Review

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Process analytical technique (PAT) miniaturization for monoclonal antibody aggregate detection in continuous downstream processing

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Abstract

The transition to continuous biomanufacturing is considered the next step to reduce costs and improve process robustness in the biopharmaceutical industry, while also improving productivity and product quality. The platform production process for monoclonal antibodies (mAbs) is eligible for continuous processing to lower manufacturing costs due to patent expiration and subsequent growing competition. One of the critical quality attributes of interest during mAb purification is aggregate formation, with several processing parameters and environmental factors known to influence antibody aggregation. Therefore, a real-time measurement to monitor aggregate formation is crucial to have immediate feedback and process control and to achieve a continuous downstream processing. Miniaturized biosensors as an in-line process analytical technology tool could play a pivotal role to facilitate the transition to continuous manufacturing. In this review, miniaturization of already well-established methods to detect protein aggregation, such as dynamic light scattering, Raman spectroscopy and circular dichroism, will be extensively evaluated for the possibility of providing a real-time measurement of mAb aggregation. The method evaluation presented in this review shows which limitations of each analytical method still need to be addressed and provides application examples of each technique for mAb aggregate characterization. Additionally, challenges related to miniaturization are also addressed, such as the design of the microfluidic chip and the microfabrication material. The evaluation provided in this review shows why the development of microfluidic biosensors is considered the key for real-time measurement of mAb aggregates and how it can contribute to the transition to a continuous processing.

Keywords: protein aggregation; continuous biomanufacturing; monoclonal antibodies; microfluidics; process analytical technology (PAT)

ABBREVIATIONS

AF4	Asymmetrical flow field-flow fractionation
ANS	Anilinonaphthalene-1-sulfonate
Bis-ANS	4-4-Bis-1-phenylamino-8-naphthalene sulfonate
CD	Circular dichroism
CGE	Capillary gel electrophoresis
CQA	Critical quality attribute
DLS	Dynamic light scattering
FFF	Field-flow fractioning
FTIR	Fourier-transform infrared spectroscopy
HDC	Hydrodynamic chromatography
HP–SEC	High-pressure size exclusion chromatography
LO	Light obscuration
mAb	Monoclonal antibody
MALS	Multi-angle light scattering
MFI	Micro-flow imaging
MS	Mass spectrometry
MVDA	Multivariate data analysis
MW	Molecular weight
PAT	Process analytical technology
PDMS	Polydimethylsiloxane
QbD	Quality by design
RMM	Resonant mass measurement
RS	Raman spectroscopy

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SEC	Size-exclusion chromatography
SERS	Surface-enhanced Raman spectroscopy
SRCD	Synchrotron radiation circular dichroism
SV-AUC	Sedimentation velocity analytical ultracentrifugation
ThT	Thioflavin T
WCRS	Waveguide-confined Raman spectroscopy

INTRODUCTION

Continuous manufacturing has been applied in many different industries, such as the chemical,[1] food[2] and pharmaceutical[3,4] industries. However, the implementation of continuous manufacturing in the biotechnological industry is still awaited, particularly in the field of biotherapeutics.[5-7] Continuous bioprocessing utilizes a continuous flow of material through the various unit operations such that, at a steady state, products of consistent quality is being manufactured as long as the operation runs. This processing mode allows for production at a smaller scale, resulting in lower capital cost, higher process automation and lower labor cost.[5] In addition to lower manufacturing costs, the benefits of switching from a batch to a continuous process include an improvement in productivity, product quality and consistency, while drastically reducing the environmental footprint.[5,8,9] Considering all the advantages of continuous biomanufacturing, regulatory agencies are eager to implement this type of processing. The development of the FDA strategic plans, quality by design (QbD) initiative, the guideline on real-time release testing (RTRT), ICH Q13 guideline and process analytical technology (PAT) framework encourage this continuous biomanufacturing.[10-13]

The biomanufacturing process of monoclonal antibodies (mAbs), an important therapeutic agent in the treatment of several diseases such as cancers and autoimmune disorders,[14] has received major focus in the last years. Due to patent expiration and rise of biosimilars, the biopharmaceutical industry aims to lower mAb manufacturing cost.[15,16] Thus several biopharmaceutical companies are currently exploring the economy of continuous processes.[7,17] For example, Walther *et al*. conducted an economic analysis into an integrated continuous biomanufacturing platform with mAb production in the business portfolio, and concluded that it would allow reduction of costs by 55% relative conventional batch processing.[18] Additionally, advances in upstream processing (e.g. improved expression levels) of these biopharmaceuticals created bottlenecks in the purification process of mAbs, which are limiting production efficiency.[8] A possible solution is implementing a continuous downstream processing, as long as the final mAb characteristics do not suffer any impact.[19]

However, to perform the entire process in a continuous mode, all the unit operations need to be fully integrated and controlled.[5] Therefore, there is a critical need for development of real-time PAT tools that are capable of identifying changes in product critical quality attributes (CQAs), such as product aggregation and glycosylation patterns. PAT needs to be integrated with appropriate global process control strategies to ensure that the continuous process remains within the defined specifications. This includes the management of process interruptions when impurities arise or, at least, adjustments in operating conditions based on the information collected.[17]

An important CQA in the production scheme of mAbs is protein aggregation, a recurrent and poorly understood phenomenon.[20] The formation of aggregates may lead to an increase in adverse immune responses or a decrease in efficacy of the biopharmaceutical.[21] In general, aggregation of proteins is often described by mechanisms where molecules assemble into stable complexes of two or more proteins, often held together by strong non-covalent contacts.[22-24] Several environmental factors and process parameters are known to induce protein aggregate formation. Environment factors, such as low pH during chromatographic and viral inactivation steps,[25-27] high temperature[28-30] or increase in protein concentration,[31,32] have been experimentally demonstrated to cause mAb aggregation. For example, mAbs are suspended in various buffer solutions that, depending on the type and concentration of the salt, and the charged groups of the protein, might have a destabilizing influence on the protein structure.[33-35] Process parameters, such as freeze–thawing and freeze-drying processes,[21,28] interaction with metal and glass surfaces,[36,37] shear stress induced from pumps[38,39] or agitation,[21,40] have been also proven to be contributors to mAb aggregation. More specifically, the use of pumps or the agitation used for homogenizing solutions (e.g. buffers added to adjust pH) creates a gas/liquid interface at which aggregation predominantly occurs.[33,38,40] For more information on how the environmental conditions and process parameters used for the purification of biomolecules can induce unwanted aggregation, several reviews are recommended[33,41-43] and will not be further discussed here. Thus the formation of aggregates is inevitable during continuous downstream processing but controlling (and even reducing) aggregate levels can be achieved by resorting to a real-time PAT tool. Protein aggregates are heterogeneous in size, morphology and other physicochemical properties, and can be categorized based on:[41]

- the type of bond, with noncovalent aggregates (such as weak electrostatic forces)[44] or covalent aggregates (caused by disulfide bridges);[45,46]
- the reversibility: reversible[47,48] *versus* irreversible[49] aggregates;
- size;[40,50] or
- the protein conformation: aggregates with predominantly native structure or predominantly non-native structure (such as partially unfolded multimeric species[50-52] and fibrillar aggregates[53-55]).

This review will focus solely on the size of these aggregates, which are classified into small soluble aggregates (oligomers such as dimers, trimers, tetramers, etc.), with the size ranging from 10 to 100 nm; insoluble aggregates/subvisible particles, from 100 nm to 100 μm; and visible particles, for aggregates with a size larger than 100 μm.[33,41] Insoluble aggregates are of great concern in the final formulation as they have been reported to cause immunogenicity.[56,57] This is reflected in regulatory guidelines provided by the United States Pharmacopeia (USP) and the European Pharmacopeia (EP), which state that all injectable solutions need to be 'practically/essentially free' of these type of particles. Regulatory agencies have also defined the maximum numbers of subvisible particles allowed in the final formulation: the number of particulates over 10 μm is ≤6000 per container, while the number of particulates over 25 μm is ≤600 per container.[58] These criteria were focused on particles originating from external sources and not specifically defined for proteinaceous particles. Additionally, only relatively large particles are considered, dismissing the more abundant smaller particles (between 10 nm and 10 μm).[58]

Furthermore, a major challenge for the analysis of mAb aggregates is that no single analytical method exists to cover the entire

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size range or type of aggregates that may appear,[59] as can be observed in Fig. 1. Each analytical method not only has its specific advantages but also its inherent limitations, such as the possibility of creating unrepresentative measurements through sample preparation by inducing or destroying the aggregates.[41] Moreover, during formulation, mAbs are produced at relatively high concentrations, but only a small fraction of the total population of molecules will form aggregates. Thus aggregate detection must be able to identify a small amount of aggregates against the background of folded mAb monomers.[60] Furthermore, quantification and characterization of each different type of aggregate is often not possible due to the heterogeneity of aggregates, both in quantity and quality, such as size, shape and morphology.[41,61]

Hence there is a demand for a PAT tool capable of detecting and quantifying aggregate formation for the implementation of continuous processing. Ideally, this PAT tool has to produce a real-time measurement, within seconds to a few minutes, to facilitate decision making and control of the process.[62] Miniaturized sensors can be a powerful solution to speed up the analytical measurements of CQAs. Employing a microfluidic environment not only results in shorter reaction times but also leads to small sample volumes (microliter or nanoliter scale), portability, lower cost, design versatility, and potential for parallel operation and for integration with other miniaturized devices.[63] Moreover, a microminiature device allows for power needs and consumable reagents reduction, while offering the possibility of high-throughput testing.[64]

In this review, the creation of a miniaturized PAT tool for detection, and possibly quantification, of protein aggregates in a continuous mAb purification process is explored. First, requirements for the implementation of a PAT tool in a continuous process are discussed, as well as the current analytical techniques employed by the biopharmaceutical industry for aggregation studies. Subsequently, design constraints necessary to develop miniaturized analytical techniques are examined and a thorough evaluation of established analytical techniques for mAb aggregation detection is provided. The last section of this review addresses additional challenges in the PAT development and implementation to facilitate the transition towards a continuous biomanufacturing.

CURRENT ANALYTICAL TECHNIQUES

The current analytical methods employed to assess and quantify protein aggregate formation in the biopharmaceutical industry are size-exclusion chromatography combined with multi-angle light scattering (SEC-MALS), sedimentation velocity analytical ultracentrifugation (SV-AUC) and light obscuration (LO). Although SEC-MALS and SV-AUC are considered sensitive and allow aggregate quantification, both techniques are time consuming, and require highly specialized operators and costly equipment.[41,65] More specifically, for SEC, the stationary phase can interact with the protein aggregates and reduce mass recovery from the column, thereby compromising the accuracy of aggregate quantification.[66,67] The estimation of aggregates by means of SEC is compromised since proteins will be diluted in the mobile phase, which can dissociate the aggregates. Higher molecular weight (MW) species (such as subvisible particles) will not pass through the frit and therefore not enter the column.[67,68] Due to the short length of the column, SEC has a limited separation ability, making the full separation of oligomeric forms difficult.[69] Additionally, molar mass calculations for large aggregates can be inaccurate as detection of large aggregates results in an intense light scattering signal despite a low concentration.[68,69] SV-AUC is the gold

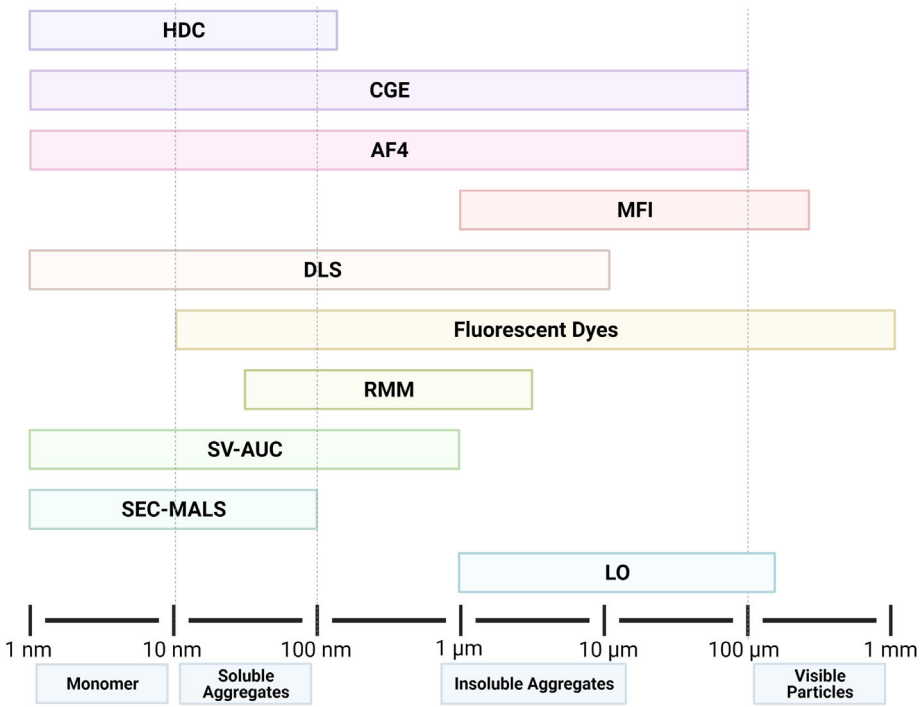

Figure 1. Schematic representation of the approximate range of protein sizes, in terms of diameter, of various analytical methods discussed in this review: hydrodynamic chromatography (HDC), capillary gel electrophoresis (CGE), asymmetrical flow field-flow fractionation (AF4), micro-flow imaging (MFI), dynamic light scattering (DLS), resonant mass measurement (RMM), sedimentation velocity analytical ultracentrifugation (SV-AUC), size-exclusion chromatography combined with multi-angle light scattering (SEC-MALS) and light obscuration (LO).

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standard in biopharmaceutical characterization due to the wide range of sample concentrations and the sensitivity to small fractions of aggregates.[70] However, the quantification of protein aggregates depends on the equipment and analysis variables controlled by the operator, leading to large and poorly understood variability between different measurements.[67,71,72] Furthermore, precision and limit of detection/quantification of SV-AUC is considered lower than for SEC.[41,73] LO allows counting of individual subvisible particles by size from approximately 1 to 150 µm, depending on the probe used.[41] Therefore, LO was the preferred method by the USP and EP regulatory agencies to define the maximum numbers of particles allowed in the final biopharmaceutical formulation.[58] Nevertheless, LO presents severe weaknesses related to limit of detection and false negative results if the particle's transparency is too high and therefore not detectable by the instrument. Moreover, LO cannot differentiate between protein aggregates, particles from extraneous source and air bubbles.[41,58,74] Hence the development of highly sensitive and robust analytical techniques that are able to detect the entire size range of aggregates is crucial for the development of a continuous mAb manufacturing platform.

DESIGN CONSTRAINTS FOR THE MINIATURIZATION OF A PAT TOOL FOR CONTINUOUS BIOMANUFACTURING

PAT is defined as 'a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality'.[11] The ultimate goal of implementing PAT in the biopharmaceutical industry is to design and develop well-understood processes that will reliably ensure a predefined quality in the final product by either real-time monitoring of the raw material or in-process product attributes to control the process.[62,75,76] Biopharmaceutical quality is then built into the process, rather than being tested before the release of the product final form.[62] The efforts made to implement QbD approaches and the development of PAT tools have put a focus on analytical method development, frequently titled analytical QbD (AQbD). With the integration of QbD principles, the analytical methods developed will be far more robust, sensitive and cost efficient.[77] A possible solution, as previously discussed, is to miniaturize the analytical method.

Several PAT applications can be defined: at-line, on-line, in-line and off-line, as illustrated in Fig. 2.[62,75,78-80] Although biomanufacturing processes, especially continuous processing, have much to gain from PAT implementation, these types of tools have been fairly unexplored for aggregation detection.[62,81,82] Though all steps in a continuous process would directly benefit from aggregate detection PAT tools, the crucial steps to obtain aggregate information would be after Protein A chromatography/viral inactivation, between and after the polishing steps, and after the ultrafiltration step (indicated in Fig. 3). During Protein A chromatography and the viral inactivation steps, mAbs are exposed to low pH conditions, which might cause structural changes.[33] Polishing steps, such as cation- and anion-exchange chromatography, are performed to eliminate impurities, including aggregates. Thus a PAT tool placed between and after the two unit operations will inform if these aggregates are eliminated from the product stream. Ultrafiltration is used for the concentration and to obtain a final formulation of the mAb. Here, mAbs will be exposed to physical stress due to pumping, which may induce aggregate formation.[33]

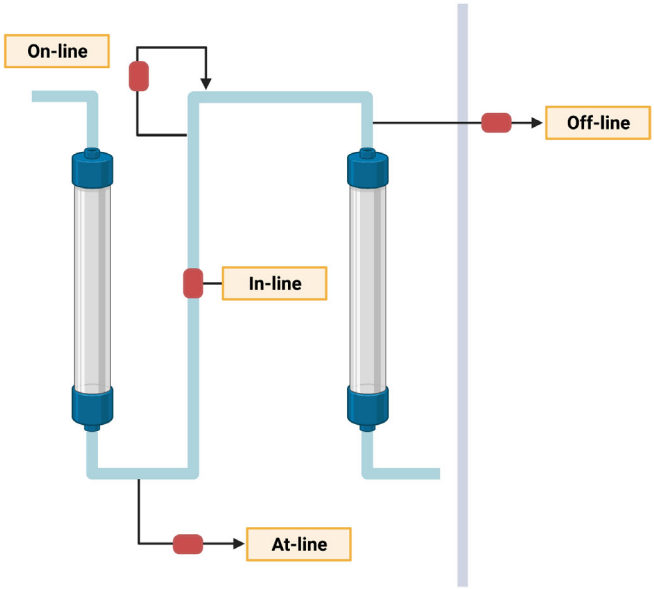

Figure 2. Schematic representation of different PAT applications in a continuous chromatographic set-up: at-line, where the sample is removed and analyzed close to the process stream, with laboratory analysis at the biomanufacturing site; on-line, where the sample is removed from the process stream, analyzed and returned to the original process stream after the measurement; in-line, where the sample is not removed, but analyzed *in situ* (the process stream may be disturbed; for example, a probe may be inserted); and off-line, where the sample is removed and analyzed away from the original process stream, at a separate site.[62,75,78-80] Figure created with BioRender.

The crucial element for PAT application in a continuous downstream process is to gather real-time information for process control.[75,82] However, especially for the chromatography steps, implementing PAT can be challenging because of the typical short process times (in the time frame of minutes).[62] Rathore *et al*. demonstrated that, with on-line analytical liquid chromatography (with the measurement performed in 30 min), continuous monitoring of the chromatography step for aggregate peaks can be achieved. An on-line high-performance liquid chromatography system was programmed to investigate the real-time pooling of an eluting product during a process chromatography step and stopped collecting when the aggregate peak started, showing the feasibility of using PAT to facilitate real-time decisions for column pooling based on CQAs.[83] Due to the inherent short reaction times provided by miniaturization of the analytical technique, the much-needed real-time detection can be achieved, delivering immediate information to regulate manufacturing conditions.

To evaluate an analytical technique's potential as a real-time miniaturized PAT tool, several constraints must be met. This includes the minimization of measurement time frame within the range of seconds to a few minutes. To reach this time frame, the technique should require minimal to no sample preparation. Furthermore, the measurement should preferably be in-line, i.e. the stream should be analyzed *in situ*. On-line and at-line measurements can also be applied as long as the result obtained is within the necessary time frame. Another important requirement is that the analytical technique should cover the broadest size

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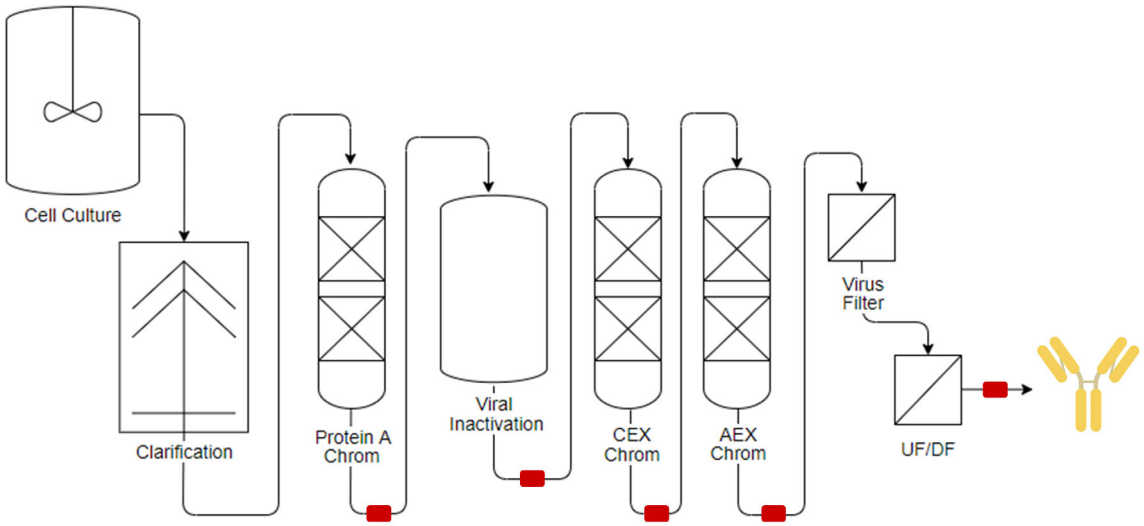

Figure 3. A typical mAb purification platform process: the first step is cell culture to produce the biopharmaceutical, followed by an initial clarification to remove the cells. Then, the downstream process begins, with a capture step, Protein A chromatography, followed by a viral inactivation step and two polishing steps: cation- (CEX) and anion-exchange (AEX) chromatography. An additional virus retentive filtration step follows and, finally, an ultrafiltration/diafiltration (UF/DF) step to formulate and concentrate the product. Regarding a PAT sensor for the detection of aggregates, all the aforementioned steps would benefit in the implementation of this tool but the crucial steps where more information could be retrieved are marked in red: after the Protein A chromatography/viral inactivation step, between and after the two chromatographic polishing steps and the final UF/DF step.

range of aggregates which can arise, ranging from the dimers to larger visible particles. If possible, the chosen method should be able not only to detect the formation of high-MW species, but also to distinguish and quantify the different types of aggregate. The analytical technique should have high sensitivity to detect even low aggregate levels in the continuous process, and robustness to provide reproducible measurements.[84] Finally, other significant conditions to consider are the overall cost of the technique and operational simplicity of the microfluidic chip, where additional training of operators is minimized.

Taking into account the design constraints, several analytical techniques were evaluated for possible miniaturization for the detection of mAb aggregates. A few analytical techniques that are able to provide a real-time measurement for mAb aggregate detection were excluded, as the technique could not be miniaturized. For example, micro-flow imaging (MFI) is a widely applied analytical technique to detect and characterize mAb aggregates.[20,85-88] MFI is a flow microscopy technology, where bright field images are captured in successive frames as a continuous sample stream passes through a flow cell.[89] MFI is more sensitive with regard to the detection of visible particles compared to other techniques and offers a better differentiation between the various subpopulations having different shapes via imaging filters.[88-90] For example, Kalonia *et al.* developed an accurate method to calculate the mass of visible protein particles using particle number and size data obtained from MFI, which was evaluated with stressed IgG solutions.[29] However, the equipment employed for this technique, a flow cell and a camera with high magnification,[89] cannot be miniaturized or the measurement itself cannot be performed within a microfluidic chip. Similarly, LO also requires a non-miniaturizable flow cell to perform the measurement.[74] Therefore, MFI and LO will not be further explored in this review. A summary of the considered analytical techniques and their characteristics can be found in Table 1. In the next section, a more in-depth explanation is provided for each evaluated technique with regard to the creation of a miniaturized PAT tool.

MINIATURIZABLE ANALYTICAL TECHNIQUES

This section provides a detailed examination of all the evaluated analytical techniques, where inherent characteristics and drawbacks for miniaturization are considered. Previous reported examples on how the respective analytical method is applied for the detection of aggregates are also presented. These techniques were divided into two categories according to the principle of aggregate detection: techniques that detect aggregates according to changes in the MW of the mAb monomer (colloidal stability) and the techniques that assess differences in the mAb monomer structure (conformational stability). Essentially, colloidal stability relates to a protein's ability to stay in a monomeric state and is influenced by protein–protein interactions (depending on the particle distance), forming small soluble aggregates. On the other hand, conformational stability is related to 3D-structural changes of the protein's native form, where the analytical technique measures conformational alterations in the denatured state (or an intermediate state) of the protein.[91,92]

Study of aggregation: colloidal stability

Fluorescent dyes

One common route for the formation of protein aggregates is through interactions of exposed hydrophobic areas. Fluorescence probes that are sensitive to the hydrophobicity of the surrounding environment, such as anilinonaphthalene-1-sulfonate (ANS), 4-4-bis-1-phenylamino-8-naphthalene sulfonate (bis-ANS), Nile Red and SYPRO Orange, can be used to study this phenomenon.[61,93,94] In the presence of hydrophobic unfolded protein structures, the dye's fluorescence will strongly increase compared to the intensity in the presence of the native monomeric mAb form,[93] providing an immediate and straightforward result. However, ANS-based probes have been shown to bind to some proteins through electrostatic as well as hydrophobic interactions, which can interfere with protein folding and unfolding pathways.

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Table 1. A summary of the analytical techniques discussed in this review, considering the size range, simplicity, in-line measurement, robustness, sensitivity, time, sample preparation, quantification of the aggregates, cost and the possible miniaturization of each technique for its implementation as a PAT tool for the detection of mAb aggregates in a continuous downstream process

Technique	Size range	Simplicity	In-line measurement	Robustness	Sensitivity	Time	Sample preparation	Quantification of aggregates	Cost	Possible miniaturization
Study of aggregates on a size level										
Size exclusion chromatography with multi-angle light scattering (SEC-MALS)	1–100 nm	●●	Yes	●●●	Medium	Slow[41]	Concentrated sample	Yes (soluble aggregates)	High	No
Analytical ultracentrifugation (AUC)	1 nm –1 μm	●	No	●	High[41]	Slow[41]	None to Minimal (dilution for high protein concentrations)	Yes	High	No
Light obscuration (LO)	1 μm –150 μm	●●●	Yes	●●	Low[41]	Immediate (few seconds)	Necessity of sample dilution	Yes	Low	No
Fluorescent dyes	10 nm– 1000 μm	●●●	No	●●●	High[61]	Immediate (few seconds)[61]	Concentrated sample	Possible	Low	[99,102-111]
Dynamic light scattering (DLS)	1 nm–10 μm	●●	Yes	●	Poor (for very low aggregates levels)	Rapid (few minutes)[41]	None to minimal (dilution for high protein concentrations)	Not suitable	Low	[121-123]
Micro-flow imaging (MFI)	1–400 μm	●●●	Yes	●	Poor[89]	Rapid	Necessity of sample dilution	Not suitable	Low	No
Asymmetrical flow field-flow fractioning (AF4)	1 nm–100 μm	●●	Yes	●●●	Medium	Slow[130]	None to minimal (dilution for high protein concentrations)	Yes (soluble aggregates)	Medium/ high	[130-133]
Capillary electrophoresis (CGE)	1 nm–100 μm	●●●	Yes	●	Low	Rapid	None to minimal (dilution for high protein concentrations)	Yes (soluble aggregates)	Low	[139-142]
Hydrodynamic chromatography (HDC)	0.7–110 nm	●●●	Yes	●	Low	Immediate	None to minimal	Yes (soluble aggregates)	Low	[114]
Resonant mass measurement (RMM)	50 nm–5 μm	●●	Yes	●●●	Medium[144]	Rapid (few minutes)[144]	None to minimal	Yes	Medium	Already performed in a microchannel[143]

Table 1. Continued

Technique	Size range	Simplicity	In-line measurement	Robustness	Sensitivity	Time	Sample preparation	Quantification of aggregates	Cost	Possible miniaturization
Study of the aggregates on a structural level										
Fourier-transform Infrared spectroscopy (FTIR)	X	●	Yes	●●●	Medium[41]	Rapid[41]	Minimal	Not suitable	Low	150,151
Native mass spectrometry		●	Yes	●●●	High[69]	Rapid	None	Yes	High	159,161,187,188
Ultrasound		●●	Yes	●●	Medium	Rapid[116]	None	Possible	Low	170
Circular dichroism (CD)		●●●	Yes	●	Poor[41]	Rapid[183]	Minimal	Not suitable	Low	185,186
Raman spectroscopy		●	Yes	●●●	Medium[171]	Rapid[41]	Concentrated sample	Not suitable	Low	178-180

●, low; ●●, medium; ●●●, high.

Additionally, Nile Red is highly sensitive to pH and to buffer type, which can impair signal detection.[93] A novel class of fluorescent molecular rotors, such as Proteostat, thioflavin T (ThT), 9-(2,2-dicyanovinyl)julolidine (DCVJ) and 9-(2-carboxy-2-cyanovinyl)julolidine (CCVJ), recently emerged as possible alternatives to the classic fluorescence probes. These novel fluorescent molecular rotors are mainly sensitive to changes in the viscosity of the environment and less to polarity.[95,96] The molecular rotors rotate freely in solution, but changes in the micro-environment will restrict the dye's movement, resulting in fluorescence emission.[96] For example, ThT will give information on aggregation due to the presence of β-sheet structures in a protein's structure since ThT binding is linked to the presence of this structural motif in fibrils and in amyloid formation, allowing dissection of its aggregation mechanism.[97,98] A summary of the application of these fluorescent dyes, sensitive to the environment's hydrophobicity and viscosity, for the detection of mAb aggregates, can be found in Table 2. Additionally, these dyes can be used in combination with other analytical techniques to develop an online fluorescent dye detection method, such as high-pressure size exclusion chromatography (HP-SEC) and asymmetrical flow field-flow fractionation (AF4), as demonstrated by Hawe *et al.* This online fluorescent dye detection for HP-SEC or AF4 is a viable method to detect both aggregation and structural changes of both monomeric and aggregated mAbs.[30,95]

However, employing fluorescent dyes requires mixing the dyes with the sample. Microfluidic systems operate in a laminar flow regime, which complicates mixing of the sample with the fluorescent dye.[99] Mixing efficiency can be achieved with microfluidic structures by inducing chaotic advection or increasing the contact area of fluidic layers. Mixing within microfluidics can be separated into two main categories, namely passive and active mixing.[100,101] Passive mixing is achieved by altering the structure or configuration of microfluidic channels and is incorporated into the system during microfabrication. For example, using distinct zigzag angles,[102] different zigzag[103] or serpentine configurations,[104] slanted wells[105] or obstructions[106] in the middle of the channel are considered passive methods. The major advantage of applying passive mixing is relatively simple fabrication and operation, as moving parts are not necessary, meaning that it is not externally controlled by users.[99] On the other hand, active mixers are activated by the user and apply external forces for stirring or agitating the fluid flow.[100] This controllable mixing may be carried out using pressure gradients, electrical voltages across the fluid or integrated mixing elements like stirring bars.[99,107] Microstirrers,[108] acoustic waves,[109] flow pulsation[110] and thermal enhancement[111] fall into this category. Even though active micromixers can reach higher mixing efficiency compared to passive mixing, the integration of peripheral devices for external power source and the complex and expensive fabrication process are severe limitations for practical applications. Furthermore, active micromixers can produce high temperature gradients, which can potentially influence mAb stability. Therefore, active mixers are not an ideal solution for biological applications.[101] For more information related to mixing in a microfluidic chip, other reviews should be consulted.[99-101,112]

Thus, due to several commercially available fluorescent dyes and recent advances in microfluidic design and fabrication, the use of fluorescent dyes in a miniaturized chip can be a reliable solution for the development of a real-time measure of the level of aggregation in a continuous process. Nevertheless, fluorescent dyes do not allow for the quantification and differentiation of the type of mAb aggregates, which might limit its implementation as a broadly applicable PAT tool.

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Table 2. Summary of available fluorescent dyes, sensitive to hydrophobicity and viscosity of the environment, applied to the detection of mAb aggregates

Fluorescent dye	Excitation wavelength (nm)	Emission wavelength (nm)	Type of mAb aggregates	Concentration range ($\mu mol\ L^{-1}$)	References
Bis-ANS	385–400	470–530	Oligomers (dimers, trimers)	1–5	30,95,189,190
ANS	350–380	505	Oligomers (dimers, trimers)	25–100	88,97,190,191
Nile Red	540–580	580–660	Insoluble aggregates and visible particles	100	120,189,192
SYPRO Orange	485–495	550–700	Oligomers, insoluble aggregates and visible particles	1×–5×*	93,96,189,193,194
Thioflavin T	415–450	480–600	Oligomers (high order) and insoluble aggregates	2–50	96,97,189,190,192,195
Proteostat	448–530	560–700	Oligomers, insoluble aggregates and visible particles	1×*–3	96,194
DCVJ	450	470–530	Oligomers and insoluble aggregates	5–100	95,189,193
CCVJ	435	450–650	Oligomers and insoluble aggregates	5	95

Excitation and emission wavelength, type of mAb aggregates and concentration ranges used are also described.*The manufacturers of these dyes do not specify the molar concentration of the product. SYPRO Orange dye is commercially supplied in 100% (v/v) dimethyl sulfoxide (DMSO) at 5000× the final working concentration, being diluted to achieve a 2×/1× SYPRO Orange final concentration. Proteostat dye is commercially supplied in 100% (v/v) DMSO at 1000× the final working concentration, being diluted to achieve a 1× Proteostat final concentration.

Hydrodynamic chromatography (HDC)

HDC provides a fast and efficient analysis as it is based on the effect of the flow profile on an analyte carried through a tube of comparable size and does not involve mass transfer. In HDC, larger molecules or particles are transported faster than smaller ones in a narrow channel (effective size ≤ 1 μm) with a laminar flow, as they cannot fully access slow-flow regions near the conduit walls.[113] A schematic representation of this process is shown in Fig. 4.

To miniaturize this technique, some constraints need to be taken into account upon the design of this chip: a long separation channel with a flat channel fluidic is required, with a large aspect ratio to increase the detection sensitivity (1 μm deep, 1000 μm wide and 8 cm long, for example), as demonstrated by Chmela *et al*. This method presents three main advantages, namely (i) high separation efficiency due to the microfluidic integration, (ii) fast analysis, with the separation occurring in 3 min, and (iii) a size range from 0.7 to 110 nm (soluble and insoluble aggregates).[114] From the same research group, Blom *et al*. used a similar planar chip configuration to separate a biopolymer mixture, ranging from 26 to 155 nm of size, within 70 s.[115]

Despite the desirable characteristics for PAT implementation, the detection of mAb aggregates by means of HDC has not yet been demonstrated and validated. Moreover, this technique only allows for the separation of molecules according to size; thus the detection of the monomer or aggregates at the end of the channel must be performed resorting to other analytical techniques. However, HDC has been coupled to a multiplicity of detection methods, such as UV or a light scattering technique, being able to provide information on the particles' MW, shape and structure.[113,115]

Light scattering technique: dynamic light scattering (DLS)

Light scattering is employed to detect and characterize soluble aggregates, with a size range from 1 nm to 5 μm.[20] Several types of light scattering methods are available and, as an example, this review will focus on DLS. DLS has been chosen based on the employment frequency in published aggregation studies and miniaturization experiments. DLS captures the Brownian motion of dispersed particles, which can be related to its hydrodynamic radius by the Stokes–Einstein equation.[41] DLS does not require sample preparation and allows for fast (few minutes) and high-throughput measurement.[41,116] Despite presenting several advantages for implementation in a continuous process, DLS lacks sensitivity, especially for low aggregate levels.[41,116] Additionally, in a continuous downstream process, the mAb solution will be highly concentrated. The high protein concentration can lead to the loss of light scattering intensity due to multiple scattering effects, making this technique only applicable as a semi-quantitative measurement.[20] However, the sensitivity of the technique can increase for a larger particle size due to the higher scattering intensity of larger particles, which is beneficial for detecting even the slightest quantity of large aggregate molecules.[117]

Regarding the use of DLS for mAb aggregate detection, a multitude of studies have been published employing this analytical tool for characterization of these types of species.[97,117-120] For example, Ahrer *et al*. showed the ability of DLS to detect small traces of aggregates in IgG samples from different process steps without sample preparation.[119] Similarly, Arosio *et al*. followed the aggregation kinetics *in situ* for IgG monomers and oligomers, characterizing and studying the stability of three different antibodies.[97]

To perform DLS measurements in a microfluidic chip, Destremaut *et al*. used low flow rates (and thus shear rates) in a tall microchannel fabricated in polydimethylsiloxane (PDMS) and glass. Dimensions for such a microchannel were length 3 mm, width 500 mm and height 700 mm. However, to perform a DLS measurement, a specific external set-up still needs to be operated. This set-up is depicted in Fig. 5. The set-up included a laser beam directed through the microfluidic device, with the alignment of the incident wave vector (k_i) of the laser orthogonal to the microfluidic chip and spatial filters collecting the scattered electric field. The measurement time of this miniaturized DLS device is approximately 300 ms, depending on the employed flow rate.[121] Even

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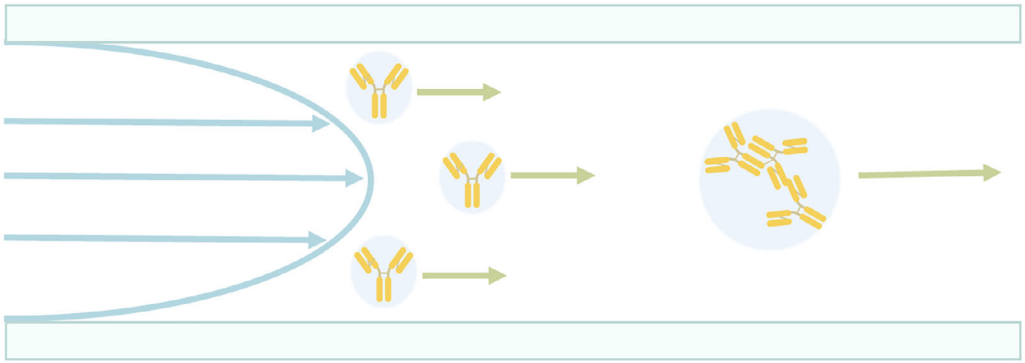

Figure 4. Physical principle of HDC separation: in a laminar flow regime, larger particles, such as mAb aggregates, are transported faster than smaller ones, mAb monomer, as these high-molecular-weight species cannot fully access slow-flow regions near the conduit walls. As such, the aggregates remain near the center of the flow profile where they preferentially experience faster streamlines.

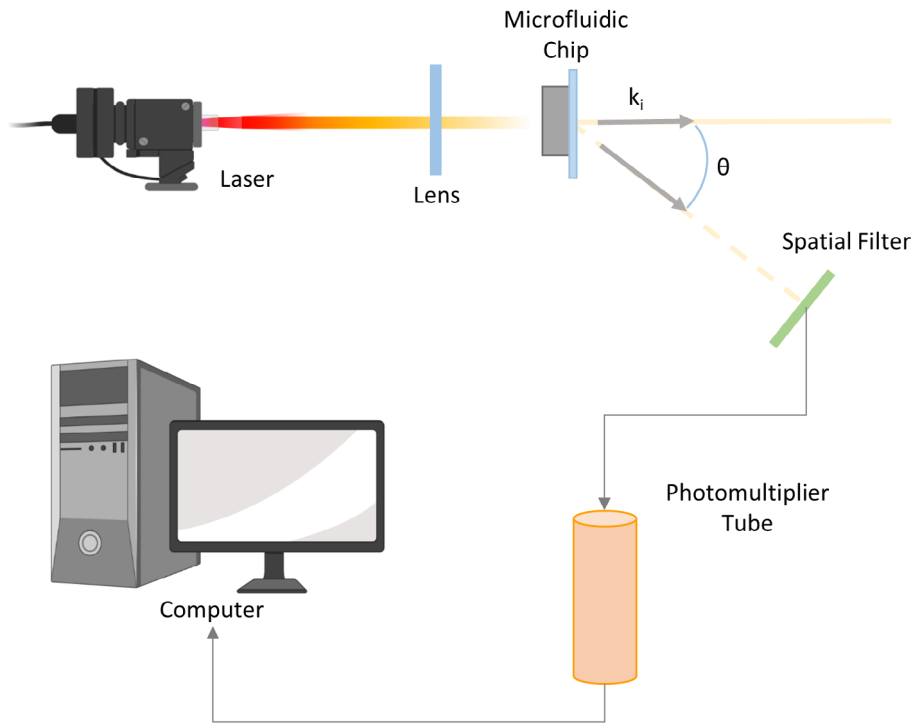

Figure 5. A simplified external set-up necessary to perform DLS in a microfluidic chip. A laser beam is directed through the microfluidic chip, and focused inside the channel through the lens. Alignment of the incident beam is performed to have the incident wave vector k_i orthogonal to the chip. The spatial filter collects the electric field scattered at angle θ by the sample in the microchannel. The collected scattered light is then directed to the photomultiplier tube, and the information is sent to a computer.[121]

though a fast and continuous measurement can be obtained with this configuration, the external set-up and alignment required complicate the implementation of DLS. Therefore, Chastek and co-workers integrated an on-line fiber optic DLS into the microfluidic chip, making the laser and detector fiber optic probes in direct contact with the sample. Thus typical problems associated with laser alignment and light refraction are minimized as the multiple scattering is reduced.[122,123]

The two aforementioned examples demonstrate that DLS is a promising on-line PAT tool to study mAb aggregation in real time in a continuous process. Nevertheless, DLS cannot be completely miniaturized as it requires external hardware, such as a laser source and scattered light detectors.

Asymmetrical flow field-flow fractioning (FFF)

FFF is a separation method able to set apart molecules ranging from the lowest nanometer range over to a two-digit millimeter range with high resolution. Separation is performed inside a narrow ribbon-like channel, with typical dimensions of approximately 50 cm in length, 2 cm in width, and between 50 and 500 mm in height. A carrier liquid is pumped through the channel, establishing a parabolic laminar flow profile that forces the sample towards the outlet. An external field is applied perpendicular to the direction of the carrier liquid flow, resulting in the sample components accumulating at one of the channel walls, the so-called accumulation wall.[124] Variations of this technique have been developed over the years, varying the type of field applied

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during separation, such as sedimentation, thermal and electrical changes.[124] The variant employing a hydrodynamic field to induce separation, the flow field-flow fractionation, is considered a well-established technique for the analysis of biomolecules.[125] A second stream of carrier liquid is pumped in a vertical direction to the axial flow stream. For asymmetrical flow field-flow fractioning (AF4) there is only one permeable wall, which means the carrier liquid can leave the channel solely via the accumulation wall to generate a cross-flow.[124]

In AF4, retention is inversely proportional to the hydrodynamic diffusion coefficient of the analyte and, consequently, to its molecular weight. AF4 is suitable for protein aggregate characterization, as it is selective and mechanical and shear stress is minimized by the absence of a stationary phase.[120,126-129] For example, Hawe *et al*. developed an AF4 method for the analysis of protein aggregates in the size range of 10 nm up to 1000 nm and used this method to evaluate aggregation in heat-stressed IgG formulations. A better separation and recovery was achieved with AF4 when compared to high-performance SEC, proving that AF4 is a valuable method for quantification of submicron protein aggregates.[126] However, Bria and Williams investigated the impact of AF4 on protein aggregate species by studying the stability of two different IgG molecules as a function of different carrier liquids, shear stress (related to sample injection) and sample dilution during separation. The results showed that the employed conditions will influence aggregate formation and detection. Nevertheless, the dilution during AF4 separation is significantly lower than in SEC, with dilution occurring mainly at the channel outlet and not during the separation. This makes AF4 a powerful alternative for SEC, as long as the AF4 retention theory is used to understand the impacts of dilution on analytes.[127]

Although AF4 presents many advantages as a separation technique for aggregate detection, its adoption has been limited. This is mainly due to the large footprint of available separation cartridges, extended analysis times and solvent consumption.[130] Miniaturization of this analytical method can address these issues, with the simplification of the AF4 cartridge, reduction of reagent consumption and analysis time. Several miniaturization approaches have already been reported.[130-133] Muller *et al*. described the fabrication and characterization of miniaturized AF4 cartridges and evaluated the separation performance using gold and silver nanoparticle standards. The obtained separation performance was comparable to or even better than a normal macroscopic AF4 cartridge, where high sensitivity and improved signal to noise ratio was achieved. This was mainly due to a reduced sample dilution and, consequently, a minimal band dispersion. However, the implementation of a miniaturized AF4 method as PAT tool in a continuous process is still a major challenge. Even though a reliable separation and a real-time measurement can be achieved, this technique only allows for the separation of molecules. Thus, similar to HDC, aggregate detection relies on other analytical techniques, such as MALS. Additionally, this type of miniaturized cartridge still needs to be explored for the specific case of mAb aggregates, which are smaller particles than the nanoparticles and molecules experimentally tested.[130,131]

Capillary gel electrophoresis (CGE)

CGE separates proteins according to their size in a fused-silica capillary with the advantages of automation, higher precision and increased throughput when compared to other electrophoresis techniques, such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The band detection is based on UV or fluorescence, with the molecules being pre-labeled with a fluorescent dye, resulting in an easier and more accurate quantification.[134,135] Coupling this technique with mass spectrometry (CGE-MS) can overcome limitations of optical detections and provide further structural information.[135] CGE can be performed under both non-reducing and reducing conditions to characterize the size heterogeneity of the intact mAb.[134,135] Therefore, CGE has been widely used for high-resolution separation and quantification of mAb aggregates and fragments to ensure the quality of mAb therapeutics.[135-138] For instance, Rouby *et al*. employed CGE, among other techniques, to study the nature and structure of aggregate species present in an unstressed formulated mAb.

The miniaturization of CGE has been developed since the 1990s, as a result of the need for a high-throughput analysis while maintaining resolution and efficiency.[139-142] More recently, especially for mAb analysis, this miniaturization has been recognized as a valuable alternative.[136] Smith *et al*. used a commercially available chip to develop and validate a microfluidic CGE method to study biopharmaceuticals size variants and purity in reducing and non-reducing conditions. Evaluation parameters such as specificity, accuracy, reproducibility and limit of detection/quantification were assessed. The microfluidic CGE platform enabled a rapid testing for product development support, due to the inherent high throughput characteristics.[142] Therefore, as CGE is already a well-established and validated technique used in biopharmaceutical industry for the detection of mAb aggregates, microfluidic CGE can easily be implemented as a PAT tool in a continuous process, allowing for a fast and robust measurement of aggregation levels.

Resonant mass measurement (Archimedes)

Resonant mass measurement (RMM) allows size particle analysis based on the Archimedes principle: any object submerged in a fluid is acted upon by an upward buoyant force, for which the magnitude is equal to the weight of the fluid displaced by the object.[74,143] The Archimedes system (Malvern Instruments, Malvern, UK) is the first and, until now, the only RMM instrument available,[74] and it has been largely employed in research and development of biopharmaceuticals. The sample solution to be studied is flushed through a microchannel inside a resonating cantilever (called a suspended microchannel resonator (SMR)), which changes its frequency depending on the mass of the particles passing the channel.[144] With a size range from about 50 nm up to 5 μm, depending on the sensor,[143,144] RMM can analyze the subvisible range. Nevertheless, possible drawbacks of RMM are the tendency of protein aggregates to stick to and clog the sensor microchannels. Thus intermediate cleaning procedures (with detergents or bleach) and regular cleanliness checks are necessary between measurements, which prevents a faster analysis and complicates the process procedure.[74]

Published studies employing RMM are still scarce, especially for the detection of mAb aggregates. However, Weinbuch *et al*. used RMM and MFI as orthogonal methods to analyze protein particles and silicone oil droplets, covering the submicron and micron size range. RMM showed a highly accurate discrimination in the size range from 0.5 to 2 μm, as long as a sufficient number of particles (>50 particles) was counted.[144] Panchal *et al*. compared RMM to DLS by measuring, among other solutions, low- and high-concentrated aggregated IgG samples (5 and 100 mg mL^{-1}, respectively). The RMM limit of detection was determined for the IgG samples of ~150 nm. Additionally, the authors showed

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that RMM did not accurately measure the particle size distribution owing to the small volume tested. However, by testing multiple samples and performing longer analysis times, this limitation can be surpassed.[143]

Since RMM measurement is already performed in a microfluidic channel, further improvements for its implementation should focus on increasing the limit of detection, especially for samples with high particle quantities, the life span of the sensor,[74] and adding a temperature control to inhibit further aggregation.[143] Moreover, additional experimental studies for the detection and quantification of aggregation still need to be performed to gain a better understanding of the RMM potential and possible limitations.

Study of the aggregates: conformational stability

Fourier-transform infrared spectroscopy

Fourier-transform infrared (FTIR) spectroscopy provides information on the secondary structure of a protein by characterizing the amide I, amide II and amide III bands and, consequently, any possible conformational changes that occur.[145] The evolution in IR instrumentation resulted in a straightforward data collection within seconds, enabling real-time monitoring.[146,147] Several studies employed FTIR spectroscopy for the detection and quantification of mAb aggregates as well as protein stability tests.[88,146,148,149] More specifically, this analytical technique was already employed as an at-line measurement in a downstream process by Capito *et al*. At a laboratory scale, FTIR was used to quantify the aggregation levels in different unit operations. The authors demonstrated that FTIR cannot be fully applicable for all processing steps, as it is only suitable for processing steps that result in higher aggregate formation (filtration and low-pH virus inactivation).[146]

A severe drawback for the miniaturization of this technique is that materials used for the fabrication of the microfluidic device, such as polymers (PDMS, more specifically), will strongly absorb mid-IR radiation.[150] A possible solution, demonstrated by Birarda *et al*., would be to fabricate the device in transparent calcium fluoride (CaF_2).[151] Another solution was presented by Srisa-Art and Furutani, where an alternative fabrication method was used to minimize the thickness of PDMS. FTIR measurements would be performed in the optically thin PDMS microchannel layer, and a supportive layer was incorporated into the device for handling and connections.[150] In addition to the issues related to the microfluidic chip materials, performing FTIR spectroscopy in a microfluidic chip requires an external hardware set-up built around the chip to collect the signal. Since IR spectroscopy is already applied as a PAT tool for real-time control of the protein A chromatography protein loading and the output concentration of an ultrafiltration step in a continuous process,[152,153] FTIR spectroscopy miniaturization might bring the needed optimization. To advance FTIR spectroscopy for real-time PAT purposes, further research should focus on instrumentation and data collection to increase accuracy and limit of detection for aggregate quantification.[145,146]

Mass spectrometry (MS)

MS is considered one of the most powerful analytical techniques, due to its high sensitivity, accuracy and high-throughput capabilities. MS provides structural information by measuring the mass-to-charge ratio (m/z) values of the charged molecules. Three elements are required to perform MS, namely (i) a ionization source, (ii), a mass analyzer and (iii) a detector.[154] For the ionization source, two options are able to ionize sample molecules with minimal fragmentation. The first is electrospray ionization (ESI), where the sample is infused through the electrospray emitter, usually in a needle-shaped structure. The second option is matrix-assisted laser desorption/ionization (MALDI), where the sample is co-crystallized with a matrix and pulses of UV laser are used to vaporize the matrix and liberate the molecules as gaseous ions.[155]

In terms of employing MS for the detection of aggregates, the hyphenation of this technique with non-denaturing liquid chromatographic (LC) modes (ion exchange (IEX), SEC and hydrophobic interaction chromatography (HIC)) has attracted attention in the last years.[156] Ehkirch *et al*. proposed a multidimensional analytical approach combining on-line SEC to ion mobility and mass spectrometry (IM-MS) for structural characterization of mAb size variants under native conditions.[157] Toth *et al*. used hydrogen exchange mass spectrometry (HX-MS) to study the stability of an IgG4 mAb and compared the data obtained with other orthogonal techniques, such as SEC and MFI. The authors demonstrated that HX-MS can be set up as a methodology to screen formulation excipients for their ability to physically stabilize mAbs.[158]

The coupling of microfluidic chips to MS can greatly improve the implementation of this technique and expand the MS analysis for applications requiring fast measurement time and enhance sensitivity.[155] Various ionization sources and mass analyzers have been developed, with the most common approach being the miniaturization of ESI and its integration with various separation and sampling units. Redman *et al*. developed a microfluidic chip incorporating CE with ESI to analyze a lysine-linked antibody drug conjugate for possible post-translational modifications and drug load variants.[159] More recently, nanoelectrospray has become the most widely exploited ionization method, a modification of ESI that enables reduction in the applicable flow rates and increase in detection sensitivity. Additionally, nanospray emitters are easily fabricated and allow a straightforward coupling to microfluidic separation devices due to the use of equal flow rates.[160] Yin *et al*. developed a microfluidic chip composed of an integrated LC column, a sample enrichment column and a nanoelectrospray tip for analysis of proteins, demonstrating the potential of microfluidic MS to be incorporated with other separation techniques in a chip.[161]

Thus a MS microfluidic chip could be a viable option as a PAT tool for implementation in a continuous process to detect mAb aggregates. Several examples can be found where direct analysis in real-time MS (DART-MS) was used as a PAT tool for monitoring processes.[162,163] Important to mention is that the detector component of MS cannot be miniaturized, so an external set-up to collect the ions still needs to be utilized. This external set-up can be an obstacle for its implementation within the biopharmaceutical industry due to the overall cost of the equipment. By comparing the instrumentation costs with the other discussed analytical techniques, MS might not be as affordable. Further improvements should focus on the ease of operating such equipment and on data treatment to extract relevant information for its implementation as a miniaturized PAT tool.

Ultrasound spectroscopy (US)

US presents the greatest potential for process control from all the presented analytical techniques for the detection of structural changes. US is considered a relatively rapid technique, with minimal sample preparation, and has been shown to detect changes as a result of aggregation and conformational instability of proteins. Moreover, US is a non-invasive technique that can be performed in-line, readily applicable as a PAT tool for monitoring

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and control.[116,164] The physical principle of US lies in the compression and decompression of the sample medium caused when ultrasonic waves pass through a sample. This (de)compression leads to change in distance between particles and molecules in the sample, which in turn represents intermolecular attraction and repulsion.[165,166] Thus the analysis of aggregate formation by the sound-scattering properties of the dispersed particles should, in theory, be possible.[41] However, changes detected by other analytical techniques can be assigned to definitive structural changes to a protein, whereas, for ultrasound, this is not currently possible. Further work is required to understand what aspects of a protein's structure can be monitored by US.[116] Furthermore, although US has been used to investigate aggregation of various biomolecules, such as red blood cells[167,168] and amyloidogenic proteins (insulin),[169] currently there are no studies reporting the detection of mAb aggregates in solution.

Although much effort still needs to be done to understand and validate this analytical method, a miniaturized chip for US has already been developed. Agrawal and Seshia were the first to report on the development of a microfluidic device using continuous-wave broadband US. This miniaturized device consisted of a set of ultrasound transducers integrated throughout a microfluidic channel and was validated by measuring various concentrations of glucose–water solutions.[170] Since US displays numerous advantages compared to other analytical techniques, the further development of US microfluidic chips can assist a better understanding of the method itself and the results obtained.

Raman spectroscopy (RS)

RS measures the inelastic scattering of light from molecules when excited by a monochromatic light source. While the majority of photons are scattered without any change in energy, a small fraction will be scattered at a different energy from that of the incident light (Raman scattering). This difference in scattered frequencies, the Raman shift, is characteristic to a chemical bond, resulting in a unique spectrum for each molecule. Thus RS is able to provide dynamic information about secondary structure, tertiary structure and aggregation mechanisms.[171] RS is robust and non-destructive, and sample preparation is not required. Raman scattering can provide simultaneous real-time information about multiple components and molecules, making it ideal for on-line monitoring of a process.[22,172,173] Nonetheless, this technique presents significant drawbacks. For example, the Raman effect is an inherently weak process, with only one out of 10^6–10^8 photons being scattered this way.[171,174] Additionally, fluorescence is also a well-known limitation of RS and the laser heat may induce changes in the sample.[116,171] Therefore, significant technological advances have been achieved in recent years with regard of RS instrumentation, ranging from the efficiency and variety of sensors and low noise detectors.[171]

RS is a well-established PAT tool in the upstream production of mAbs, where it is widely used as an in-line measurement to perform a real-time control of multiple attributes in a cell culture bioreactor, such as consumption of nutrients and the production of metabolic waste products.[172,173,175] However, regarding the downstream processing of mAbs, RS has remained fairly unexplored, especially for the detection of mAb aggregates. Zhang *et al*. presented a method combining RS with a multivariate analysis that could differentiate between aggregation levels in mAb samples,[176] demonstrating the feasibility of using this technique for the detection of aggregates. Additionally, McAvan *et al*. demonstrated, for the first time, the ability of RS to differentiate between degraded samples of IgG4 with differing fragmentation and mixed aggregate species.[177]

In recent years, great effort has been placed in the miniaturization of this technique to solve limitations regarding the intrinsically weak scattering signal.[174] For example, Ashok *et al*. developed a novel RS detection scheme: waveguide-confined Raman spectroscopy (WCRS). A major limitation of the implementation of WCRS at the micro-scale is that the weak Raman signal can be overwhelmed by a strong spectral background from the material in which the microfluidic chip is fabricated. Thus, to fabricate this microfluidic structure, it is crucial to minimize the interference of the background. This can be achieved by embedding fibers throughout the chip's geometry, confining the Raman excitation and collection region and ensuring maximum Raman signal collection.[178] Another approach to enhancing the weak Raman signal is bringing the target molecules into the proximity of metallic nanostructures, denominated surface-enhanced Raman spectroscopy (SERS).[174] Zhou *et al*. created a SERS microfluidic platform that was fabricated in PDMS and contained gold nanoparticles. This microfluidic SERS platform was able to detect bovine serum albumin, down to a picomolar level. Choi *et al*. fabricated a microfluidic device using gold nanoparticle-based SERS-active substrate to study the aggregation of an amyloid β-peptide. This microfluidic device was sensitive enough to detect the aggregates at concentrations much lower (from 10 fmol L^{-1} up to 1 μmol L^{-1}) than the limit of detection of existing instrumentation.[180] Therefore, the miniaturization RS can be the driving force to implement RS as a downstream PAT tool since it will allow for a more sensitive and accurate measurement of the intrinsically weak Raman signal.

Circular dichroism (CD)

CD is a light absorption spectroscopy method based on the difference in absorbance of left- and right-circularly polarized light. Optically active chiral molecules, such as proteins, will preferentially absorb one direction of the circularly polarized light. CD has been widely used to analyze the secondary structure of optically active biomacromolecules.[181] Even though CD does not provide information on the secondary structure of specific residues, the method has the advantage that the measurement is sensitive (≤20 μg of protein sample[182]) and can be collected in real time and *in situ* for the monitoring of molecular aggregation.[181]

In the past years, numerous publications have used CD as an orthogonal method to study and characterize mAb aggregation.[88,97,120,183,184] Furthermore, Joshi *et al*. employed this analytical method as a high-throughput screening tool for examining mAb stability in various buffers, proving that CD is a reliable method for rapid monitoring of mAb conformational changes.

Regarding the miniaturization of CD, a major challenge was the incompatibility of conventional materials used in the fabrication of these devices, such as PDMS, with the far-UV measurement needed in CD.[185] Kane *et al*. reported a microfluidic chip employing synchrotron radiation circular dichroism (SRCD), where an intense light source is used for these measurements to study protein refolding kinetics of cytochrome C. The mixing devices used were made of fused silica with the beam light focused on a masked slit.[186] Bortolini *et al*. tried to overcome the polymer incompatibility problem by using conventional PDMS-based soft lithography to fabricate the CD microfluidic chip. The authors studied two architectures with measurement chambers of different height, to allow an SRCD measurement within the chip. Heterogeneous protein mixtures were used to validate the chips. By

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taking advantage of the laminar flow properties inherent to microfluidic devices, these complex mixtures were separated into well-resolved size-dependent fractions, using an H-filter configuration. This design allows for a more accurate and sensitive measurement per component of the mixture since only average spectroscopic features can be resolved.[185] Thus, with these promising breakthroughs, an affordable CD microfluidic chip can be developed. This affordable chip can be used to obtain further understanding of the results acquired by CD and push its implementation as an accurate and reliable PAT tool for the detection of mAb aggregates.

Comparison of the colloidal and conformational stability methods

The miniaturization of colloidal methods, where aggregation is detected according to changes in the mAb monomer size, can be considered less complex to implement compared to conformation stability methods. In general, the design of the microfluidic chip is rather modest, with a single channel used to separate the particles (HDC, AF4 and CGE). An external set-up is not necessary, except for DLS, where a laser beam, lenses and spatial filters are required to perform the measurement. Many of the techniques can be miniaturized using affordable fabrication material, resulting in a lower overall cost. An example of a miniaturized colloidal stability method being employed is RMM, where the sample measurement is performed in a microchannel.[143] The simplicity and affordability of a miniaturized device of colloidal methods might be the ideal solution for PAT implementation in a continuous downstream processing. However, the sensitivity to detect low aggregate levels and the robustness are inferior when compared to conformational stability methods. Additionally, none of the analytical methods discussed can completely cover the entire size range of aggregates that might be formed during a continuous process. The quantification of aggregates resorting to the majority of the colloidal methods will also be possible, but only for soluble aggregates.

Conformational stability methods will allow for in-line measurement with minimal sample preparation and present overall better sensitivity and robustness. These characteristics make conformational stability methods a better fit for process control. However, the design of the microfluidic chip will be more complex and an external set-up will be required to complete the measurement. Even though several efforts have been made in the design and fabrication of these devices in affordable polymers, the overall cost is higher when compared to colloidal stability methods. Further advances in the design, fabrication material and necessary external set-up are still required to obtain a competitive alternative.

Even though the miniaturization of a PAT tool speeds up the analytical measurement, not all miniaturized devices can be easily implemented in a continuous process. By performing the measurement in a microfluidic chip, the PAT tools are transformed from in-line to on-line applications, with a reduced flow stream split from the main stream sending a sample to the microfluidic device. Although in-line aggregation measurement is preferred, on-line and at-line measurements can also be applied if results are obtained within the necessary time frame. From the analytical techniques discussed, only slow methods, like AF4, are not suitable for on-line control. Analytical techniques that provide immediate results will be ideal for process control, such as HDC and fluorescent dyes. In particular, a microfluidic device employing fluorescent dyes for aggregate detection will work at-line since the split stream cannot return to the main stream. However, the time range to perform this technique is minor, with measurement taking place within seconds,[61] making fluorescent dyes a viable alternative for miniaturization.

ADDITIONAL FUTURE CHALLENGES FOR PAT IMPLEMENTATION

A major challenge towards the transition of an integrated and continuous mAb production is the development of real-time and in-process analytics for the detection of high-MW species.[82] The particle size of these aggregates can range from several nanometers to micrometers, making it impossible for any analytical method to offer accurate, qualitative and quantitative characterization over the complete size range. A possible solution could be the combination of multiple orthogonal analytical techniques in a single microfluidic chip, thereby covering a broad size range of aggregates. At the lab scale, Bansal *et al*. combined three analytical tools to characterize and quantify aggregates over the complete size spectrum (1 nm to 300 μm).[20] However, combining two or more different techniques in a microfluidic chip, the complexity of fabrication, handling and instrumentation set-up drastically increases. Yet the increase in complexity when performing the measurement will be balanced out if more relevant information can be collected. Another benefit of combining different techniques is the ability to overcome inherent limitations. For example, by combining HDC (a separation method) with DLS (a light detection technique), the accuracy of molecular weight determination will increase since one of the drawbacks of using DLS for mAb aggregate detection is low sensitivity.

The implementation of the PAT framework is often accompanied by the application of multivariate mathematical approaches to better understand and extract relevant information from large quantities of multivariate measurements or raw data.[145] Infrared, ultrasound, CD and Raman spectroscopy are attractive methods to implement as a PAT tool, as these analytical techniques rapidly and non-destructively provide chemical and physical information. However, the use of the appropriate data analytical procedures is essential to extract the maximum amount of information from a spectrum. Multivariate data analysis (MVDA), such as partial least squares and principal component analysis, are commonly used methods in pharmaceutical and bioprocessing to create soft sensors. Biological therapeutics, such as mAbs, often show only subtle changes or structural rearrangements as a result of the aggregation mechanism, which are difficult to detect in a large molecule and interpret in a spectrum. By using MVDA, these subtle structural differences can be drawn out, as shown by McAvan *et al*.[177] Therefore, in addition to instrumentation and limit of detection of each analytical technique, data collecting and handling need to be addressed.

CONCLUSION

MAbs are currently produced almost entirely using batch operations. Recent advances in downstream processing technology for the purification of these molecules has created significant opportunities for the realization of fully integrated continuous processes. Continuous processing could provide unique opportunities for the production and delivery of low-cost mAbs. One of the major challenges for the transition towards continuous processes is the implementation of PAT tools that provide information on the status of the process, especially on the formation of

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aggregates. The ability to measure CQAs makes it possible to have immediate feedback on process performance. This could help significantly improve the implementation of continuous bioprocessing by allowing proactive decision making based on real-time process data. Real-time measurement of CQAs will be beneficial in all types of processes (batch or semi-continuous). Although batch processes are more flexible in terms of the measurement time range – up to a few minutes –real-time PAT tools developed for continuous processes could easily be implemented. Thus the development of real-time in-process analytics plays a central role in ensuring product quality. By miniaturizing the already well-established analytical techniques for the detection of mAb aggregates, an attractive solution for the creation of a PAT tool can be achieved. A microfluidic chip will speed up the measurement, allowing to gain control over the manufacturing process. Advances in design and fabrication of these micro devices are still necessary, especially for the conformational stability methods. Here, the focus should be on reducing the fabrication material costs and simplifying the design and equipment set-up.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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