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Single-use mixing systems for protein products: Evaluation of dissolution performance and mechanical stress



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ABSTRACT

Sterile manufacturing (fill & finish) of biologic drug products involves critical operations like freezing/thawing, mixing, filtration, and filling. These processes must be well characterized to understand their impact on product quality. While single-use systems offer efficiency and flexibility, they also introduce challenges related to aseptic handling and material compatibility with biological formulations. For the unit operation “mixing”, equipment configurations vary, each with distinct advantages and limitations. In this study, we systematically compared the performance of two single-use mixing systems using drug substance bottles, equipped with a top-mounted impeller (TMM), a novel bottom-mounted impeller (BMM), and a conventional magnetic stirrer (MS). Mixing efficiency, measured by changes in conductivity reported as dissolution time, was evaluated across scale-up parameters, including container size, fill volume, and impeller tip speed. Dissolution time was dependent on mixer type and inversely correlated to tip speed and impeller diameter/bottle diameter ratio. BMM and TMM showed consistently lower dissolution times and less challenges compared to MS, which showed insufficient mixing quality for some configurations. Mechanical stress during mixing was assessed using a model protein, with results corroborated through computational fluid dynamics (CFD) simulations. Mechanical stress was dependent on mixer type and fill volume and lead to adverse product quality impact of the model protein, which became less pronounced with higher fill volume. The adverse impact on product quality was generally higher and more variable when using the MS than with the BMM. These findings provide formulation and process development scientists with data-driven guidance for selecting appropriate mixing technologies for biologic drug manufacturing and scaling-up processes.

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Introduction

Single-use (disposable) bioprocessing materials are routinely used for manufacturing across biopharmaceutical industry. Integration of such systems across various stages of biopharmaceutical production, particularly in fill-finish operations, offer several advantages. These include the elimination of cleaning and sterilization-in-place procedures and their associated validation, reduced risk of cross-

contamination, and enhanced operational flexibility through plug-and-play capabilities that may enable faster batch changeovers and hence, prolonged production times.¹ Multi-use materials, with stainless steel being often traditionally used, bears some additional disadvantages compared to single-use systems such as potential interaction of the material/formulation with metal surfaces, including potential risk of metal leachables or subsequent oxidation risks.² However, disposable systems also present challenges. They must undergo rigorous evaluation to meet regulatory requirements, including extractables/leachables, and potential contaminations such as by endotoxin and particulate must be minimized and controlled. Furthermore, their functionality, product compatibility, and integrity must be thoroughly assessed and validated.¹ However, single-use materials contribute significantly to contaminated (plastic) waste, hence options for circularity in its use or alternative materials are required.³

Sterile drug product manufacturing (fill & finish) of biological drug products involves multiple unit operations, typically comprising

Abbreviations: A, Area; ACN, Acetonitrile; ANOVA, Analysis of variance; AQL, Acceptable quality level; BMM, Bottom mounted mixer; CFD, Computational fluid dynamics; CQA, Critical quality attributes; d, Impeller diameter; D, Bottle diameter; DS, Drug substance; HPLC, High-performance liquid chromatography; Imp, Impeller; LOD, Limit of detection; m, Meter; MS, Magnetic stirrer; NaCl, Sodium chloride; PC, Polycarbonate; PET, Polyethylene terephthalate; PS80, Polysorbate 80; RANS, Reynolds-Averaged Navier–Stokes; RP, Reverse-phase; s, Second; TFA, Trifluoroacetic acid; TMM, Top mounted mixer; VIF, Variance Inflation Factor; VOF, Volume of Fluid.

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thawing (of drug substance), pooling, potentially final formulation/dilution, homogenization (mixing) in mixing vessels or containers, (bioburden reduction and sterile) filtration, filling, stopper setting, possibly capping, and visual inspection as well as AQL (acceptable quality level) inspection. Each of these unit operations' process parameters must be characterized and their impact on critical quality attributes (CQAs) must be assessed.⁴

Mixing is a critical unit operation. It may include dissolution of formulation excipients, and/or pooling of different final or partially formulated drug substance solutions followed by mixing in a respective container. Mixing needs to ensure homogeneity of the compounded material (solution) by eliminating any potential gradients of concentrations of any component (excipient or active substance(s)) - whilst maintaining product quality. Optimal mixing processes target a homogenous mixture at minimal process time, causing minimal mechanical stresses to the product with no or negligible product quality impact. For example, different mixer types such as Rushton turbines, pitched-blade impellers, and marine-blade impellers can generate different mechanical stress, depending on their design.⁵ Relevant testing of mixing hence includes both the homogeneity (e.g., by testing a surrogate parameter like API, excipient concentration or osmolality) as well as potentially impacted CQAs. For biologics such as antibodies or recombinant proteins, this typically includes protein content (concentration), aggregates (oligomers/multimers), (proteinaceous) precipitation (visible and sub-visible particles, possibly sub-micron particles) and turbidity/opalescence.⁶

Nakach et al. assessed mixing time by systematically varying stirrer speed, fill volume, and mixing duration by using a NaCl surrogate solution. They also investigated the impact of mechanical stress during mixing, using an IgG1 monoclonal antibody formulation in square disposable vessels (Levmixer® systems from Sartorius Stedim) at multiple scales (4–650 L). They established a correlation for mixing time under turbulent flow, showing that mixing time decreases with increasing rotation speed and that, at high Reynolds numbers, the product of mixing time and rotation speed remains mostly constant. However, for a given vessel, mixing time increased with increasing filling volume. To assess potential protein degradation, a lab-scale stress test was conducted under corresponding turbulent stress but extended mixing duration (4 h). No impact on product quality, including increase of protein aggregates, subvisible and submicron particles, changes in protein concentration, and osmolality, were reported, and a large-scale verification run under worst-case industrial conditions confirmed these findings.⁴

Gikanga et al. investigated four different bottom-mounted mixers and evaluated how their mechanism and design impacted drug product quality using a monoclonal antibody formulation. They assessed changes in product quality by measuring size variants (high molecular weight formation), particle counts, and turbidity, as well as examining the effect on process performance, particularly sterile filtration after mixing (filter clogging due to proteinaceous particles). The study found that mixers designed to operate without physical contact between the impeller and the bearing, such as those with magnetic levitation, were the most favorable, resulting in fewer particles. In contrast, mixers with contact or narrow clearances between the impeller and bearing had increased particle counts, whilst no protein aggregation or fragmentation was detected by other methods. The authors speculated that the particles were generated due to the contact between the bearing and the impeller. Subsequently, they further developed a small-scale screening model using two types of magnetic stir bars, one representing contact and the other non-contact, which validated their hypothesis about the impact of contact of bearing and impeller on particle formation with less particles generated for the magnetic stirrer with less contact.⁷

Gikanga et al. elucidated the mechanistic pathways underlying particle formation in mAb formulations during mixing processes employing specific types of BMM, presented in a comprehensive

review.⁸ Lysozyme inactivation and aggregation in a stirred-reactor (top-mounted Rushton turbine) was evaluated by Colombié et al. They found the inactivation kinetics to follow a first order kinetic (for concentrations between 0.1 and 0.8 mg/mL), for a set of stirring speed range, whereas they detected that the inactivation constant is proportional to the power input by the impeller.⁹

Hydrodynamic conditions in biologic processing systems have been increasingly assessed by using computational fluid dynamics (CFD) to evaluate the potential impact on protein stability. Ladner et al. assessed shear induced by bottom-mounted magnetic stirrers in monoclonal antibody formulations using flow-analysis, created by CFD. They evaluated local shear exposure, as well as the pathway of fluid through critical mixer regions, whereas they found that mixer designs minimizing physical contact between moving components, reduced particle formation and improved product quality.¹⁰ Kreitmayer et al. has shown CFD-based and experimental data assessing a single-use bioreactor, evaluating mixing time, flow behavior, vortex formation, turbulence dissipation and hydrodynamic stress for different operating conditions. They were able to characterize mixing behavior, namely vortex formation and local stress environments in single-use systems with non-classical geometries.¹¹ However, hydrodynamic parameters, as well as shear stress simulations, are not sufficient for explaining protein instability, as this is a interaction of multiple stress mechanisms, typically referred to as 'mechanical stress'.¹² Therefore, there is a need to complement CFD-based characterization with experimental assessments such as particulate matter to analyze the impact of mixing systems on protein stability¹¹

With new single-use mixing systems becoming available, the current challenges and limitations of different mixing systems need to be assessed. In the present study, we compared the mixing performance and mechanical stress and its impact on product (surrogate) quality of different single-use systems in bottle configurations, a top-mounted mixer (TMM), a novel bottom-mounted mixer (BMM), and a standard magnetic stirrer (MS). Mixing performance was assessed by dissolving sodium chloride (NaCl) in water and monitoring conductivity for a variety of bottle sizes, fill volumes, and process parameters. Differences in mechanical stress resulting from mixing and its impact on product quality were evaluated using a lysozyme surrogate solution. Enzyme inactivation rates and precipitation were assessed depending on the mixers' dimensional characteristics, fill volume and rotational speed. To elucidate significant process parameters for mixing performance and mechanical stress, experimental results were fitted with multilinear models. Experimental findings from the mixing performance and mechanical stress testing study were further complemented using CFD simulations.

Materials and methods

Material

Mixer set-up

A bottom-mounted mixer (BMM) and top-mounted (TMM) mixer (M4SB and M4ST from the Mixed4Sure™ line, SaniSure, Camarillo, CA) were compared to a magnetic stirrer (MS) in a PharmaTainer™ set-up (SaniSure, Camarillo, CA) in the 0.5, 2, 5, and 20 L format, whereas for the TMM only 5 and 20 L configurations exist, which were tested. For all mixers, bottles were available of either polycarbonate (PC) or polyethylene terephthalate (PET). Mechanical stress tests used only PC bottles, while mixing performance tests employed either PC or PET bottles.

As seen in Fig. 1, the BMM consists of a four-blade impeller (mixing head), which is centrally mounted and coupled via a shaft bearing, and the impeller is made to operate in a range of 200–600 rpm. The TMM features a large, bar-style impeller, including a baffle. The MS has a cylindrical stir bar with rounded ends without a ring. The bottle's main body is roughly rectangular with four rounded corners,

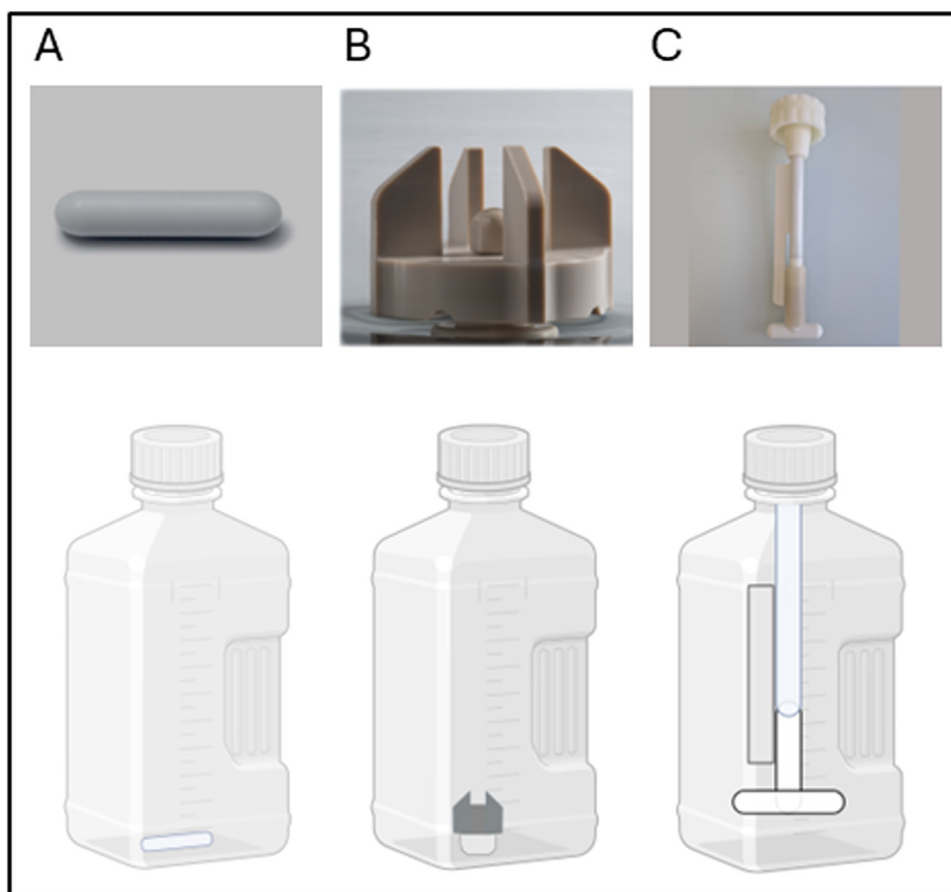


Fig. 1. Real-life pictures of mixers and corresponding schematic pictures of the tested bottles in their corresponding set-up. From left to right: (A) Magnetic stirrer in a PharmaTainer™, (B) bottom mounted mixer (Mixed4Sure™, M4SB) and (C) top mounted mixer (Mixed4Sure™, M4ST).

tapering to a circular neck. The mixers themselves were all magnetically coupled with suspended mixing devices. All experiments were executed on a magnetic stirrer plate (IKA RET basic, Staufen DE), with the function to set the speed (in 5 rpm intervals) and the temperature (with one decimal place as precision for settings).

Surrogate solutions

Mixing performance experiments were executed by adding 10 g sodium chloride $\geq 99.0\%$ (NaCl) (Sigma Aldrich, St. Louis, MO) to 1 L Milli-Q water.

Product quality assessments of a model protein following mechanical stress testing was performed using a lysozyme (Thermo Scientific, Waltham, MA) solution at a concentration of 0.1 mg/mL. The model protein was chosen due to its known sensitivity towards mechanical stress, following lysozyme inactivation. Lysozyme was formulated in a surrogate solution consisting of a 0.1 M phosphate buffer pH 6.2 (NaH_2PO_4 H_2O and Na_2HPO_4 from Merck, Darmstadt, DE), with the addition of 0.04% Polysorbate 80 (PS80) (J.T. Baker, Radnor, PA), based on Colombié et al., who tested lysozyme inactivation in a stirred-reactor.⁹

Methods

Mixing performance

Mixing performance was evaluated as summarized in Table 1, where we tested three different mixers: BMM, MS and TMM in four different bottles with different nominal fill, fill volumes, and at different speeds. Table 1 further lists fill volume to nominal fill volume ratio, the tip speed ($\pi \cdot \text{impeller diameter} \cdot \text{rotational speed}$), and the

dimension of the impeller and the bottle, respectively. The tip speed is defined as the tangential velocity of the impeller at the tip and it is a function of RPM and diameter of the impeller.¹³ As the vessels are not cylindrical but identical in geometry, an equivalent diameter was used as an approximation to enable the calculation of characteristic mixing parameters. All bottle dimension parameters are listed in the supplement, in Table S1.

It is important to note that the minimal fill volume for the BMM does not fully submerge the mixer head, while minimal fill volume for the MS and TMM were always setup in a way that the mixer remained fully submerged. As the minimal fill volume for TMM was higher than for BMM, BMM and MS, setups with the minimal fill volume considering the TMM were also evaluated. The level of submergence can be seen in Fig. 2.

Mixing performance was evaluated assessing the dissolution of NaCl, by monitoring the (increase in) conductivity, as a function of time. NaCl was added into a defined amount of water yielding a concentration of 10 g/L of NaCl, using a funnel. To monitor the dissolution process of the NaCl (until no visible traces of NaCl remained and conductivity remained stable), conductivity was recorded every 10 s using the conductivity probe InLab 731 – ISM, four-pole graphite cell, with integrated temperature sensor (Mettler Toledo, Columbus, OH), which was calibrated before each use. A 10 s sampling interval was selected because mixing and dissolution occurred on the scale of several minutes, ensuring that all relevant changes in conductivity were appropriately captured. Temperature was reported between 21 °C and 25.5 °C. The conductivity probe was positioned close to the bottle wall and in the upper region of the working volume, with the sensing element fully submerged but placed as far as possible from the impeller. This location was selected to minimize flow disturbance

Table 1
Mixer test set-ups for mixing performance studies.

Mixer	Nominal fill [L]	Fill volume [L]	Rotational speed [rpm]	Tip speed [m/s]	Bottle diameter [mm]	Impeller diameter [mm]	Impeller height [mm]
BMM	0.5	0.1/0.25/0.5	200/600	0.3/1.0	86.0	31.8	23.6
	2	0.25/1/2			136.3		
	5	0.43/2.5/5		0.5/1.5	186.5	48.3	36.3
	20	1.12/10/20			273.2		
MS	0.5	0.1/0.5		0.3/1.0	86.0	30.3	6.04
	2	0.25/2			136.3		
	5	0.43/5		0.5/1.5	186.5	50.8	10.0
	20	1.12/20			273.2		
TMM	5	1.5/5	130/400		186.5	76.2	25.4
	20	4.5/20	80/240		273.2	127.0	

Three different mixer types were compared: Magnetic stirrer (MS) vs. bottom-mounted mixer (BMM) vs. top-mounted mixer (TMM), in four different bottles sizes with nominal fill volumes between 0.5 and 20 L, at different fill volumes/fill volume/nominal fill volume ratios, and at different speed (rpm) and tip speed (m/s), respectively. Dimensions of the bottle, as well as from the impeller are listed. The mixing performance was evaluated using minimal and maximal fill volume (additionally mid-fill volume for BMM) as well as minimum and maximum rotational/tip speed. The minimal fill volume tested was based on recommendations by the supplier. Rotational speed was adjusted to have comparable values of tip speed, which was calculated as the product of impeller diameter, rotational speed and π . Bottle dimensions and calculated parameters used for further analysis can be found in the Supplement, Table S1.

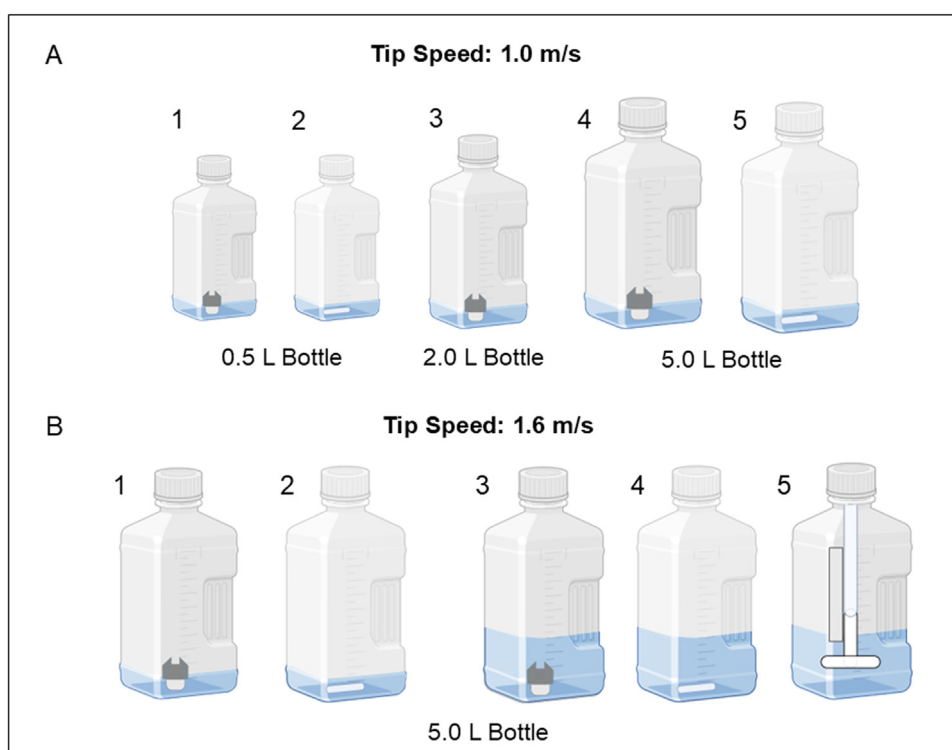


Fig. 2. Experimental design for assessing mechanical stress for different mixers including bottom mounted mixer (BMM), magnetic stirrer (MS) and top mounted mixer (TMM). (A) Tip speed = 1.0 m/s and (B) 1.6 m/s (2.4 m/s for TMM). For configuration (A) 1–5 the following minimal fill volumes were used: (A1, A2) 0.05 L; (A3) 0.123 L; (A4, A5) 0.217 L. For configurations (B) 1–5 the following minimal fill volumes were used: (B1, B2) 0.217 L; (B3, B4, B5) 1.5 L corresponding to the minimal fill volume of the TMM.

caused by the probe itself and to capture the region where concentration changes were expected to occur last (worst case). The probe was mounted in a fixed vertical orientation throughout all experiments. The dissolution time was defined as the time at which the conductivity signal first entered and subsequently remained within $\pm 5\%$ of its final steady-state value. This criterion was applied separately to each individual experimental trace, as described by Nienow et al.¹⁴ A schematic of the experimental design can be seen in the supplement, in Fig. S1.

Mechanical stress testing

Table 2 shows the set-ups which were assessed to evaluate the mechanical stress and its impact on product quality in different mixer types. We evaluated three different mixers: BMM, MS and TMM in three different bottle sizes, with different fill volumes and at different

rotational speeds. Additionally, the tip speed (calculated from impeller diameter and rotational speed) and the bottle, respectively impeller, dimensions are listed. As the vessels are not cylindrical but identical in geometry, an equivalent diameter was used as an approximation to enable the calculation of characteristic mixing parameters. The setup of the experiments, including the level of impeller submergence, are displayed in Fig. 2.

To assess the differences in mechanical stress generated by different mixer types, 0.1 mg/mL lysozyme was used as a model protein, in a formulation buffer containing 0.04 % polysorbate 80. The formulation was filled into the bottle, which was placed on a magnetic stirrer plate and heated to $72 \pm 2^\circ\text{C}$, based on the study of Colombié et al.⁹ To note, local temperature fields for each mixer type were not measured. While the bulk fluid was maintained at $72 \pm 2^\circ\text{C}$ and no significant thermal gradients were expected, we acknowledge that local variations may exist. This assumption of nominally isothermal

Table 2

Mixer set-ups for mechanical stress testing.

Mixer	Nominal fill [L]	Fill volume [L]	Rotational speed [rpm]	Tip speed [m/s]	Bottle diameter [mm]	Impeller diameter [mm]	Impeller height [mm]
BMM	0.5	0.05	600	1.0	86.0	31.8	23.6
MS						30.3	6.0
BMM	2	0.123			136.3	31.8	23.6
TMM	5	1.5		2.4	186.5	76.2	25.4
BMM				1.6		48.3	36.3
MS						50.8	10.0
BMM		0.217				48.3	36.3
MS						50.8	10.0
BMM			400	1.0		48.3	36.3
MS						50.8	10.0

Three different mixer types were compared to each other: Magnetic stirrer (MS) vs. bottom-mounted mixer (BMM) vs. top-mounted mixer (TMM) in three different bottles sizes (0.5 vs. 2 vs. 5 L). Mechanical stress was evaluated using minimal fill volume and maximal rotational speed. The dimensions of bottles and impellers are listed, as well as tip speed, which is calculated from impeller diameter and rotational speed. Bottle dimensions and calculated parameters used for further analysis can be found in the Supplement, Table S1.

conditions needs to be considered when interpreting the relative effects of different mixing configurations. Once the temperature was reached, the mixing process was started, and the experiment was performed for a period of four hours. Twelve samples were taken over four hours, by using a long stainless needle and a one milliliter syringe. Fill volume was adjusted prior to the experiment, by considering sampling volume, to never fall below the minimal volume (as specified by the manufacturer) for each mixer. A control setup was done in the PharmaTainer™, replicating each experimental condition and processed under identical conditions, but without agitation. The mean of three replicate control setups was used as the final control value. The samples were stored at 2 – 8 °C in crimped 2R glass vials until measuring the concentration, purity, and activity of lysozyme. Stability of the samples until time of analysis was verified with references stored in the same manner.

Concentration and purity measurements (for mechanical stress testing experiments)

The concentration and purity of lysozyme in the formulation after mechanical stress testing was determined with high-performance liquid chromatography (HPLC) (Agilent 1200, Santa Clara, CA) using a reverse-phase (RP) column (XBridge Peptide BEH C18 Column) (Waters, Milford, MA). The previously established method of Liao et al. was used, where a 50 µL sample of the enzyme solution was applied to the C18 column, which was set to 25 °C and eluted at a flow rate of 0.5 mL/min.¹⁵ A gradient of acetonitrile (ACN) (0.1 % trifluoroacetic acid, TFA) in water, using the procedure described by Liao et al. was applied and the concentration was detected at a wavelength of 220 nm.¹⁵ A calibration curve was run with every sequence, using concentrations from the limit of detection (LOD) up to 1 mg/mL. Remaining lysozyme concentration in the samples was calculated by using the calibration curve. As described by Colombié et al., the inactivation rate of lysozyme in a mixer follows first-order kinetics.⁹ Therefore, the collected concentration data for each setup were transformed to a logarithmic scale, and the slope of the linear fit was considered as the inactivation rate for the corresponding setup. Purity was assessed by integrating areas (A) of pre- and post-peaks relative to the total chromatographic area. In addition, the fraction of protein precipitated was calculated as $1 - (A_{\text{total, 4h}}/A_{\text{total, 0}})$ and all solutions were visually inspected for particles. Precipitated fraction of the protein was fitted against the inactivation rate with a single-phase exponential saturation model, constrained to reach the maximum inactivation rate at full precipitation.

Protein activity measurements (for mechanical stress testing experiments)

Lysozyme activity was defined as the ability to decrease the absorbance at 450 nm by 0.001 per minute during the catalytic hydrolysis

of a 2 mg/mL *Micrococcus lysodeikticus* suspension. This reaction was conducted at 37 °C in a 0.1 M phosphate buffer (pH 6.2). The EnzCheck™ assay (Thermo fisher Scientific, Waltham, MA) was used, which involved preparing a DQ lysozyme substrate and creating dilutions of known lysozyme concentrations within the range of 1 – 15.9 U. The mixtures were incubated at 37 °C for 30 min, protected from light. Fluorescence was measured using a SpectraMax iD5 plate reader (Molecular Devices, San Jose, CA) at an excitation/emission wavelength of 485/530 nm. Lysozyme activity was calculated by using the calibration curve adjusted for the dilution factor.

Statistical analysis

Data analysis was performed using JMP 18.0.0 (Cary, NC). For both the mixing performance and mechanical stress experiments, multiple linear regression models were built using the *Fit Model* platform. The relevant response variables were treated as interdependent variables, and the input parameters as independent variables. Interaction terms were initially included to assess potential parameter interactions, but were removed if found non-significant to improve model fit. Model performance was evaluated using 5-fold cross-validation. To minimize multicollinearity, Variance Inflation Factors (VIF) were monitored (targeting VIF < 5), and parameters were selected accordingly. All graphs were generated using OriginPro 2023 (Northampton, MA).

To identify significant parameters influencing mixing performance, a regression model was created with dissolution time as the response variable. The following parameters were included as predictors: mixer type, nominal fill, fill volume, fill volume-to-nominal fill ratio, tip speed ($\pi \times \text{impeller diameter} \times \text{rotational speed}$), and $d_{\text{impeller}}/D_{\text{bottle}}$ ratio (impeller diameter divided by bottle diameter). Mixer type and bottle size/nominal fill were treated as categorical variables; all other variables were continuous.

A square root transformation was applied to the dissolution time to reflect the diffusion-controlled nature of the process, consistent with the Noyes-Whitney equation (Eq. (1)):

$$\frac{dm}{dt} = A * \frac{D}{l} * (C_s - C_b) \quad (1)$$

With dm/dt is the solute dissolution rate ($\text{kg} \cdot \text{s}^{-1}$), m the mass of dissolved material (kg), t the dissolution time (s), A the surface area of the solute particle (m^2), D the diffusion coefficient ($\text{m}^2 \cdot \text{s}^{-1}$), l the thickness of concentration gradient (m), C_s the surface saturated concentration of solute particle ($\text{kg} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$) and C_b the concentration in the bulk solution ($\text{kg} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$).¹⁶

For the mechanical stress experiments, a separate regression model was developed to evaluate factors influencing the lysozyme inactivation rate. The same set of parameters was used as predictors as for the previous model. Given that inactivation under mechanical stress is often influenced by hydrodynamic forces in a nonlinear fashion, a reciprocal transformation was applied to the inactivation rate

to linearize the relationship. This approach is analogous to classical enzyme kinetics (e.g., the Lineweaver–Burk plot), where reciprocal transformations are used to represent inverse relationships between reaction rate and substrate concentration.¹⁷

Computational fluid dynamics (CFD) simulations

CFD simulations were performed using the *SolidWorks Flow Simulation software, Professional 2023 SP4.0 with Flow Simulation 2023 SP4.0* (Dassault Systèmes, Waltham, MA) analyzing (shear) dynamics in the mixing systems. The computational domain consists of the entire internal volume of the bottle, including the liquid and air phases. The model integrated a liquid domain representing water and a free-surface air boundary to capture surface interactions. To simplify the simulations, water was modeled at 21 °C (viscosity: 0.001002 Pa·s, density: 998.2 kg/m³), with gravity applied at −9.81 m/s² in the negative z-direction. Standard atmospheric conditions were used; all walls were defined as adiabatic with no-slip boundary conditions.

Transient simulations were run with a base time step of 0.02 s. Adaptive time-stepping was used to maintain numerical stability. Simulations were evaluated at 30 s, which refers to the simulated physical time. This duration was selected as a fully developed vortex was established within this time, and extending simulations beyond 30 s did not result in further qualitative changes in flow structure. The mesh was a Cartesian grid with adaptive refinement, typically consisting of ~ 50,000 elements. This cell count refers to the entire internal domain (air and liquid), with local mesh refinement applied to the fluid region and the impeller vicinity. Higher mesh densities were evaluated but introduced numerical instability without improving accuracy. Mesh refinement studies were conducted over a range of approximately 4000 to 100,000 cells; finer meshes frequently led to solver instability or crashes, and the final mesh density was selected as a balance between numerical stability and result robustness. SolidWorks does not output detailed mesh quality metrics, but the grid strategy aligned with best practices for this system type. Turbulence was modeled using the standard *k*– ϵ model with 2% turbulence intensity and a characteristic length scale of 0.0686 in. The same turbulence model and parameters were applied consistently across all simulated cases; solver settings were not varied between configurations. Convergence was achieved when residuals stabilized within 10^{−5} to 10^{−6}, as defined by the solver. In addition, key output quantities exhibited stable plateau behavior over time.

The key output parameter evaluated from the CFD simulations was shear stress (Pa), representing the force exerted per unit area within the fluid. Post-processing outputs included shear distributions visualized as color-mapped contour plots, and velocity vector fields illustrating flow patterns and mixing dynamics within the fluid domain. Rotating components were handled using a local rotating reference frame, which provides time-averaged solutions for impeller-driven flows. We used a RANS (Reynolds-Averaged Navier–Stokes) turbulence model, which efficiently captures mean flow patterns and shows shear characteristics underlining our studies. As RANS resolves only averaged fields, detailed turbulent structures and fluctuation related quantities are not directly represented. We therefore show results which characterize large-scale mixing behavior rather than fully resolved turbulence, which we deem appropriate to compare the data generated in this work. To assess the flow regime and support interpretation of CFD results, Reynolds numbers were calculated for each mixing condition. Reynolds numbers were calculated using the standard stirred-tank formulation and shall be seen as comparative indicators of flow intensity. Because the bottle geometry and impeller designs differ from classical cylindrical vessels, the absolute regime boundaries (laminar vs. turbulent) should be interpreted with caution. In this context, we intend that Reynolds number primarily serves to support relative comparisons between operating

conditions regarding the same system rather than defining precise flow regimes. In general, the Reynolds number (R_e), is a dimensionless parameter commonly used to characterize fluid flow, and the extended version of R_e for impellers (R_{eimp}) is defined as the following equation (Eq. (2)):

$$R_{eimp} = \frac{\rho * N * D^2}{\mu} \quad (2)$$

Where ρ is density of the liquid (water for simplicity purposes = 1000 kg/m³), N the speed of the impeller, D the diameter of the impeller and μ the viscosity of the fluid under mixing operation (water for simplicity purposes = 0.001 Pa·s). $R_{eimp} < 10$ indicates a laminar flow pattern and $R_{eimp} > 10^4$, turbulent flow and everything in between a transitional flow pattern.¹³

Results

We established experimental setups to assess mixing performance and mechanical stress in three different single-use mixers, magnetic stirrer (MS) in a single-use PharmaTainer™, a bottom-mounted mixer (BMM), and a top mounted mixer (TMM) in different configurations. CFD simulations were additionally performed to support our findings.

Mixing performance

Mixing performance of the three mixers was evaluated by assessing dissolution times of dissolving solid sodium chloride in water. The dissolution time was defined as the point at which the conductivity signal stabilized within $\pm 5\%$, as this was the range where the signal consistently leveled off. An example of mixing performance results is shown in Fig. 3, illustrating the minimal fill volume for the 5 L and maximal fill volume for the 20 L, which are the configurations available for all three mixer types, highlighting dissolution times under two extremes: minimal fill volume with maximal impeller tip speed, and maximal fill volume with minimal tip speed. The shown graph represents the averaged signal across three replicates and is only included for visualization purpose; the reported dissolution times were calculated individually from each replicate and then averaged. Fig. 3 (A) shows that BMM and TMM exhibit faster dissolution times for the conditions tested compared to the MS, additionally, it is notable that the MS consistently shows higher standard deviations considering the replicates ($n = 3$). When looking at the 20 L bottle configuration (Fig. 3 (B)), BMM and TMM exhibit faster dissolution times than the MS, whereas the TMM shows a slightly steeper increase. The MS with maximal fill volume and minimal tip speed, shows no change in conductivity, which means not reaching the plateau that typically indicates complete dissolution of the entire amount of NaCl. This means that in this configuration, for the same dimension of the impeller and therefore tip speed as BMM, the MS does not sufficiently agitate the entire fill volume and therefore was not able to dissolve the NaCl under the test conditions during the tested time. Another mixing issue was observed with the 2 L MS bottle (lower speed = 200 rpm), where the magnetic stirrer lacked sufficient power to agitate the entire volume, as also reflected in the data of Fig. S2, in the supplement. As a result, in the magnetic stirrer configuration, the conductivity probe registered values approximately twice the expected fully mixed conductivity. This elevated signal is attributable to the probe sampling a locally concentrated region caused by limited upward convective transport and transient density stratification, rather than reflecting the global salt concentration in the vessel.

For the tested configuration as provided in Table 1, individual results of mixing performance are summarized in the supplement in Table S2. Based on these data, we have generated a statistical model

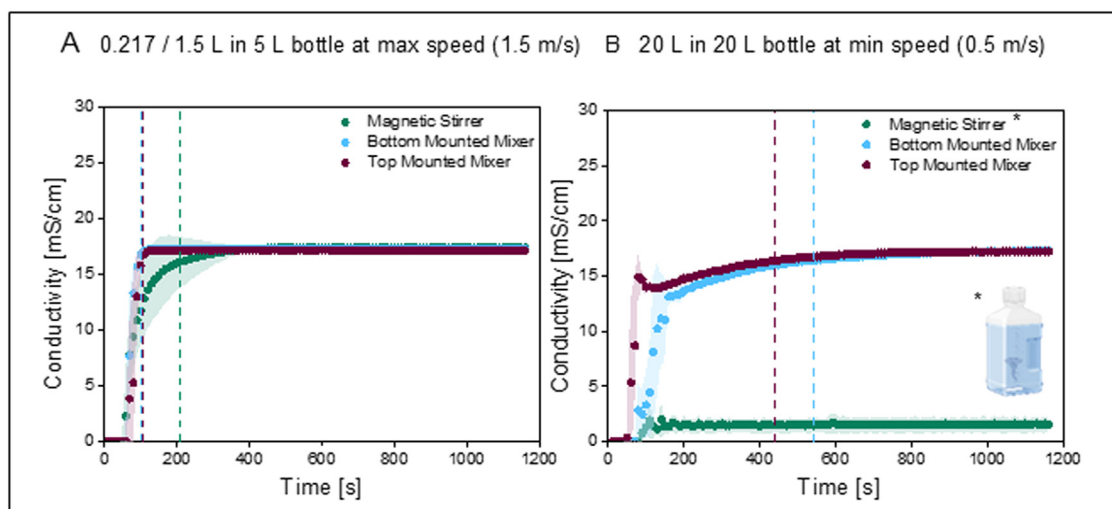


Fig. 3. Conductivity as a function of dissolution time of dissolving sodium chloride in water in the 5 L and 20 L container size for the two single-use mixing systems bottom mounted mixer (BMM) and top mounted mixer (TMM), as well as for the magnetic stirrer (MS). (A) 5 L bottle filled with minimal fill volume (0.217 L for BMM/MS and 1.5 L for TMM) at maximal tip speed (1.5 m/s) (B) 20 L bottle filled with maximal fill volume (20 L) at minimal tip speed (0.5 m/s). The dissolution time as indicated by vertical dashed lines is defined within a 5 % stabilization of the conductivity value. Conductivity was reported every 10 s; the error bands show the standard deviations of three runs. *The tested amount of NaCl did not dissolve when mixing with the magnetic stirrer at low speed in the 20 L bottle. The mixing was insufficient as a small vortex above the mixer formed (schematic representation in graph).

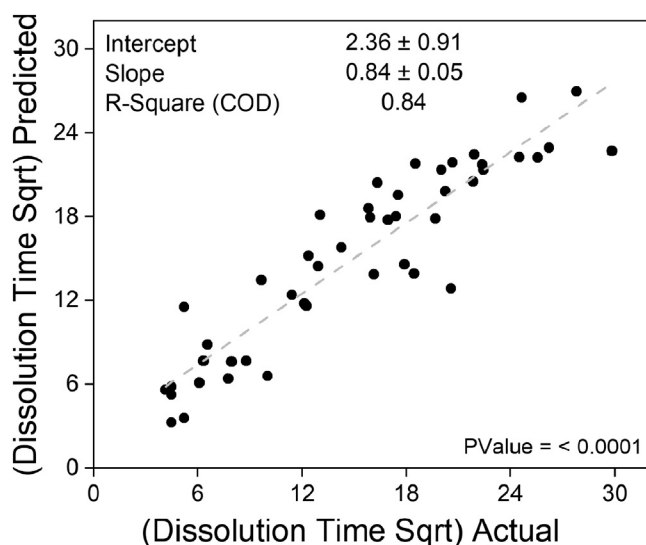


Fig. 4. Multiple linear regression fit applied to the mixing performance data, showing an R^2 value of 0.84 and a p value < 0.0001. The figure illustrates the model fit to the generated dataset and is included for visualization purposes (not for prediction of physical parameters).

for mixing performance using multiple linear regression analysis to assess the statistically significant parameters. The statistical model was generated using the JMP software, with dissolution time as response variable. To avoid multicollinearity and improve model fit, some parameters were excluded from the model, as described in the method section. Mixer type, fill volume-to-nominal fill ratio, tip speed, $d_{\text{impeller}}/D_{\text{bottle}}$, and their interactions were retained in the final model based on their contribution to model performance, even if not all were statistically significant. The final model was achieved with an R^2 of 0.84 and a significance of < 0.0001, as seen in Fig. 4. For the cross-validated R^2 a value of 0.81 was determined, indicating a strong predictive performance also beyond the data obtained.

The results indicate that the mixer type (only BMM being significant), tip speed and $d_{\text{impeller}}/D_{\text{bottle}}$ ratio exert a statistically significant effect on mixing performance, as seen in Table 3. Analysis of variance (ANOVA) revealed significant differences among group means ($p < 0.05$), confirming the influence of these factors. The

absence of a statistically significant effect for MS and TMM may be attributed to the limited number of observations resulting from the experimental design: BMM was tested at three fill volumes (minimum, medium, and maximum), whereas MS and TMM were evaluated only at the minimum and maximum levels. Additionally, TMM was only available in large-volume configurations, further restricting data variability and reducing the model's ability to reliably estimate its effect.

Fig. 5 illustrates the dependence of statistically relevant parameters on dissolution time for each mixer. It should be noted that the TMM can only be applied for larger volumes, exceeding the smaller volume that were tested of the corresponding configurations for the MS and BMM stating a limitation for the TMM. We found that for all mixers and configurations, an increasing tip speed resulted in a significant reduction in dissolution time. A similar inverse relationship was observed for the $d_{\text{impeller}}/D_{\text{bottle}}$ ratio, with larger impellers (higher d/D) corresponding to shorter dissolution times. When comparing mixers at equivalent tip speeds, the BMM consistently exhibited superior mixing performance, evidenced by shorter dissolution times, relative to the MS across all tested conditions. Compared to the BMM, the TMM achieved similar dissolution times; however, at a tip speed of 0.53 m/s, the TMM showed more consistent dissolution times, indicating a more consistent performance. The dissolution times depending on tip speed when comparing MS to BMM, reduced on average by 42 %, with individual reductions ranging from 19 to 68 %. Whereas when comparing MS to TMM we have seen a reduction of 58 %, reductions ranging from 29 to 87 %. Regarding the influence of $d_{\text{impeller}}/D_{\text{bottle}}$ on dissolution time, the BMM again outperformed the MS and, in this context, also the TMM. While the mean dissolution times for the BMM and TMM in this context were comparable, impellers with larger $d_{\text{impeller}}/D_{\text{bottle}}$ ratios exhibited reduced variability, particularly when looking at the two configurations (5 L and 20 L nominal fill), available for all mixers. Whereas, the dissolution times when comparing MS to BMM, reduced on average by 32 %, with individual reductions ranging from 13 to 54 %.

Mechanical stress testing

Product quality was assessed after mixing using lysozyme as a model protein, which was subjected to stirring at 72 °C, and by monitoring residual protein concentration in solution. The temperature

Table 3

Results of a multiple linear regression analysis to assess the statistically significant parameters on mixing performance.

Predictor	Estimate	Std. error	p value	Significance
Mixer type [BMM]	-5.13	0.80	<.0001	***
Mixer [MS]	-0.52	0.82	0.5285	-
Fill volume ratio	-1.06	1.19	0.3788	-
d/D	-56.35	7.50	<.0001	***
Tip speed	-11.80	0.95	<.0001	***
Fill volume ratio * d/D	10.12	13.32	0.4521	-
Fill volume ratio * tip speed	-4.56	2.36	0.0610	-
d/D * tip speed	-9.82	10.00	0.3321	-

The estimate represents the regression coefficient for each predictor, whereas the standard error reflects the precision of the estimate. The parameters with a p value ≤ 0.05 are considered statistically significant. The level of significance is shown in the last column, indicated by stars: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

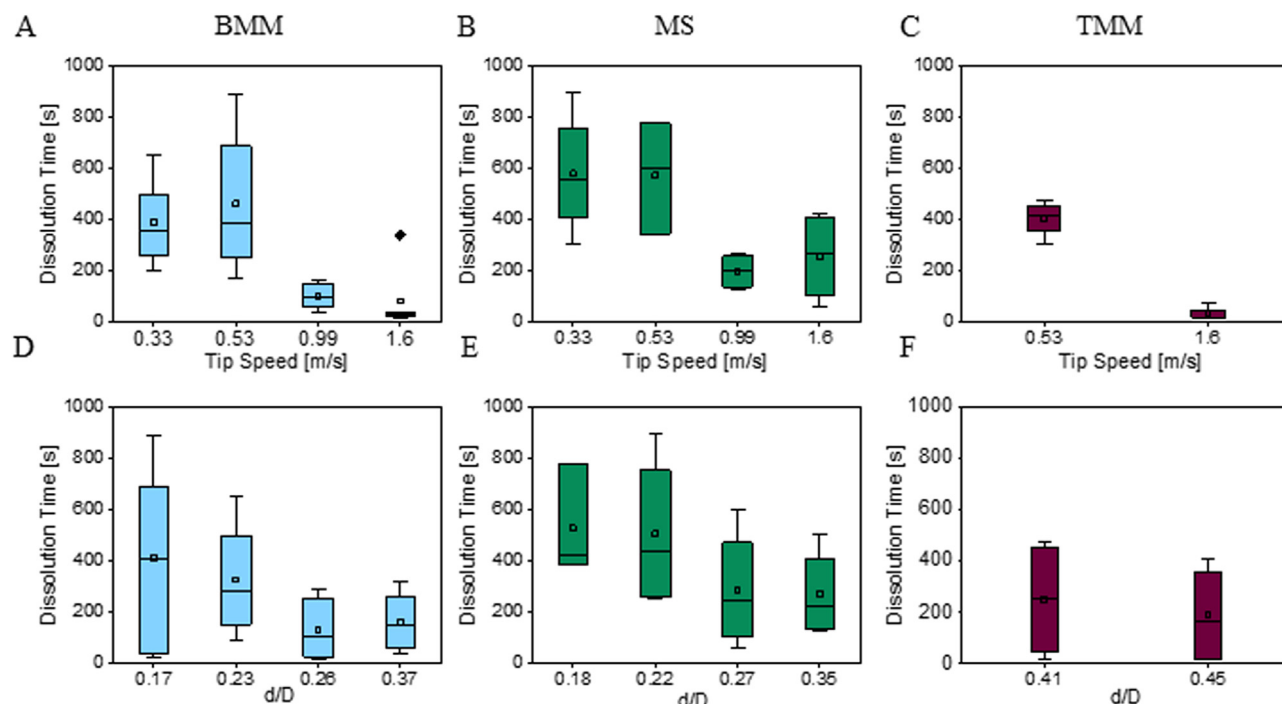


Fig. 5. Dissolution time for bottom mounted mixer (BMM), magnetic stirrer (MS) and top mounted mixer (TMM), for all tested configurations, depending on tip speed and $d_{\text{impeller}}/D_{\text{bottle}}$ ratio, split into different mixers and shown as box plots (A – F). The box plots were created with 25 – 75 % quartiles and additionally show: the ranges within a 1.5 IQR, a median line —, the mean shown as a squares and outliers. Outliers are determined using the 1.5 IQR rule, whereas data points below $Q1 - 1.5$ IQR or above $Q3 + 1.5$ IQR are considered mild outliers presented as a circle and everything beyond are extreme outliers displayed as a diamond shape.

was chosen according to a study conducted by Colombié et al. as lysozyme is known to be sensitive to mixing at that temperature.⁹ An identically processed control setup was included to show the effect of the thermal stress without agitation (mixing). Since lysozyme inactivation under these conditions follows first-order kinetics,⁹ concentration data were logarithmically transformed (natural log) and plotted against process time. The inactivation rate was then determined from the slope of the resulting linear fit. Concentration measurements were chosen because similar results for inactivation rate obtained from activity measurements exhibited higher standard deviations, reflecting the variability of this assay. A correlation between inactivation rates determined by concentration measurements and those based on activity can be observed in the supplement, in Fig. S4.

Fig. 6 shows the inactivation rate for the different bottles at varying nominal fill volume, actual fill volume, and the tip speed. The results show that with an increase in fill volume, the inactivation rate decreases. At higher mixing volumes like 1.5 L fill volume in a 5 L bottle there were no relevant differences between the mixers, nor to the control, which exhibited minimal inactivation of the model protein over the tested period, as seen. When comparing the mixers, we

found that for comparable setups of the BMM and MS, especially at lower volumes the BMM shows lower inactivation rates than the MS setups in all cases.

Similar results were obtained when assessing protein purity by visual inspection and HPLC analysis. For all setups with the highest fill volume, no particles were observed. However, for all remaining MS configurations, visible particles were detected. For the BMM, visible particles were observed as well but to a much smaller extent. Precipitation was estimated by calculating the fraction of protein remaining at the end of the experiment. A correlation between the inactivation rate and the precipitated protein fraction is shown in Fig. 7 with the raw data in Table S3, in the supplement. following the trends observed for lysozyme inactivation rate (Fig. 6).

In addition, lysozyme purity was monitored by HPLC throughout the process, and chromatograms from multiple time points over the four hour period were analyzed for the appearance of new peaks or other changes indicative of structural changes or degradation products. Representative chromatogram are provided in Fig. S5 in the supplement. To note, precipitated protein remained undetectable in the HPLC chromatogram. As a result, changes in pre- and post-peaks across all mixer setups were smaller than those observed in the

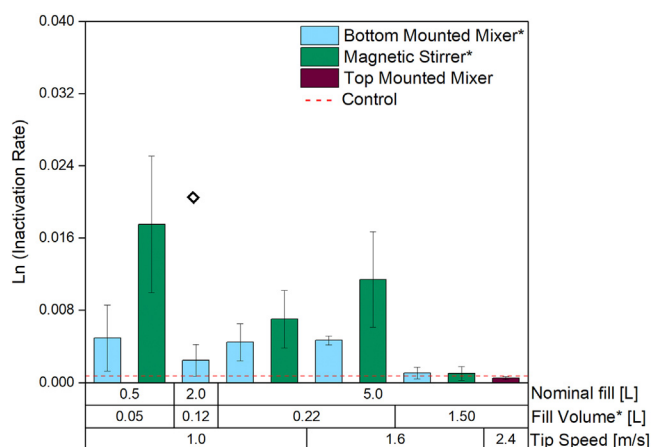


Fig. 6. Protein inactivation rate in the different mixers depending on bottle volume, fill volume and tip speed. All experiments were executed in triplicate ($n = 3$), standard deviations are depicted accordingly. Outliers are shown as diamond shaped. Parameters marked with an asterisk (*) are statistically significant ($p < 0.05$).

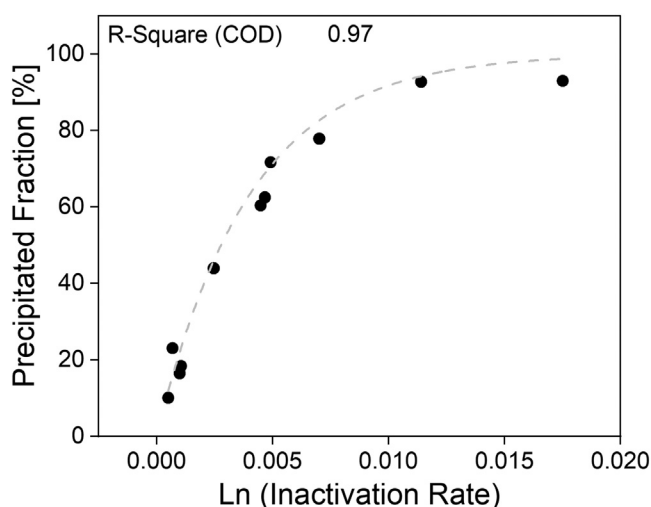


Fig. 7. Correlation between lysozyme inactivation rate and the fraction of protein precipitated determined from HPLC peak area decrease. A single-phase exponential saturation model was applied to guide the eye.

control (Table S3 in the supplement). TMM and BMM configurations showed no or small differences in pre-peaks compared to the control, whereas the MS configuration exhibited a greater reduction in pre-peaks reflecting enhanced lysozyme precipitation under higher mechanical stress, in line with inactivation rate results.

To assess the statistical significance of individual parameters on the lysozyme inactivation rate (output variable Y) across different mixing systems, a statistical model was fitted using JMP software. To avoid multicollinearity and improve model fit, some parameters were excluded, as described in the method section. Mixer type, bottle

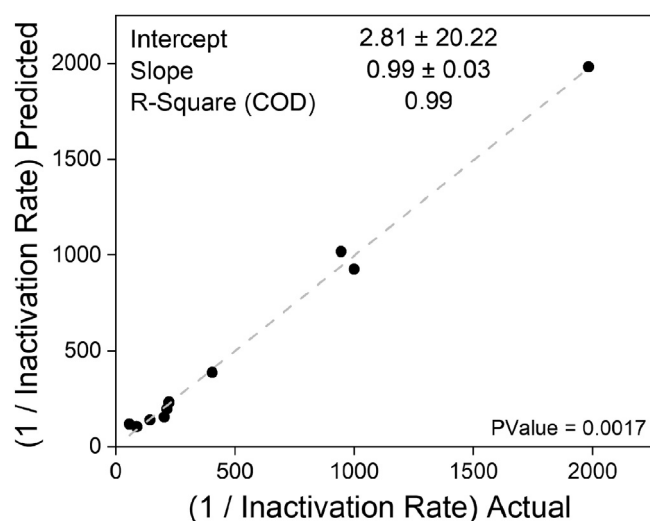


Fig. 8. Multiple linear regression fit applied to the mechanical stress evaluation data, showing an R^2 value of 0.99 and a p value of 0.0017. The figure illustrates the model fit to the generated dataset and is included for visualization purposes (not for prediction of physical parameters).

nominal fill, fill volume, tip speed, and $d_{\text{impeller}}/D_{\text{bottle}}$ were retained in the final model based on their contribution to model performance, although not all were statistically significant.

Mixer type was treated as a categorical variable, while all other parameters were continuous. The fit of the model was satisfying with an R^2 of 0.99 and a significance of 0.0017, as seen in Fig. 8. The results show that the BMM and MS mixers and fill volume have a statistically significant influence on the inactivation rate, as seen in Fig. 6 (indicated with a star) and Table 4. For the cross-validated R^2 a value of 0.95 was determined, indicating a strong predictive performance also beyond the data obtained. Lastly, ANOVA has shown a statistically significant difference between the group means ($p < 0.05$).

Computational fluid dynamics simulations

CFD simulations were employed providing mixing profiles for the setups summarized in Table 2, as seen in Fig. 9. Contour plots were generated to analyze areas of elevated shear (Fig. 10).

For all mixer types, the bottle geometry shows a specific flow pattern, including upward flow along the sidewalls and downward recirculation along the central axis, as well as strong radial dispersion (Fig. 9). Analysis of the mixing profile generated by the stir bar revealed pronounced radial flow in smaller volumes, with velocity vectors primarily oriented parallel to the vessel base, seen in Fig. 9 (A). In these conditions, particularly with the MS, limited fluid motion near the vessel corners indicated the presence of dead zones, i.e. zones characterized by poor circulation. Although axial flow was detectable, the velocity vectors in the central region displayed a lack of directional coherence, suggesting reduced mixing efficiency and

Table 4

Results of a multiple linear regression analysis to assess the statistically significant parameters on mechanical stress.

Predictor	Estimate	Std. error	p value	Significance
Mixer [BMM]	−408.09	73.81	0.0117	**
Mixer [MS]	−481.98	61.89	0.0044	**
Nominal fill	−51.37	25.68	0.1393	-
Fill volume	640.07	60.55	0.0018	**
d/D	−1882.32	930.08	0.1362	-
Tip speed	−65.81	143.74	0.6782	-

The estimate represents the regression coefficient for each predictor, whereas the standard error reflects the precision of the estimate. The parameters with a p value ≤ 0.05 are considered statistically significant. The level of significance is shown in the last column, indicated by stars: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

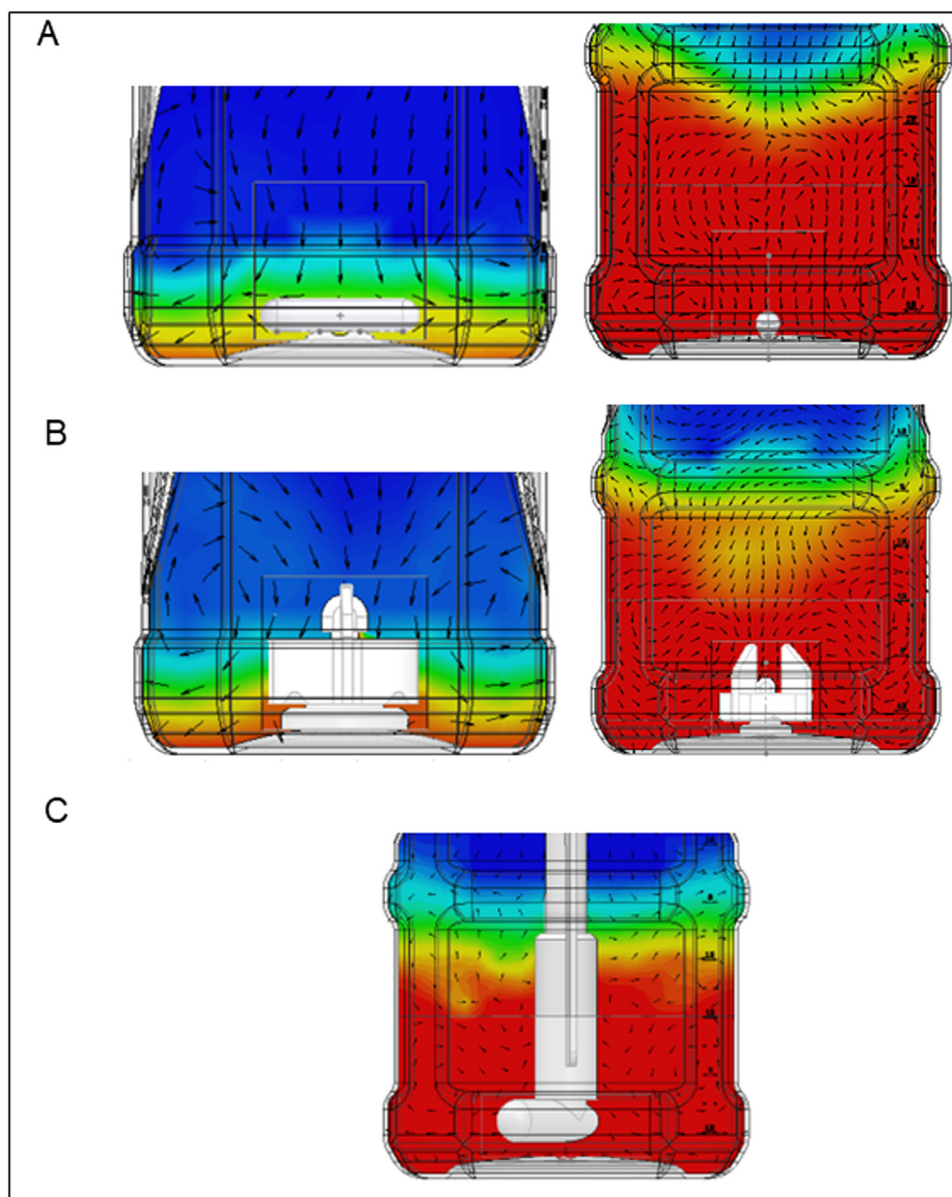


Fig. 9. Mixing profiles of (A) MS, (B) BMM, and (C) TMM, shown as color maps with velocity vectors. For MS and BMM, the left panels represent minimal fill volume, and the right panels show 1.5 L in a 5 L bottle. For TMM, only the 1.5 L condition is shown. The color scale represents the local volume fraction (VOF) of the fluid phases (red = water, blue = air), while the velocity vectors illustrate the direction and large-scale structure of the time-averaged flow and recirculation around the impeller. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compromised flow uniformity in this zone when using the MS. The mixing profile of the BMM (Fig. 9 (B)) displays combined axial and radial flow, with axial mixing being dominant. Velocity vectors point downward and outward, indicating strong axial drawing and peripheral circulation. In smaller-volume bottles, radial forces enhance outward flow, while the curved vessel walls guide the fluid upward, establishing a stable recirculation loop. This pattern persists in larger volumes, though smaller bottles exhibit more uniform and less turbulent flow, resulting in gentle yet consistent mixing. The TMM mixing profile (Fig. 9 (C)) is characterized by dominant tangential flow, especially when not filled to the nominal fill. The floating stir bar induces localized circular mixing, while the integrated fixed baffle disrupts this motion, promoting enhanced fluid motion near the central vortex. The baffle redirects tangential flow into a vertical component.

In addition to flow patterns, CFD simulations were performed to evaluate the distribution of shear, and identify regions of elevated shear stress as displayed by contour plots (Fig. 8). At minimal fill

volume in the 0.5 L bottle, the MS exhibited predominantly localized shear zones, concentrated around the stir bar, with reduced shear intensity observed toward the bottle walls, as seen in Fig. 10 (A). Notably, areas of elevated shear stress are present at the contact points between the stir bar and the bottle surface. When assessing the 0.5 L BMM at minimal fill volume, the highest shear stress was observed around the tips of the bladed wings. Additionally, elevated shear stress is present near the bottom of the bottle, where the mixer induces both axial and radial flow, directing fluid downward and outward, as seen in Fig. 10 (A).

The contour plot of the mixing pattern for the 5 L TMM incorporating a baffled stir bar, seen in Fig. 9 (C), illustrates enhanced vortex formation and dominant tangential flow throughout the vessel. The baffle effectively disrupts circular symmetry, intensifying fluid rotation and promoting more vigorous mixing near the center. As a result, broader shear zones are evident, particularly within the central and mid-radius regions, indicating improved mixing efficiency in these areas, as seen in Fig. 10. While the inner zones show dynamic

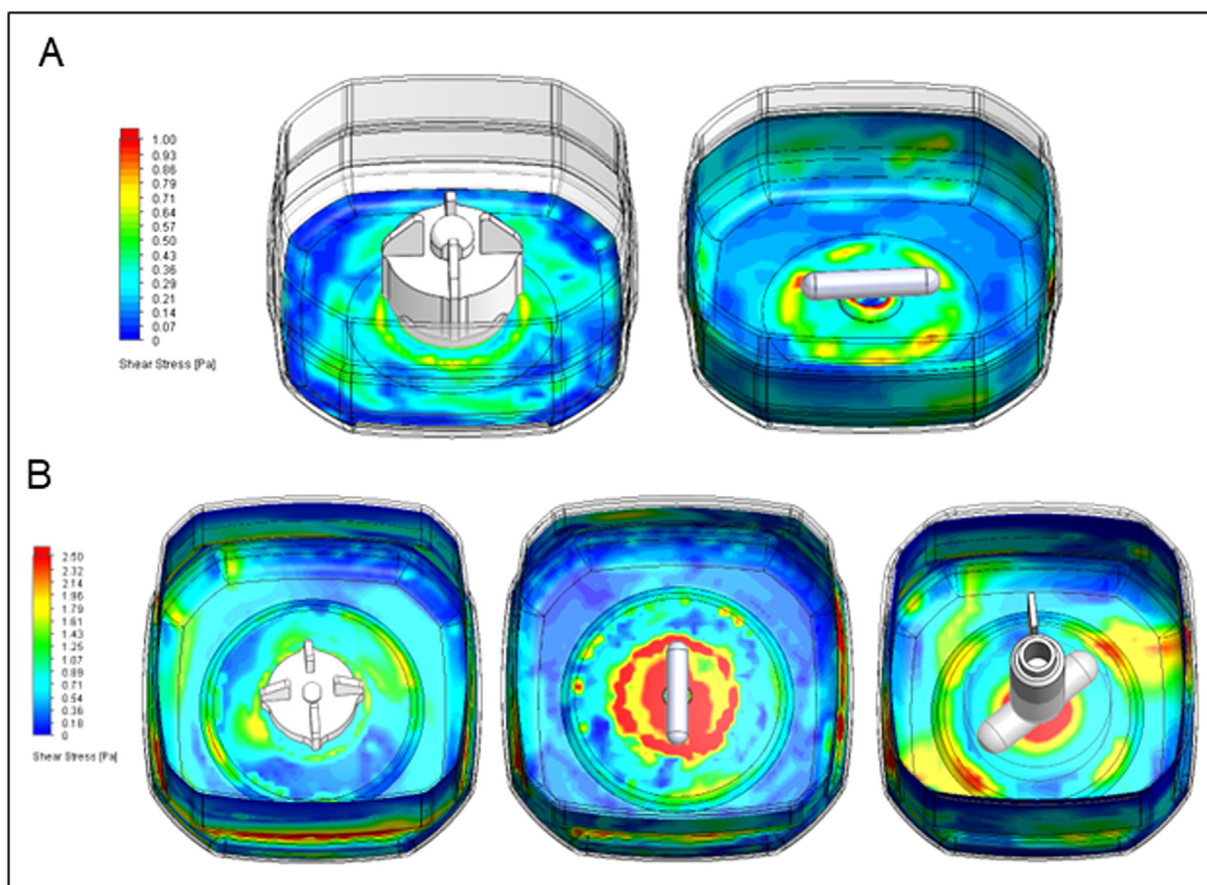


Fig. 10. (A) Mixing shear stress presented as contour plot of (A) the 0.5 L BMM and MS bottle at the minimal fill volume (0.05 L in 0.5 L bottle) and (B) 5 L BMM, MS and TMM bottle at the minimal fill volume adjusted to the TMM specifications (1.5 L in 5 L bottle). The scale bar and legend show the level of shear stress, from blue (lower shear) to red (higher shear), at (A) from 0 to 1.0 Pa and at (B) from 0 to 2.5 Pa. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

circulation, the flow near the outer vessel walls remains comparatively calm, suggesting reduced momentum transfer to the periphery. Regarding the contour plot for the higher fill volume of the MS and BMM (Fig. 10 (B)), adjusted to the fill volume of the TMM, we can see higher zones of higher shear stress values in the mixing area. In the BMM, strong mixing around the edges of the wings is present and we can see higher shear stress where the bottle shows shifting geometry, as well as the corners. Whereas for the MS, we can see that the shear stress is higher in the mixing field of the magnetic stirrer and low around the edges of the bottle. The minimal fill volume in the 5 L bottle for BMM and MS at lower and higher tip speed (1.0 vs. 1.6 m/s) show similar trends regarding the generated shear stress in comparison to the adjusted higher fill volume.

When calculating the Reynolds number for the different impeller configurations across various rotational speeds, the observed flow regimes for the MS/BMM setups with the smaller impeller (0.5 and 2 L bottle) at all speeds (200 – 600 rpm) and also at low rotational speed (200 rpm) for the larger impeller (5 and 20 L bottle), were transitional ($Re_{imp} = 3307.5\text{--}9922.5$). Whereas for the MS/BMM setups operating at 400 or 600 rpm with the larger impeller, the flow entered a fully turbulent regime ($Re_{imp} = 17204.3/25806.4$). In contrast, the TMM configuration consistently exhibited turbulent flow patterns ($Re_{imp} = 464,515\text{--}9677,400$) across all tested impeller sizes and rotational speeds (80–600 rpm).

For the comparative analysis of mechanical stress this implies that turbulent flow conditions were present in all setups with the larger impeller (5 L bottle) but not with the smaller impeller (0.5 L) at the applied rotational speeds (400–600 rpm). Although the Reynolds number exceeded the turbulent threshold in most cases, qualitative and quantitative differences in flow behavior were still observed

between the mixer types. For instance, the MS configuration exhibited localized dead zones and reduced flow coherence under specific conditions, indicating that a high Reynolds number alone does not guarantee effective bulk mixing. These observations underline the importance of both global flow regime metrics and local hydrodynamic characteristics when evaluating mixing performance.

Although the reason for inactivation and precipitation is diverse and shear is only one of them, the experimentally evaluated inactivation rates were correlated to calculated shear stress data from CFD simulations. We used the ratio of shear stress (Pa)/fill volume (L) to account for differences in shear depending on fill volume. When plotting the inactivation rate vs. the shear stress per volume (Pa/L), we obtain a linear correlation with an R^2 of 0.72, as seen in Fig. S6, in the supplement.

Discussion

In the present study, we examined the mixing performance of three different single-use mixing systems by studying the dissolution time of dissolving NaCl in water, depending on various process parameters and study conditions. We further evaluated the three mixing systems for mechanical stress during mixing, using lysozyme as a model protein. Lastly, CFD simulations were performed visualizing mixing patterns and areas of elevated shear in order to discuss experimental results from mechanical stress testing.

Mixing performance

When assessing the dissolution times for the different mixers, we found that despite mixer type, two parameters significantly influence

the dissolution time; which were, tip speed and $d_{\text{impeller}}/D_{\text{bottle}}$. We also found that the BMM performance was superior (lower dissolution time) compared to the MS for all setups, especially low volumes, and comparable to the TMM setups, as high fill volumes. In this regard, we have identified challenges for some mixing configurations, especially at high fill volumes when using the MS. For the two configurations tested, nominal fill 2 L and 20 L maximally filled and stirred at the lowest rotational speed, the MS failed to fully dissolve NaCl, even after extended mixing times. For the 2 L bottle, visual observation revealed that agitation occurred primarily in the lower portion of the vessel. A clear dividing line was observed, above which there was minimal or no fluid movement (see Fig. S2 in the supplement), indicating ineffective mixing in the upper regions. In the 20 L bottle, a formation of a small vortex above the stirrer, extending approximately to the midpoint of the fill volume, could be seen visually. While this swirling pattern may indicate fluid motion, it does not equate to effective mixing. Such vortex formation leads to circular motion that largely recirculates fluid near the stirrer without promoting adequate radial or vertical exchange.

The reduced mixing efficiency observed with the MS can be attributed to the flow patterns revealed by computational fluid dynamics (CFD) simulations, seen in Fig. 9. The geometry and dimensions of the stir bar appear not ideal for use in taller vessels, as evidenced by limited fluid motion near the corners and the presence of dead zones, indicating inadequate circulation. Furthermore, the flow induced by the stir bar lacked directional coherence, which likely contributed to lower mixing performance and reduced homogeneity throughout the vessel. In contrast, the BMM demonstrated improved mixing efficiency, characterized by the formation of a stable recirculation loop that enhanced fluid motion and provided more uniform mixing throughout the volume. This coherent flow pattern likely minimized dead zones and promoted better overall mixing. TMM also exhibited a high mixing efficiency whereas, the presence of a fixed baffle effectively disrupted the circular flow commonly associated with vortex formation, promoting increased local shear and enhancing axial mixing. This disruption of dominant swirling flows facilitated more effective top-to-bottom mixing, leading to improved homogenization and reduced stratification. Comparing the mixing performance to unbaffled configurations, where the flow behavior is led by vortex formation and rotational bulk motion,¹⁸ the integrated baffle in the TMM can likely disrupt flow patterns and redistribute the flow within the vessel. This potentially leads to the improved axial and radial mixing of the baffled system. Overall, these findings illustrate that the MS generated non-uniform mixing patterns, particularly at high fill volumes, where dead zones and limited bulk movement reduce mixing efficiency. This further highlights the limitations of the MS for large-scale or high-volume mixing applications, where BMM and TMM outperform.

With respect to the influence of tip speed on mixing performance, Parvizi et al. have shown that with an increase in impeller speed, the dead zones around the impeller decrease and are more so stuck to the mixer wall, which we could also see with the mixing profiles (Fig. 9) generated by CFD simulations.¹⁹ In line with our results, Nakach et al. established in an extensive study that under turbulent flow conditions, mixing time decreases with increasing rotational speed and then remains mostly constant at higher Reynolds numbers.⁴ Zhou et al., have also shown that the energy dissipation rate is directly proportional to the rotational speed (tip speed) and that in turbulent mixing systems with constant geometry, velocity fluctuations scale proportionally with the impeller's tip speed.²⁰ In our study, we observed a similar trend regarding the influence of rotational speed on dissolution time, with dissolution time decreasing as rotational speed, and consequently Reynolds number, increases. Furthermore, our data support the conclusion that once the Reynolds number reaches the turbulent regime (achieved at higher tip speeds for BMM and MS and for all setups of TMM) dissolution time

stabilizes and remains relatively constant, consistent with the reported behavior and as seen in Fig. 5.

Nakach et al. further observed that mixing time increases with filling volume for a given vessel.⁴ To explore the influence of fill volume on dissolution time in the BMM, an intermediate fill level was tested, seen in Fig. S3, in the supplement. The results revealed differing trends depending on bottle size, suggesting a complex interplay between fill volume and flow structure. While fill volume did not show a statistically significant effect overall, these variations may stem from changes in flow patterns, which we have shown to impact mixing performance. Alberini et al. reported increased power consumption and altered vortex and recirculation structures at intermediate fill levels, which can enhance mixing efficiency.²¹ Such flow phenomena, alongside variations in Reynolds number and velocity profiles, may explain the divergent trends observed across bottle sizes.

We additionally reported that the $d_{\text{impeller}}/D_{\text{bottle}}$ ratio has a significant influence on mixing performance revealing that a higher ratio, decreases the dissolution time. This effect is particularly evident in small-volume bottles, where a larger impeller generates more vigorous fluid motion and improved circulation. The trend is clearly reflected in the mixing profiles (Fig. 9), which show increased fluid movement and more homogeneous mixing with larger impellers. Wu et al. demonstrated that mixing performance improves with increasing $d_{\text{impeller}}/D_{\text{bottle}}$, as evidenced by greater velocity, improved suspension homogeneity, and more uniform flow patterns, up to an optimal ratio of 0.46. Beyond this threshold, mixing performance tended to plateau or decline, likely because of increased wall interference and energy loss. In the present study, the highest $d_{\text{impeller}}/D_{\text{bottle}}$ investigated was 0.41 (as seen in Table S1, in the supplement), which remains within the previously reported optimal range for enhanced mixing efficiency. Therefore the observed increase in mixing performance indicated by faster dissolution times can be attributed to a higher $d_{\text{impeller}}/D_{\text{bottle}}$ ratio because it enhances energy input and fluid velocity while reducing dead zones and promoting more efficient mixing.²²

To summarize, conductivity-based measurements provided reproducible and robust methods to comparatively assess dissolution and therefore mixing behavior in the investigated systems. The experiments helped identify the differences in flow behavior, mixing efficiency, as well as the influence of operational parameters. Complementary orthogonal methods, including pH tracer measurements or optical visual techniques may provide additional information on mixing dynamics, especially for systems with alternate formulations or non-conductive components. These approaches could further support mechanistic characterization of mixing performance in future investigations.

Evaluation of mechanical stress

Mechanical stress generated from the different mixers was evaluated by quantifying the inactivation rate of a model protein (lysozyme) calculated from remaining lysozyme concentration, as well as by lysozyme purity assessment. When testing statistical significance using the lysozyme inactivation rate data, our statistical model revealed the mixer type, namely BMM and MS and the fill volume as statistically significant parameters. The absence of a statistically significant effect for TMM may be due to the limited number of observations in this category, as this mixer type is only available and suitable in larger volume configurations. This restriction likely reduced the model's ability to reliably estimate its effect.

Product quality assessments across the different mixing systems revealed that protein precipitation was most pronounced in the MS configurations, whereas the BMM and TMM setups exhibited comparatively less precipitation. This trend follows the results from the protein inactivation analysis and is reflected by the correlation of the

precipitated fraction and inactivation rate following exponential kinetics.

The impact of mechanical stress on protein stability has been studied by Kiese et al. The authors examined the impact of agitation, specifically stirring and shaking, on a mAb (IgG1) formulation. They concluded that the impact on product quality as a result of mechanical stress from stirring is a result of multiple factors: interfacial effects between the protein and surfaces such as the stirrer, container bottom, or hydrophobic materials like Teflon; cavitation; local thermal effects due to friction between the stirrer and the container bottom; and the rapid transport of aggregated or adsorbed species into the bulk solution, thereby facilitating further aggregation. Their findings also suggested that shear stress alone does not cause protein unfolding and aggregation; rather, interfacial phenomena and other localized stresses contribute significantly to aggregation processes under these conditions.¹² Therefore the effects on protein quality were assessed experimentally through enzyme inactivation analysis as well as by characterization of protein precipitation by assessment of visible particle formation. Colombié et al. have discussed the inactivation of lysozyme itself in a stirred tank and they concluded that simultaneously to the inactivation of lysozyme, aggregation was proportionally occurring, induced by the collision between native and inactive enzymes. This observation aligns with our findings, where changes in purity, over four hours of mechanical stress, suggest scrambled lysozyme species²³ and visual inspection indicated aggregation processes occurring during the experiment, even leading to protein precipitation. Also, Gikanga et al. have discussed that besides grinding stress as found for their BMMs, other stress factors such as mechanical stress, interfacial stress and cavitation may impact protein degradation.⁸ Eshraghi et al. recently confirmed that cavitation at vapor - solution interfaces (through bubble nucleation, oscillation, and collapse) induces significantly more subvisible particles than shear stress or air - solution interfacial stresses, by using controlled stressing experiments on bovine serum albumin (BSA).²⁴

When performing CFD simulations regarding shear stress, we focused on fluid dynamics. Analyzing the correlation between the experimentally evaluated inactivation data and shear stress calculations, we observed an R^2 of 0.72 (seen in Fig. S6 in the supplement), indicating a moderately good fit, and we can assume additional factors beyond shear likely contribute to protein inactivation in the mixers, as suggested by Kiese et al.¹² As emphasized previously, protein instability cannot generally be attributed to shear stress alone. Mechanical stress in protein formulations is influenced by a combination of factors; therefore, the CFD results generated in this study are primarily used to compare the mixing environments of different mixer configurations rather than to directly predict protein stability. Nevertheless, the CFD-based contour plots of shear stress and flow patterns provide insight into the hydrodynamic behavior of the systems and aid in the interpretation of differences observed between the mixer types.

Regarding the influence of mixer type on protein quality, Gikanga et al. reported that increased contact between impeller and the vessel surface, such as in formerly designed BMMs, can promote particle formation in mAb formulations. They concluded that particles generate particles because of mechanical contact between mixer parts which can cause shear, grinding and cavitation.⁷ Similarly, Ladner et al. combined experimental investigations with CFD-supported hydrodynamic characterization of bottom-mounted magnetic stirrers and demonstrated that mixer designs minimizing physical contact between moving components were associated with reduced particle formation and improved product quality in monoclonal antibody formulations.¹⁰ Transferring their conclusions to our data, this may imply that the MS in our set-up, which has the most contact with the bottom of the bottle, has the highest inactivation rates, in comparison to the BMM, which does not have the same contact surface and thus performing superior in mechanical stress. This finding is supported

by both the contour plots of shear stress as well as by the mixing profiles seen with CFD simulations, as we have observed a much more chaotic and less uniform profile for the MS, whereas we see more gentle and uniform mixing for the BMM. The latter may also explain the impact of increased rotational speed, shown as increase in tip speed in Fig. 6, on increased protein inactivation rate for comparable setups for the MS setups, which was not seen for other setups, BMM and TMM.

The findings of this study have further shown that with a higher fill volume in the tested bottle, regardless of the used mixer, the inactivation rate has decreased significantly. In a second publication of Colombié et al. have also suggested that the observed decreased inactivation rate of lysozyme in larger volumes can be attributed to lower mechanical stresses, including reduced specific interfacial area per unit volume. In smaller volumes, the same mixer generates higher power density, increasing molecular collisions and interactions at air - liquid and impeller - liquid interfaces, which promote enzyme unfolding, aggregation, and inactivation. Consequently, lysozyme inactivation proceeds more slowly in larger volumes under identical mixing conditions.²⁴

In summary, as protein quality in mixing systems is influenced by a combination of hydrodynamic effects, interfacial exposure and flow induced transport phenomena, the present work focused on a comparative evaluation of the resulting protein stability under different mixing parameters. The CFD analysis provided additional insight into the underlying mixing behavior and hydrodynamic differences between mixer configurations, supporting interpretation of the experimentally observed trends. However, as protein instability cannot generally be attributed to shear stress alone, the CFD results should be interpreted as a characterization of the mixing environment rather than a direct predictor of protein stability. Furthermore, the CFD studies were performed using aqueous systems with comparable fluid properties to enable a controlled comparison of the influence of mixing parameters. Studies including viscosity variations and additional protein systems could further increase the applicability of the analysis across a broader formulation design space.

Conclusion

Choosing adequate mixing systems for biopharmaceutical manufacturing remains challenging and mixing performance and the mechanical stress-related impact on product quality must both be carefully evaluated when choosing (single-use) systems and process parameters for this unit operation. In this study, we compared single-use mixing systems, BMM, TMM, and MS in a bottle configuration, commonly used in biological manufacturing. The MS consistently exhibited longer dissolution times across varying parameters with technical challenges at higher mixing volumes, compared to BMM and TMM demonstrating comparable performance, while MS and BMM can be applied to very low mixing volumes. Based on a statistical model, mixing performance was found to be dependent on mixer type, tip speed, and the ratio of the impeller diameter to bottle diameter, but not on other process or dimensional parameters. Mixer type and the fill volume were identified as statistically significant parameters that impacted the model protein (lysozyme) product quality (inactivation rate). We report that a higher mechanical stress related quality impact was found for the conventional MS compared to the BMM in a comparable set-up. At higher fill volumes, there was no difference between the three mixers in comparison to the control in this regard. CFD simulations further complemented the mixing performance as well as mechanical stress testing results. In summary, these findings may provide an approach for choosing appropriate single-use mixing systems in biopharmaceutical manufacturing and assessing the risk for potential product impact.

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Declaration of competing interest

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Schemes were created with BioRender.com (<https://BioRender.com/rpsg9dw>)

Supplementary materials

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