to develop, produce and characterize antibody cocktails from the perspective of a drug developer. With this minireview, we summarized the most recent progress in the field of antibody FDCs and gave an overview of the benefits and challenges of developing such products with a focus on analytical techniques that have already been applied to characterize antibody mixtures.

Different combination strategies (e.g., co-formulation, co-administration, sequential administration) have been explored to ensure the therapeutic benefits of combining mAbs. Each of these strategies has advantages and disadvantages that have to be considered during the development of a new antibody combination. The co-formulated FDCs offer benefits like reduced risk of dosing errors, easier handling, convenient administration, and improved patient compliance. The development of FDCs is a particularly attractive approach to enable the subcutaneous administration of antibody combinations outside hospitals.

To facilitate the discovery of mAbs with synergistic activity, it will be important to establish platforms for the identification of mAbs with complementary modes of action. To de-risk the drug development, predictive assays will have to be implemented to find compatible antibody combinations from a physicochemical and stability perspective. The preliminary identification of detrimental cross-interactions between antibodies could be based on biophysical techniques that require small sample volumes.[41] During the later stages of development, orthogonal analytical methods can be applied to verify the compatibility of the antibodies.

Ideally, the co-formulated mAbs will not exhibit cross-interactions in solution. However, an important question during the development of FDCs is whether some degree of cross-interactions between the co-formulated mAbs is acceptable. It is important to mention that not only destabilizing but also stabilizing interactions could occur.[121] The impact of potential cross-interactions on the quality and safety of the DP will have to be assessed on a case-by-case basis.

Understanding the degradation pathways of mAbs in antibody mixtures and comparing this data to the degradation of individual mAbs will be important.[42] There will be always a possibility that a degradation product from one mAb cannot be detected by a certain analytical technique due to an overlap with the other mAbs in the mixture. Such scenarios will have to be considered as a part of the risk assessment strategy. Comparing measurements on the individual antibodies and their degradation products to the antibody mixtures will demonstrate confidence in the analytical strategy.

The analysis of antibody FDCs may also open an avenue for novel analytical approaches. Hybrid methods that combine different modes of separation and detection will be particularly useful. Such methods (e.g., SEC-MS, EC-IEX-MS or cIEF-MS)[122–124] are already emerging and can be very valuable during the development of co-formulated antibodies.

Finally, the opportunities to develop FDCs span far beyond the combinations of conventional mAbs. Novel modalities like engineered fusion proteins or bispecifics could also make their way into the FDC field.

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Table 1. *A comparison between mAbs, pAbs and FDCs of mAbs*

	single mAb	**mAb FDC**	**pAb**
active components	1	≥ 2	multiple
targeted epitopes	1	≥ 2	multiple
targeted antigens	1	≥ 1	1
therapy failure due to a single epitope mutation	possible	unlikely	unlikely
risk for cross-reactivity	low	low	high
analytical characterization	well established	challenging	very challenging
manufacturing variability	low	low to medium	high

689 ***Table 2*** *Overview of co-formulated antibody FDCs. CF – co-formulated, CA - co-administration. *The BRII-196/BRII-198 combination is also*

690 *approved by the China National Medical Products Administration.*

Name	Company	Combined mAbs	Format	Indication	Development stage	Reference
Approved or under EUA						
Phesgo®	Roche/Genentech	trastuzumab, pertuzumab	CF	HER2-positive breast cancer	approved by FDA and EMA	[39,46,58]
Inmazeb ®	Regeneron	atoltivimab, maftivimab, odesivimab	CF	*Zaire ebolavirus* infections	approved by FDA	[50–52]
Opdualag™	Bristol Myers Squibb	relatlimab, nivolumab	CF	advanced melanoma	approved by FDA	[53]
REGN-COV, Ronapreve™	Regeneron	casirivimab, imdevimab	CF, CA	prevention and treatment of COVID-19	EUA by FDA, approved by EMA	[54]
In clinical trials						
-	Brii Biosciences	amubarvimab (BRII-196), romlusevimab (BRII-198)	-	treatment of COVID-19	Phase III*	[59]
Sym004	Symphogen (Servier)	futuximab, modotuximab	CF	metastatic colorectal cancer	Phase II	[60,61]
Sym015	Symphogen (Servier)	Hu9006, Hu9338	CF	solid tumors	Phase II	[62–64]
Sym013	Symphogen (Servier)	6 mAbs	CF	advanced epithelial malignancies	Phase I/II	[65]
rozrolimupab	Symphogen (Servier)	25 mAbs	CF	primary immune thrombocytopenia	Phase I/II	[37]

NTM-1632	National Institute of Allergy and Infectious Diseases	XB10, XB18, XB23	CF	botulism	Phase I	[66]
NTM-1633	National Institute of Allergy and Infectious Diseases	XE02, XE06, XE17	CF	botulism	Phase I	[67]
NTM-1634	Ology Bioservices	XC-a, XC-b, XC-c, XC-d	CF	botulism	Phase I	[35]
MM-151	Merrimack Pharmaceuticals	3 mAbs	CF	colorectal cancers	Phase I	[68]

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Table 3 Overview of analytical methods used to analyze combinations of antibodies and antibody-FDCs

Technique	Used to assess	Reference
ELISA	potency, non-competitive binding to the target	69,70,110,111
SPR	non-competitive binding to the target	71,112,113
BLI	non-competitive binding to the target	72,111,114,115
CD	secondary and tertiary structure	42
FTIR	secondary structure	42
DSC	structure and thermal stability	42,69,78
nanoDSF	thermal stability	41,116
fluorescence spectroscopy	tertiary structure probed by intrinsic protein fluorescence	83
DLS	colloidal stability and cross-interactions, aggregates	41,42,69,81,83,98,116
SLS	colloidal stability and cross-interactions	81,82
AC-SINS	colloidal stability and cross-interactions	83
ITC	cross-interactions	42
AUC	analyze aggregate formation due to cross-interactions	42,83,86
CIC	cross-interactions	87
NMR	cross-interactions	88,89
viscosity measurements	cross-interactions at high protein concentration	81,93,116
HP-SEC	small soluble aggregates	42,69,78,83,98,100,116
CE-SDS	aggregates	38,42,69,78
IEX-HPLC	charge variants	38,42,69,86,98,100,105,116,117
cIEF	analysis of charge variants	42,100,105
HIC	separation of mAbs for content determination	42,100
RP-HPLC	separation of mAbs for quantification	69,98,100
peptide mapping	analysis of post-translational modifications	38,42,78,105
focused peptide mapping	deamidation in the complementarity-determining regions	105