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ORIGINAL ARTICLE

Development of 48-condition buffer screen for protein stability assessment

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Abstract

The determination of a suitable buffer environment for a protein of interest is not an easy task. The requirements of advanced techniques, the demands on the biological material and the researcher time needed for buffer optimization, as well as personal inflexibility, lead frequently to the use of sub-optimal buffers. Here, we demonstrate the design of a 48-condition buffer screen that can be used to determine an appropriate environment for downstream studies. By the combination of several techniques (differential scanning fluorimetry, dynamic light scattering, and bio-layer interferometry), we are able to assess the protein stability, homogeneity and binding activity across the screen with less than half a milligram of protein in 1 day. The application of this screen helps to avoid unsuitable conditions, to explain problems observed upon protein analysis and to choose the most suitable buffers for further research. The screen can be routinely used as a primary screen for buffer optimization in labs and facilities.

Keywords Protein stability · Buffer · Screening · Differential scanning fluorimetry · Dynamic light scattering · Bio-layer interferometry

Introduction

The vast majority of processes in living organisms require a water-based environment. This applies not only to enzymatic reactions but to biomolecular interactions in general. Since the experimental work in a natural environment, such as cell cytoplasm or body fluids, faces multiple complications (complexity of the solution, non-repeatability of experiments, inaccessibility of material, price), most of the experiments are conducted in a defined environment—a buffer (Stoll and Blanchard 2009). The buffer is a solution that usually contains up to four kinds of molecules: water as a solvent, buffering substance (weak acid or base) maintaining desired pH, salt for ionic strength adjustment and additives for specific purposes.

While the simplicity of the used buffer is highly advantageous over the complex natural environment, it is known that individual macromolecules may act differently in different environments. Some of them even require quite strict conditions for their function (Sambrook and Russell 2001; Ferreira et al. 2015). Therefore, the use of a single buffer for all purposes is not reasonable. Commonly used buffers, e.g., the phosphate-buffered saline (PBS) at pH 7.4, combine the so-called physiological neutral pH and isotonic osmotic pressure. However, it was shown in many cases that PBS is not entirely suitable buffer for particular purposes (Blanchard 1984). On the other hand, it is not possible to perform each experiment in numerous buffers, especially in case of demanding techniques, such as cryo-electron microscopy, X-ray diffraction, and nuclear magnetic resonance. As a compromise, a search for an "optimal" buffer for molecule(s) of interest is usually done, focusing mainly on macromolecular stability and activity.

There exists a number of commercially available buffer screens nowadays (Vedadi et al. 2006; Boivin et al. 2013;

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Grøftehauge et al. 2015). They are intended for various purposes, e.g., crystallization, enzymatic assays, and stability. They are usually designed as 96-condition screens due to the compatibility with a standard ANSI/SLAS plate layout (slas.org), pipetting robotics and experimental machines, e.g., RT-PCR thermocycler or fluorescence reader. However, several disadvantages can also be found: (i) a relatively high number of tested conditions results in an issue of the sample consumption; (ii) many instruments are not directly compatible with 96-well design; (iii) a higher number of conditions disfavors manual setup that is still a common approach in many cases; (iv) the precious experimental time is directly proportional to the number of conditions tested for serial measurements. All these issues increase the cost of the experiment, and also raises the question of comparability of results, considering that there might be multiple hours (or even days) between the first and the last condition tested for a particular sample. This problem could be partially prevented using multiplicates, which, however, increases the time and sample consumption even further. All the above-mentioned aspects favor screens of a lower number of individual conditions to be used while keeping the broad variability.

Here, we describe a design of the 48-condition buffer screen, that covers a broad area of pH values, ionic strength and common additives. Using two examples of sugar-binding proteins, the lectins AFL from *Aspergillus fumigatus* and BC2L-C-Nterm from *Burkholderia cenocepacia*, we demonstrate its applicability in the determination of temperature stability, protein aggregation, and binding properties. We show a possibility to obtain all necessary data with as low as 0.4 mg of the protein in less than 1 day of experimental time. We also discuss the obtained results with a focus on protein quality assessment.

Materials and methods

Screen preparation

Buffer screen components were purchased from Sigma-Aldrich, Penta, AppliChem and Carbosynth companies in high purity (> 98%) and dissolved in demineralized ultrapure water to form 5x-concentrated stock solutions. pH was adjusted where appropriate. Resulting solutions were filtered using 0.2 µm sterile filter, aliquoted and stored frozen at − 20 °C. Prior to usage, the aliquots were thawed and used immediately for subsequent experiments.

Protein preparation

The recombinant lectin AFL from *Aspergillus fumigatus* was prepared as described previously (Houser et al. 2013). In brief, *Escherichia coli* BL21(DE3)Gold cells harboring pET29 vector with inserted *afl* gene were cultivated in the standard low salt LB medium with 50 µg ml^{-1} kanamycin at 37 °C until OD_{600} reached 0.5. After the induction by 0.5 mM isopropyl-β-thiogalactoside and additional 3-h cultivation at 30 °C, the cells were harvested using centrifugation (10 min/4 °C/6000 g), and the pellet was resuspended in 20 mM Tris/HCl, pH 7.3. Cells were disintegrated by sonication, insoluble fractions were removed by centrifugation for 40 min at 4 °C at 21,000 g, and AFL was purified by affinity chromatography on a mannose-agarose column (Sigma-Aldrich) using FPLC system Äkta purifier (GE Healthcare) with isocratic elution. Fractions containing pure AFL were combined, desalted by dialysis against water and lyophilized for long-term storage prior to other experiments.

The recombinant lectin BC2L-C-Nterm from *Burkholderia cenocepacia* was prepared as described previously (Šulák et al. 2010). In brief, *Escherichia coli* BL21(DE3) cells harboring pET25 vector with inserted gene *bc2l-C-Nterm* were cultivated in the standard low salt LB medium at 37 °C until OD_{600} reached 0.5. After the induction by 0.5 mM isopropyl-β-thiogalactoside and additional 3-h cultivation at 30 °C, the cells were harvested using centrifugation (10 min/4 °C/6000 g), and the pellet was resuspended in 20 mM Tris/HCl, 100 mM NaCl, pH 7.4. Cells were disintegrated by sonication, insoluble fractions were removed by centrifugation for 30 min at 4 °C at 21,000 g, and BC2L-C-Nterm was purified by affinity chromatography on an Ni–NTA agarose column (Sigma-Aldrich) using FPLC system Äkta purifier (GE Healthcare). Protein was eluted using step gradient of imidazole (0–250 mM) in the running buffer and the fractions containing pure BC2L-C-Nterm were combined and dialyzed against 10 mM Tris/HCl pH 7.5. The protein was used immediately after the dialysis for the subsequent experiments.

Buffer screening

The lyophilized protein AFL was dissolved in demineralized ultrapure water to 0.5 mg ml^{-1} concentration of the stock solution. BC2L-C-Nterm was used directly after preparation, with a stock solution concentration of 0.12 mg ml^{-1}. Each protein was mixed with the buffer screen solutions in PCR plate in 4:1 (v/v) ratio (16 µl protein + 4 µl 5x-concentrated buffer stock solution) and centrifuged (4000 rpm/1 min). The resulting 20 µl of protein:buffer solution was used for all subsequent experiments. Initial concentration (0.4 mg ml^{-1} for AFL, 0.1 mg ml^{-1} for BC2L-C-Nterm) was used for differential scanning fluorimetry and dynamic light scattering experiments. For bio-layer interferometry, the solution was further 40 × diluted (10 µg ml^{-1} for AFL, 2.5 µg ml^{-1} for BC2L-C-Nterm) in corresponding 1x-concentrated buffers.

Differential scanning fluorimetry

Protein temperature stability was determined using differential scanning fluorimetry (nanoDSF) on Prometheus NT.48 (Nanotemper). The initial concentration of AFL of 0.4 mg ml^{-1} (0.1 mg ml^{-1} for BC2L-C-Nterm) in corresponding protein:buffer solution was used to fill one standard capillary (Nanotemper) per condition. The temperature stability measurement was conducted in the range of 20–95 °C at a heating rate of 1 °C min^{-1}, and the intrinsic tryptophan fluorescence at 350 and 330 nm was recorded. The protein stability was evaluated based on T_m (melting temperature, calculated as an inflection point of the $F_{350/330}$ vs. temperature curve) and T_{on} (temperature onset, calculated as a point where two state fit of the $F_{350/330}$ vs. temperature curve deviates from the baseline by more than 0.5%) parameters, as defined by PR.ThermControl evaluation SW (Nanotemper) with a manual check.

Dynamic light scattering

The tendency of the protein to form aggregates was determined by dynamic light scattering (DLS) in the plate using SpectroLight 600 (Xtal Concepts). Two 0.5 µl drops of each of the protein:buffer solutions (0.4 or 0.1 mg ml^{-1} for AFL or BC2L-C-Nterm, respectively, identical solutions as those for temperature stability) were pipetted under paraffin oil (Molecular Dimensions) into the 96-well plate (Vapor Batch Plate Silver, Douglas Instruments). Twelve scans of 10-s data acquisition for each well was performed in 1 h intervals to examine the sample stability in time. Data were collected and processed by in-built SW (Xtal Concepts). The presence of aggregates was determined based on a regularization fit of obtained autocorrelation functions of scattered light. The data were evaluated as: (i) the hydrodynamic radius (R_h) of the main peak in expected protein range (2 nm < R_h < 10 nm for AFL, 1.7 nm < R_h < 10 nm for BC2L-C-Nterm); (ii) the polydispersity index (PDI) of the main peak; (iii) the qualitative analysis of the presence of aggregates (the presence of peaks of R_h > 10 nm) for intensity-based data. The theoretical sizes of the AFL monomer and dimer and of the BC2L-C-Nterm monomer and trimer were calculated using the known 3D structures (PDB 4agi and 2wq4) and SW Hydropro (Ortega et al. 2011). The expected size of the unfolded monomer was calculated using an online Hydrodynamic Radius Converter (Fluidic Analytics). For the unfolded monomer maximum size, a theoretical calculation was done, multiplying the length of extended sheet conformation (0.35 nm) by the number of protein residues (315 for AFL and 187 for BC2L-C-Nterm).

Bio-layer interferometry

For the determination of AFL and BC2L-C-Nterm binding abilities at various conditions, the technique of biolayer interferometry (BLI) on Octet RED96e (ForteBio) was employed. For each condition, 5 µl of the initial protein:buffer solution was diluted with 195 µl of 1x-concentrated buffer solution in 96-round-well flat-bottom black plate (Corning) resulting in 200 µl of working solution of 10 µg ml^{-1} AFL or 2.5 µg ml^{-1} BC2L-C-Nterm, respectively. 200 µl of 1x-concentrated buffer solution was used for dissociation for each condition. SA biosensors (ForteBio) bearing immobilized streptavidin were initially loaded by 5 min immersion into 0.25 mM α-L-fucose-biotin solution (Lectinity), washed for 5 min in ultrapure water and subsequently used for the BLI experiment. Six sensors were used in parallel in the following procedure for each condition tested: 180 s association in protein:buffer solution, 180 s dissociation in the corresponding buffer, two cycles of 20 s regeneration in 50 mM NaOH and 20 s water wash and final 60 s stabilization in water. Six SA biosensors bearing immobilized streptavidin with no sugar immobilized were used as a blank in the subsequent experiment with the same setup. Data were processed using Data Analysis 11.1 evaluation SW (ForteBio). Obtained binding curves were blank-subtracted and fitted by a 1:1 binding model. Resulting k_{obs} (rate of association), k_{off}, $K_{D(apparent)}$ and R_{max} values were examined.

Results

Buffer screen design

The 48-condition buffer screen was designed so to cover a broad range of conditions in a relatively small number of experiments (Table 1). It evaluates the effect of pH while allowing for a separation of the influence of pH and the buffering substance. It covers a broad range of ionic strength for buffers of pH 6–8 (most commonly used range for protein analyses). It includes several common additives that are being used during protein purification or storage. The four positions are designated for commonly used buffers. Those were chosen based on our core facility experience and may be adjusted to meet the demand of a particular protein or a lab.

The buffers in the screen were prepared as 5x-concentrated stock solutions, so to minimize protein dilution and allow the analysis also for unconcentrated samples. The concentrations chosen should allow for usage with proteins dissolved in up to 20 mM buffers, which is the case for most of the protein preparation routines. Chosen methods require

Table 1 Buffer screen conditions—final working concentrations

1x	1	2	3	4	5	6	7	8	9	10	11	12
A	REFERENCE	BROAD pH RANGE										
	-	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0	pH 10.0	pH 11.0	pH 12.0
	H_2O	maleate	glycine/HCl	formate	citrate	cacodylate/ HCl	HEPES/ NaOH	bicine/ NaOH	CHES/HCl	borate	CAPS/ NaOH	Na-phosphate
	-	50 mM*	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	50 mM*	100 mM	100 mM
B	DETAILED pH RANGE											
	pH 4.0	pH 4.5	pH 5.0	pH 5.5	pH 6.0	pH 6.5	pH 7.0	pH 7.5	pH 8.0	pH 8.5	pH 9.0	pH 9.5
	acetate	acetate	acetate	MES/NaOH	MES/NaOH	MES/NaOH	Na-phosphate	K-phosphate	Tris/HCl	Tris/HCl	glycine/ NaOH	glycine/ NaOH
	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM	100 mM
C	IONIC STRENGTH											
	pH 6.0				pH 7.0				pH 8.0			
	100 mM MES/NaOH				100 mM Na/K-phosphate				100mM Tris/HCl			
	100 mM NaCl	200 mM NaCl	500 mM NaCl	1000 mM NaCl	100 mM NaCl	200 mM NaCl	500 mM NaCl	1000 mM NaCl	100 mM NaCl	200 mM NaCl	500 mM NaCl	1000 mM NaCl
D	COMMON BUFFERS				ADDITIVES							
	pH 7.5	pH 7.5	pH 7.5	pH 7.5	pH 7.5	-	-	-	-	-	pH 7.0	pH 8.0
	buffer 1	buffer 2	buffer 3	buffer 4	imidazole/ HCl	Tween20	glycerol	βME	DMSO	D-Trehalose	L-Arg + L-Glu	EDTA
	-	-	-	-	200 mM	0.05% (v/v)	5% (v/v)	5 mM	5% (v/v)	5% (w/v)	20 + 20 mM	5 mM

*concentration lowered due to solubility limitation of stock solution.

a small amount of the protein and, when combined, give an overview of protein characteristics that meet the minimal sample characterization criteria (Fig. 1).

Thermal stability

One of the basic protein properties is its thermal stability. Therefore, we employed the technique of differential scanning fluorimetry (nanoDSF). We analyzed the thermal stability of the model proteins AFL (Fig. 2a) with moderate thermostability (published T_m around 50 °C) (Houser et al. 2013) and BC2L-C-Nterm (Fig. 3a) for which the T_m was not published so far.

We observed a general AFL preference for neutral or slightly acidic pH with citrate pH 5 being the most stabilizing buffer ($T_m = 51$ °C). An extreme pH below 4 or above 10 leads to a substantial decrease in the protein stability; the ΔT_m drop of up to 20 °C for pH 12 compared to the best condition. This is also accompanied by the decrease in signal strength and may cause failure in automated data processing. Furthermore, the results revealed a mild stabilization by higher ionic strength that was also pH-dependent (lowest to highest tested ionic strength difference of $\Delta T_m < 1$ °C for pH 6 and $\Delta T_m > 3$ °C for pH 8). None of the common buffers showed a significant deviation from the other conditions at similar pH. Among additives, glycerol stabilized the protein the most with the highest T_m overall of 53.9 °C. A similar

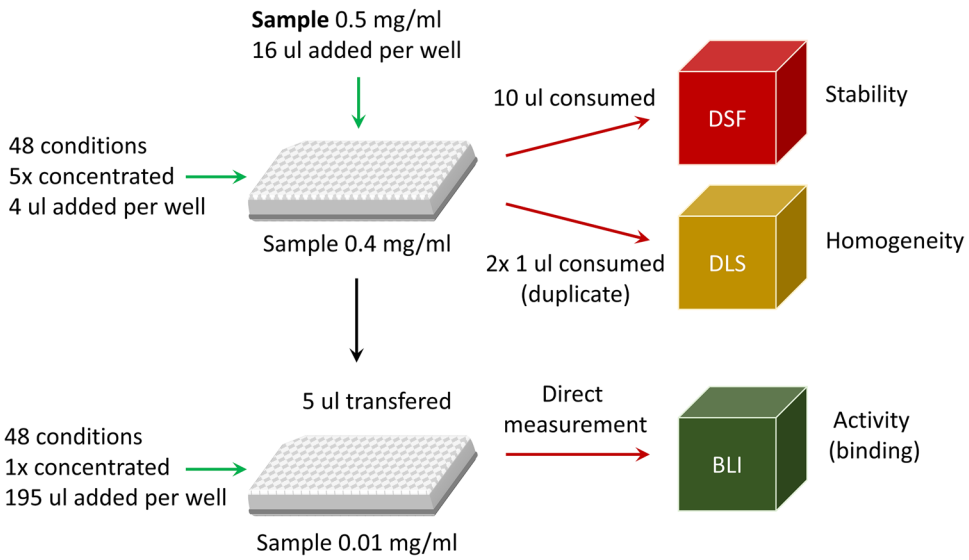

Fig. 1 Buffer screen application for protein characterization—overall scheme. Displayed concentrations were adjusted based on AFL properties. For BC2L-C-Nterm, 4-times lower concentrations were used. Volumes are based on recommended procedures for instruments used

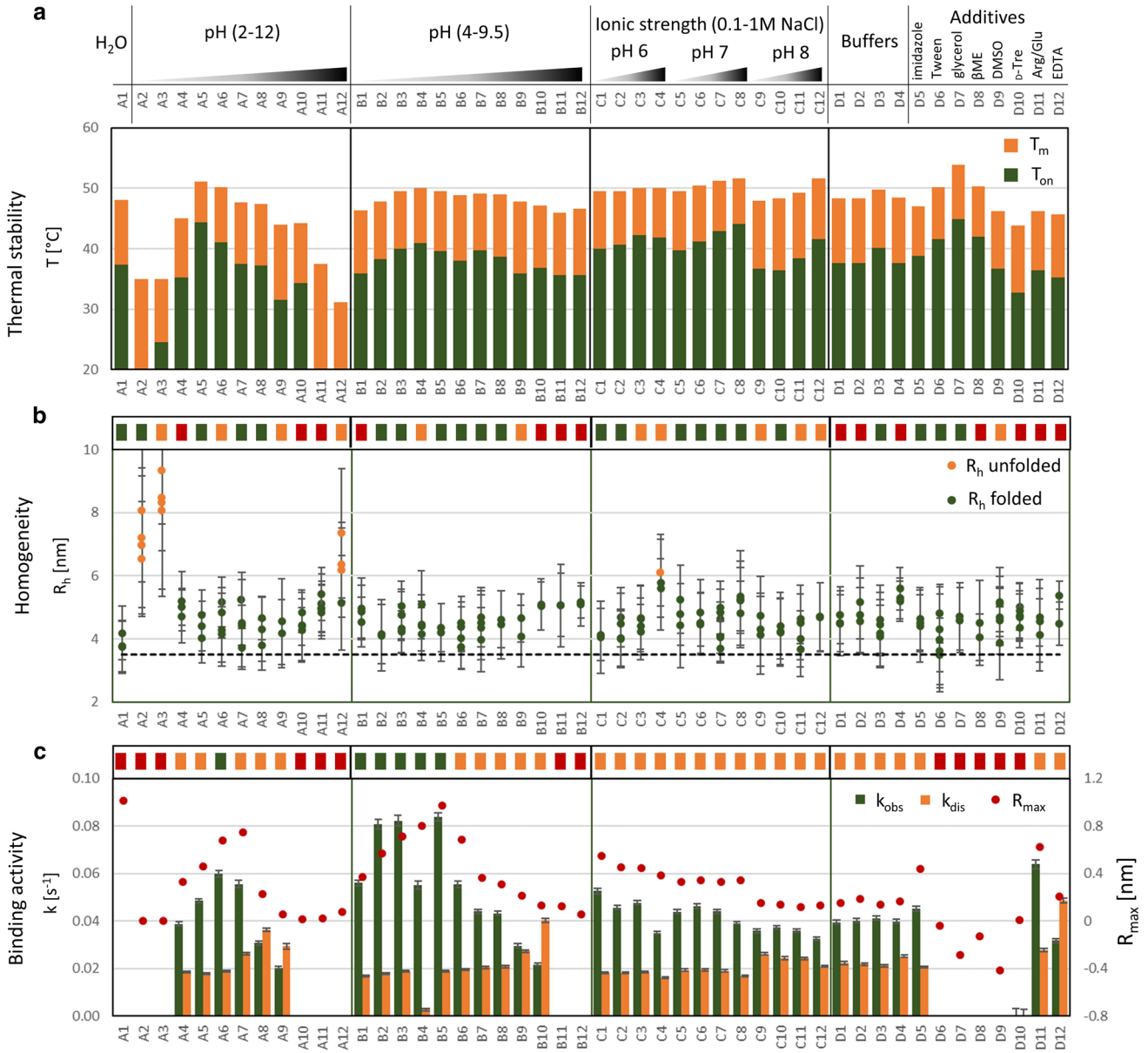

Fig. 2 Protein AFL characterization by buffer screen. **a** Thermal stability determined by nanoDSF. Green columns represent T_{on}, orange columns T_m. **b** Protein homogeneity assessed by DLS. Upper panel: tendency to form aggregates (see Fig. 4) represented in three-color scale. Green stands for negligible aggregates formation, orange for slight tendency to form aggregates, red for clear formation of aggregates. Lower panel: hydrodynamic radius (R_h) of protein peaks. All calculated peaks from both duplicates in range 2–10 nm depicted as dots. Green stands for size compatible with folded monomer/dimer size, orange stands for highly unfolded protein/microaggregates. Error bars represent calculated polydispersity of each peak. Dashed line shows theoretical size of AFL dimer calculated from X-ray structure. **c** Binding activity determined by BLI. Upper panel: apparent affinity represented in three-color scale. Green stands for high relative affinity ($K_{D(best)} - 10\times K_{D(best)}$), orange for lower relative affinity ($> 10\times K_{D(best)}$) and red for no affinity or unclear result (K_D cannot be determined). Lower panel: calculated association (k_{obs}) and dissociation (k_{dis}) rates shown on left axes as green and orange columns, respectively. Error bars stand for fitting error from single measurement. Maximum response (R_{max}) depicted as red dots on right axes

trend was observed when analyzing the T_{on}. Here, the stabilization effect of citrate pH 5 and glycerol was even more dominant, accompanied by high-salt phosphate at pH 7. In these three cases, the T_{on} for AFL reached over 44 °C.

In case of BC2L-C-Nterm, we determined relatively high stability with T_m reaching over 70 °C for majority of the conditions. We observed a drop of stability at acidic pH below 4, while at alkaline pH above 9, the T_m was not possible to determine. We can expect that the protein becomes (at least partially) unfolded at high pH values even at the lowest temperature used (25 °C). The highest stability was observed for high ionic strength (1 M NaCl) reaching $T_m > 80$ °C. T_{on}

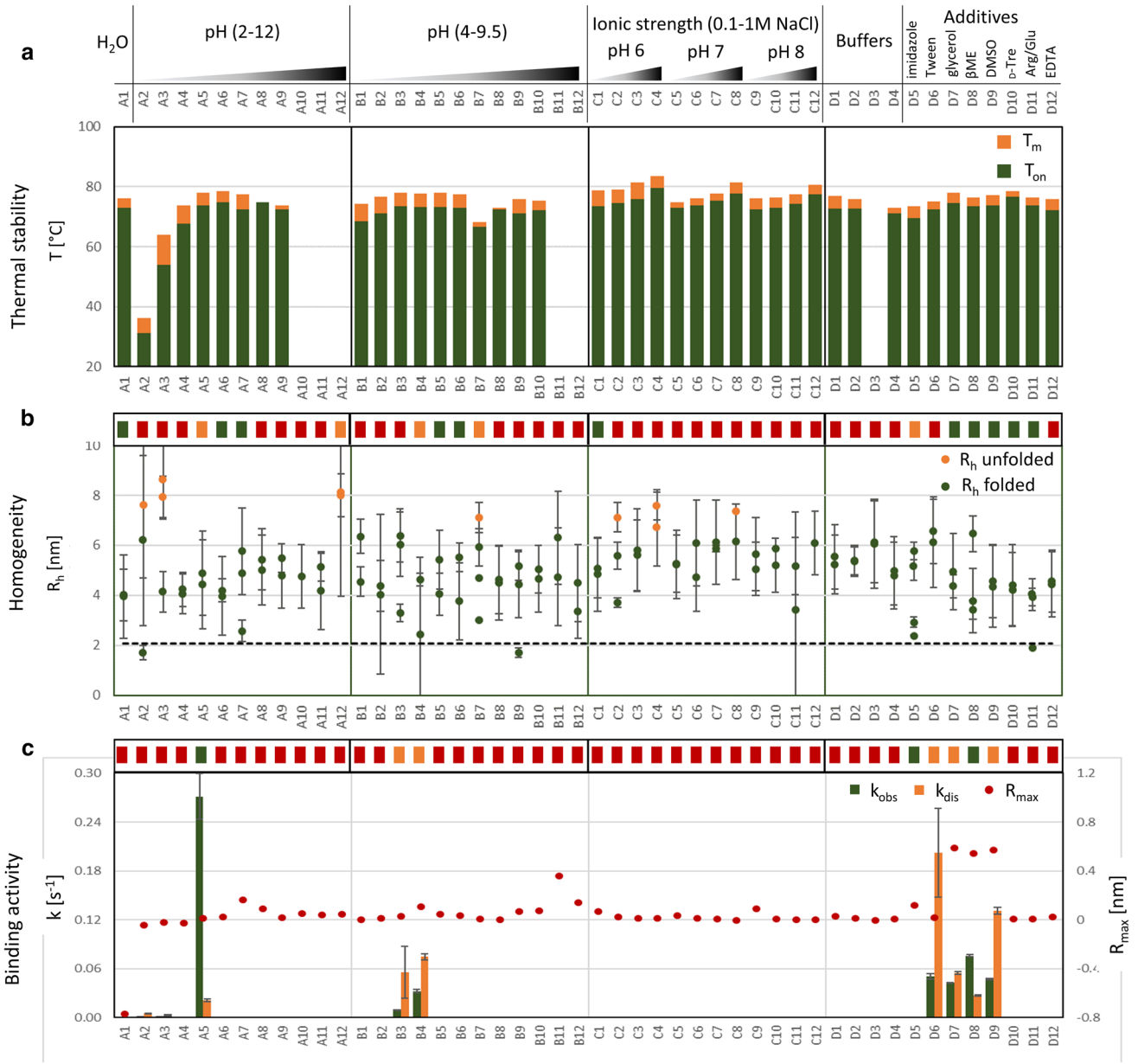

Fig. 3 Protein BC2L-C-Nterm characterization by buffer screen. **a** Thermal stability determined by nanoDSF. Green columns represent T_{on}, orange columns T_m. **b** Protein homogeneity assessed by DLS. Upper panel: tendency to form aggregates (see Fig. 5) represented in three-color scale. Green stands for negligible aggregate formation, orange for slight tendency to form aggregates, red for clear formation of aggregates. Lower panel: hydrodynamic radius (R_h) of protein peaks. All calculated peaks from both duplicates in range 1.7–10 nm depicted as dots. Green stands for size compatible with folded monomer/dimer size, orange stands for highly unfolded protein/micro-aggregates. Error bars represent calculated polydispersity of each peak. Dashed line shows theoretical size of BC2L-C-Nterm trimer calculated from X-ray structure. **c** Binding activity determined by BLI. Upper panel: apparent affinity represented in three-color scale. Green stands for high relative affinity ($K_{D(best)} - 10 \times K_{D(best)}$), orange for lower relative affinity ($> 10 \times K_{D(best)}$) and red for no affinity (K_D cannot be determined). Lower panel: calculated association (k_{obs}) and dissociation (k_{dis}) rates shown on left axes as green and orange columns, respectively. Error bars stand for fitting error from single measurement. Maximum response (R_{max}) depicted as red dots on right axes

values were close to the melting point, corresponding to a sharp transition. Generally, BC2L-C-Nterm is a protein with high thermostability based on both T_m and T_{on} values.

Homogeneity

For the protein homogeneity analysis, the method of dynamic light scattering (DLS) was used. The samples were measured repeatedly in the period of 12 h to evaluate the

time effect on sample quality (Figs. 4 and 5). The experiment was run in a duplicate to exclude random fluctuations in the samples (e.g., dust particles in the air) or pipetting errors. For all the conditions tested, a major peak corresponding to a particle radius within the range of 2–10 nm was observed (Figs. 2b and 3b), which corresponds to sizes of AFL dimer ($R_h = 3.5$ nm), BC2L-C-Nterm trimer ($R_h = 5.3$ nm) or to unfolded monomer ($5.9\ \text{nm} < R_h < 11$ nm for AFL, $4.2\ \text{nm} < R_h < 6.5$ nm for BC2L-C-Nterm) rather than folded monomer ($R_h = 2.6$ or 1.7 nm, respectively). The increase of R_h value was observed at extreme pH values of 2–3 or 12, where the protein was most probably dominantly in the unfolded state.

Considering the presence of aggregates in AFL solution, their total calculated mass was lower than 0.1% of total protein mass in all cases, demonstrating the stability of AFL in solution. However, due to the high sensitivity of the DLS technique, a tendency to form aggregates was observed for multiple conditions (Figs. 2b and 4). Similarly to nanoDSF, neutral to mild acidic conditions are favorable for avoiding the protein aggregation. All buffers with pH > 8 caused the formation of aggregates as well as several additives and two of the common buffers (HBS and PBST). The concentration of salt did not affect aggregation much; however, a higher ionic strength might stimulate the occasional formation of bigger particles.

The DLS analysis of BC2L-C-Nterm revealed a tendency to form small aggregates of approximately 30 nm radius at pH 8 or higher and at the higher ionic strength (Fig. 5). This was observed also for all 4 common buffers and for addition of Tween20. At pH 5 and lower, occasional formation of aggregates was also observed. The best behavior was seen for pH 6–7 or for various additives (e.g., glycerol or DMSO). Overall, BC2L-C-Nterm aggregates easily and the amount of suitable conditions is quite limited.

Sugar-binding activity

As the AFL and BC2L-C-Nterm proteins are both fucose-specific lectins, their activity is given by the ability to bind L-fucose. Therefore, we used a bio-layer interferometry technique (BLI) with immobilized α-L-fucoside on the sensor surface. The parameters of total response (R_{max}), association (k_{obs}) and dissociation (k_{off}) rates were evaluated (Figs. 2c and 3c). In addition, the apparent K_D value was calculated

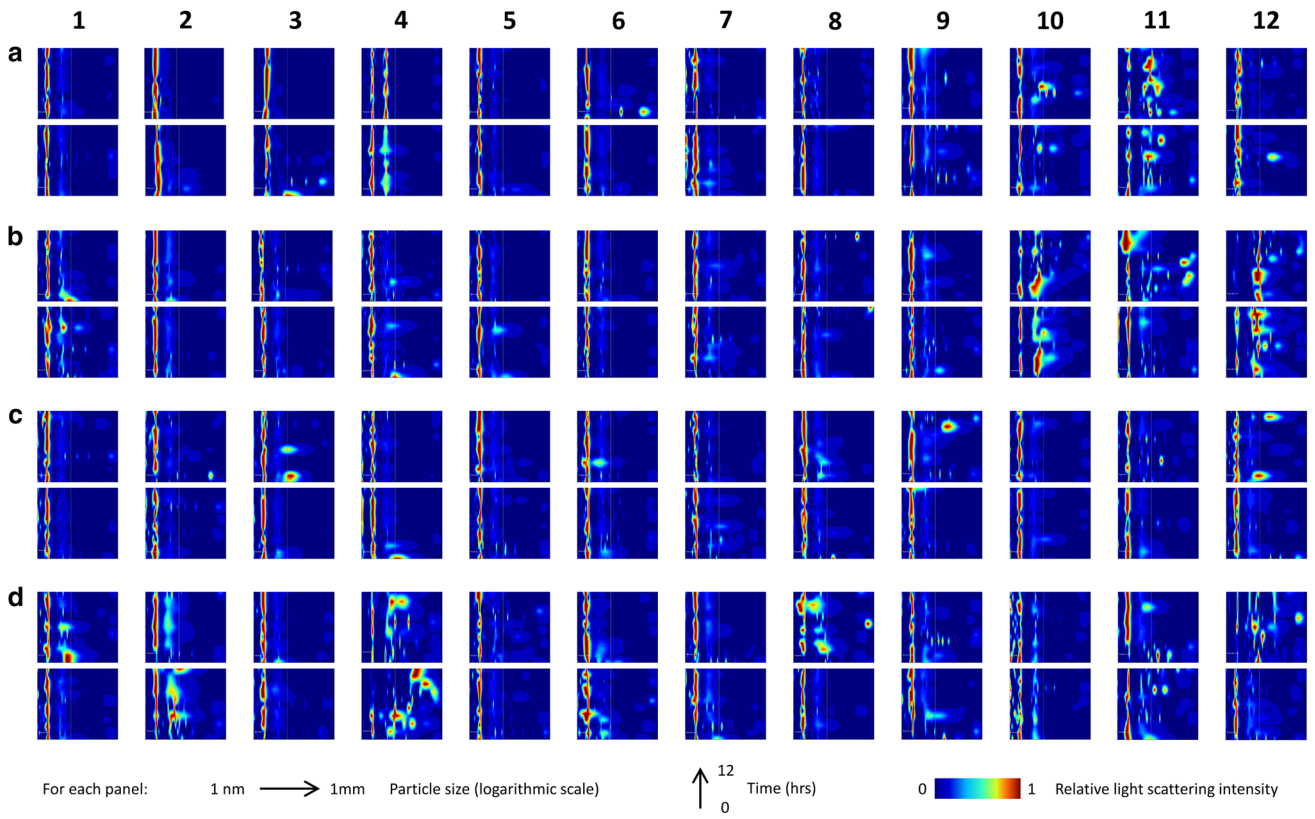

Fig. 4 Dynamic light scattering of AFL in buffer screen. For each condition, duplicate is shown. For each panel: X-axes—hydrodynamic radius in logarithmic scale (10^{-9}–10^{-1} m), Y-axes—time (bottom to top, 12 scans over 12 h range), color—observed relative intensity of dynamic light scattering from dark blue (0) to red (1)

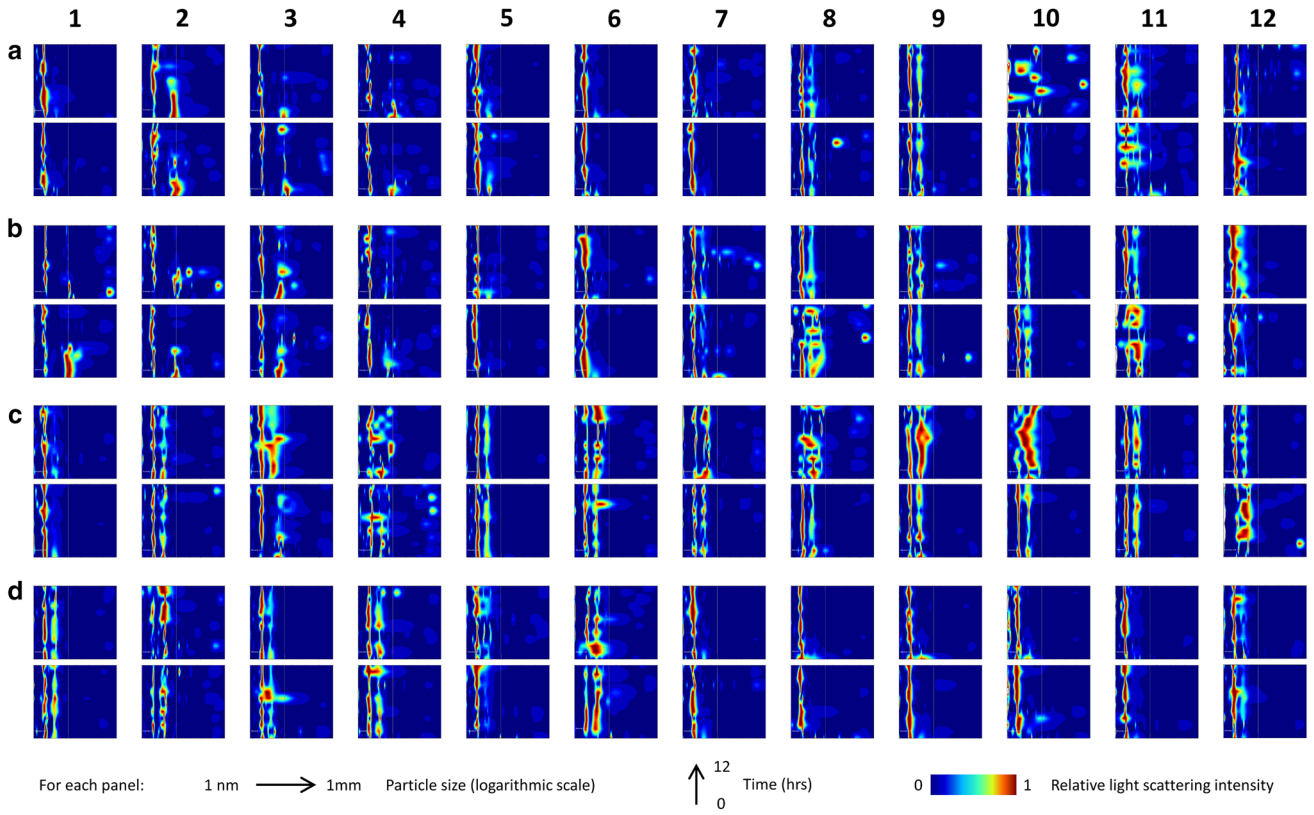

Fig. 5 Dynamic light scattering of BC2L-C-Nterm in buffer screen. For each condition, duplicate is shown. For each panel: X-axes—hydrodynamic radius in logarithmic scale (10^{-9}–10^{-1} m), Y-axes—time (bottom to top, 12 scans over 12 h range), color—observed relative intensity of dynamic light scattering from dark blue (0) to red (1)

to roughly compare the influence of individual conditions on protein binding.

For AFL (Fig. 2c), the total response was strongly pH-dependent with the highest response reached at pH 6 for both pH series (lines A and B of the screen). Highly acidic or alkalic pH resulted in no binding. Increase of ionic strength at pH 6 resulted in a slight decrease of the response, while for pH 7 and 8, it did not affect the binding response significantly. In the low ionic strength environment (water reference, additives with no charge), the response was high on both active and blank channels, corresponding to non-specific binding. For the uncharged additives, this resulted in negative value after blank subtraction. For water, the binding response was the highest of all conditions tested, however, the shape of the curve was affected, and the curve fitting by the binding model was unsuccessful. The observed association and dissociation rates did not differ by more than fourfold throughout all conditions, except for MES buffer pH 5.5, where the calculated k_{dis} was approximately tenfold lower than the average. Generally, the highest k_{obs} value was reached for slightly acidic conditions in acetate or MES buffers, the lowest k_{obs} and highest k_{dis} values were achieved for high pH values. The fast dissociation at high pH also corresponds to the fact that sodium hydroxide was efficiently used as a regeneration solution during the BLI experiment.

BC2L-C-Nterm (Fig. 3c) displayed mostly weak binding to the sensor surface and also slow dissociation, if any. This makes the comparison of individual conditions awkward. However, the binding was possible to be evaluated for a few conditions around pH 5 and also for several additives. The fastest association was seen in citrate pH 5, followed by imidazole pH 7.5 and β-mercaptoethanol. Binding and dissociation were possible to analyze also for the addition of glycerol, DMSO or D-trehalose but not for most of the buffers. With respect to the results from DLS, we can assume that the problems with the evaluation of BC2L-C-Nterm binding could be caused by the inhomogeneity in the solution. This could affect the binding and dissociation processes in a similar way that was observed by BLI measurement.

Discussion

The protein quality is an ongoing issue in biochemistry, molecular biology and related fields (Geerlof et al. 2006; Oliveira and Domingues 2018). The Association of

Resources for Biophysical Research in Europe (ARBRE-Mobieu; arbre-mobieu.eu) together with the Production and Purification Partnership in Europe (P4EU; p4eu.org) recognize protein quality, alongside with correct data management, as one of the crucial factors in biophysical research. Protein quality assessment includes multiple analyses with various techniques employed: identity (mass spectrometry (Qu et al. 2017)), conformational stability—frequently defined as the temperature stability (differential scanning fluorimetry (Senisterra and Finerty 2009), differential scanning calorimetry (Spink 2008)), and homogeneity (dynamic light scattering (Stetefeld et al. 2016), size exclusion chromatography (Fekete et al. 2014), and analytical ultracentrifugation (Pekar and Sukumar 2007)). Apart from basic characterization, the temperature stability helps to understand the results of long-time experiments (e.g., NMR), while the homogeneity analysis can be highly beneficial for protein crystallization or cryo-electron microscopy. For this study, we have chosen the DSF method with intrinsic Trp fluorescence detection that allows for high-throughput experiment without adding of external dye. Alternatively, a common DSF with an addition of external fluorescence dye may be used instead. Regarding the homogeneity analysis, DLS is probably the only available high-throughput technique. For many proteins, it is also important to analyze their activity. For enzymes, this is usually defined as the reaction rate, however, in the case of non-enzymatic proteins, the activity means usually the ability to bind a ligand. For this, several techniques can be employed, such as ITC, MST, SPR, BLI, NMR, SwitchSENSE (Kairys et al. 2019). While some of these techniques cannot be really run in a high-throughput mode (e.g., ITC), BLI and MST are well suited for quick measurements under various conditions.

Many problems with protein analysis lay in the identification of a suitable buffer for the experiment. There is not a single criterion for being the best (highest T_m does not mean the presence of the active state, a small amount of aggregates may interfere strongly with subsequent experiments, e.g., immunogenicity or precipitate formation) and sometimes it is not even possible. The real aim should be rather to choose a set of conditions, where the obtained results can be compared to each other, as well as those conditions, that are highly unsuitable and should be avoided. In addition, the well-chosen buffer-screening test gives many additional pieces of information that can be used to guide further research. For example, the behavior in extreme pH can be used for choosing the regeneration condition in the binding assays. Ideally, one wants to analyze as many conditions as possible while keeping sample consumption and analysis time low. However, the protein availability and pressure towards fast results result in the need to examine several criteria at once and find a suitable compromise. Commonly used buffer optimization screens contain 96 conditions (Reinhard et al. 2013; Boivin et al. 2013). However, the smaller 48-condition screen might be favorable due to better machine compatibility, shortening of the experiment time or the possibility to run multiplicates in the 96-well format.

One of the critical points preventing scientists from running the buffer optimization is the amount of protein needed for the test. This is true especially for proteins produced in mammalian cells or isolated from rare natural sources. In our demonstration, we used in total 0.4 mg of the AFL protein or 0.1 mg of BC2L-C-Nterm protein for a complete buffer screening. That is an amount that is usually affordable, especially regarding the potential reduction of irreproducible or unsuccessful experiments that would follow. Even though the exact amount of the protein might vary case to case due to differences in extinction coefficient or size, with the current development of new experimental techniques, it is highly probable, that the sample consumption can be further lowered. The second parameter that is of importance is the time required. Using the chosen techniques, we were able to collect all data within a day, including sample preparation and the 12-h stability measurement. With the top high-throughput machines available today, the pure experimental time should not exceed 1 h per analysis, meaning that the real bottleneck here is the desire to evaluate the protein stability in time.

In our case study, we confirmed the previously reported stability parameters for the AFL lectin (Houser et al. 2013, 2015). Similarly, the previously observed presence of dimeric AFL in solution under various salt concentration (Houser et al. 2013) is in good agreement with our DLS data. The binding characteristics were earlier determined in HBS buffer at pH 7.4 by SPR (Houser et al. 2015). As seen from buffer screening, this is the upper edge of the high-activity region, and possibly a shift towards pH 6–6.5 might result in slightly higher affinity. However, we can conclude that AFL is a relatively stable protein with a broad range of conditions retaining the protein active state. On the other hand, BC2L-C-Nterm reveals itself as a difficult protein. Even though its temperature stability is high throughout most of the conditions tested, the absence of aggregates was seen only for a quite narrow range of conditions. It seems that BC2L-C-Nterm generally prefers lower ionic strength what corresponds to the previous works (Šulák et al. 2010, 2011), where low to medium salt buffers were used. Similarly to AFL, a shift towards more acidic conditions could favorize BC2L-C-Nterm binding activity. However, it needs to be mentioned that both studied lectins are multivalent proteins, and therefore, the exact behavior cannot be simply reduced to one-to-one binding in the fast screening.

There are also obvious drawbacks of the presented buffer screen. Reduction of the number of conditions may always result in missing of the sweet spot for the protein of interest, especially if the ideal conditions are rather unusual, e.g.,

presence of a specific cofactor for enzymes. In addition, proteins that naturally form heterogenous mixtures, e.g. actin (Masai et al. 1986), could be easily misjudged in the DLS experiment. The most tricky part of the screening is the determination of protein activity. This usually requires some, if not quite specific, idea about the protein function. Then the binding partner needs to be available for immobilization on a sensor surface for BLI or for labeling for MST experiments, otherwise a different technique needs to be chosen. For enzymatic reactions, the broad range of pH used in the screen might cause false negatives and positives, depending on the enzymatic assay. In some cases, a different screen concentration might be desired, depending on the initial protein buffer and on the specificities of alternative techniques, if they are used. In all cases, a detailed examination of the results is advisable. Despite possible pitfalls, the here-described method can be a suitable first-approach technique for many proteins to determine possible ways of further optimization or even directly give hints on what conditions to choose.

Conclusion

We prepared a 48-condition buffer screen for fast and easy protein characterization. We demonstrated its suitability to determine protein stability, homogeneity and binding activity. Since the screen is composed of mainly easy-to-obtain chemicals, we assume that it can be routinely used as a primary screen for buffer optimization in other labs and facilities.

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