Original article

Aggregation and conformational stability evaluation of myoglobin in the presence of ionic surfactant

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ABSTRACT

Sodium lauroyl sarcosinate (SLS) is frequently used for the solubilization of inclusion bodies *in vitro* due to its structural similarity to lipid plasma membrane. There are many factors that could influence protein aggregation propensity, including overall protein surface charge and hydrophobicity. Here, the aggregation pathway of myoglobin protein was studied under different conditions (pH 3.5 and 7.4) in the presence of varying concentrations of SLS to evaluate the underlying forces dictating protein aggregation. Data obtained from Rayleigh light scattering, ThT binding assay, and far-UV CD indicated that SLS have different effects on the protein depending on its concentration and environmental conditions. In the presence of low concentrations of SLS (0.05–0.1 mM), no aggregation was detected at both pH conditions tested. Whereas, as we reach higher SLS concentrations (0.5–10.0 mM), myoglobin started forming larger-sized aggregates at pH 3.5 and not pH 7.4. These results suggest that electrostatics interactions as well as hydrophobic forces play an important role in SLS-induced myoglobin aggregation.
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1. Introduction

Studying protein-surfactant interactions is a very important subject to grasp due to the repeated use of these surfactants in protein isolation steps after production and expression (Matsumiya et al., 2017; Kumar et al., 2015). Many studies have previously evaluated the effect of surfactants at low concentrations on the aggregation and denaturation propensities of proteins and compared those surfactants against other chemical denaturants (e.g. guanidine hydrochloride and urea) (Singh et al., 2015; Khan et al., 2016a,b). The continuous use of surfactants mandate deeper evaluation of the nature of surfactant-protein interactions to further understand the underlying mechanism of protein aggregation and unfolding. Surfactants are found in two states (monomer or micellar) depending on the concentration used (Rub et al., 2016;

Kumar et al., 2017), the concentration in which Micelles start forming is termed critical micelle concentration (Rub et al., 2017a,b,c). Monomeric and micellar surfactant interacts differently with proteins (Khan et al., 2017). Monomeric surfactants are known to promote amyloid fibril formation in proteins while micellar surfactants are not capable of inducing protein aggregation (Gospodarczyk and Kozak, 2015). Among monomeric surfactants, anionic ones such as sodium dodecyl sulphate (SDS) have been evaluated before in terms of promoting amyloid fibril formation in proteins (Khan et al., 2014a,b). Similar to SDS from a structural and functional prospective, sodium lauroyl sarcosinate (SLS), used commonly in protein isolation from inclusion bodies, is less studied in terms of its potential effect on protein aggregation. Structurally, SLS contains a negatively charged hydrophilic carboxylate head that is attached to a 12-carbon hydrophobic tail through an amide linkage and its critical micellar concentration is found to be around 14.5 in water (Fig. 1). SLS and SDS surfactants have their similarities in structure as both have a negatively charged head group, however, the nature of the head functional group between the two is different where SDS harbors a sulphate group and SLS has a carboxylate group. SDS and SLS share the same hydrophobic tail. Many studies have evaluated and demonstrated the effect of SDS on the amyloid-like aggregates formation of proteins, however, plenty of work remains to be done on SLS to evaluate similar phenomenon.
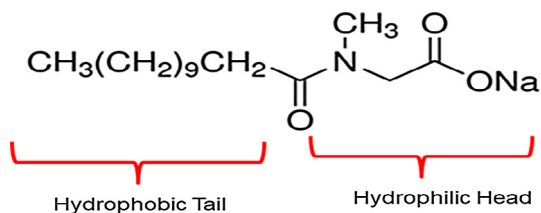
Fig. 1. A molecular model of negatively charged sodium lauroyl sarcosinate (SLS).

Protein aggregation is directly linked to several neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's disease (Dobson, 2003; Chiti and Dobson, 2006). The pathogenesis of these neurodegenerative diseases is still unclear and effective treatments are momentarily lacking. Previous studies have reported that other proteins not linked with any amyloid diseases can also form amyloid-like aggregates under adverse stresses (Litvinovich et al., 1998). These adverse stresses include extreme temperatures and pH conditions as well as the presence of proteins around different solvents and biological or synthetic molecules (Stefani and Rigacci, 2013; Uversky and Fink, 2004). The induction mechanism of protein aggregation by lipids from biological membranes is still not clear (Roberts, 2014; Jelinek and Sheynis, 2010). In this paper, lipid represented by SLS tail is used as a model system to evaluate myoglobin aggregation propensity.

Myoglobin is a colored protein that contains heme as a prosthetic group. Myoglobin is a monomeric, oxygen-binding protein that is largely found in muscle cells and highly used for conformational stability studies. The heme group exists in a hydrophobic pocket and is attached directly to the imidazole groups of proximal and distal histidines available for oxygen coordinate binding. Structurally, myoglobin belongs to the alpha class of proteins as it is composed of close to 75% alpha helices in its conformation. The study aim to understand the main forces governing the interactions between SLS and myoglobin under different pH conditions (pH 3.5 and 7.4) for safer use of SLS during protein isolation and production. These two pH conditions were chosen as a result of the isoelectric (pI) point of myoglobin ($\approx$6.2). Myoglobin retains a total net charge of +26.6 at pH 3.5 and a total net negative charge of -7.3 at pH 7.4 (calculated through PROTEIN CALCULATOR v3.3 software).

2. Material and methods

Myoglobin from equine heart muscle lot no SLBD8797V, sarkosyl lot no L9150 and Thioflavin T (ThT) were procured from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. All other reagents were used in analytical grade. Milli-Q water was used throughout the experiments.

2.1. Methods

Myoglobin concentration was measured spectrophotometrically after dissolving it in 20 mM Tris–HCl buffer at pH 7.4. The dissolve myoglobin was filtered through PVDF 0.45 mm pore sized syringe filters (Millipore Milex-HV). The concentration of filtered myoglobin was calculated by bicinchoninic acid kit.

2.2. pH measurements

The pH of buffers was measured by Mettler Toledo pH meter (seven easy S 20-K) with least count of 0.01 pH units, using an Expert Pro3 in 1′ type electrode. All buffers were filtered through PVDF 0.45 mm syringe filters before use.

2.3. Light scattering measurements (RLS)

RLS was measured using Carry Eclipse fluorescence spectrofluorometer attached to Peltier and a stirrer at constant rpm in room temperature. RLS measurements were analyzed to characterize the myoglobin aggregates in buffered solution. Samples containing myoglobin alone and with various concentrations of SLS (0–10.0 mM) at pH 3.5 and 7.4 were excited at 350 nm and emission spectra were recorded in the range of 300–400. The emission maxima at 350 nm were plotted against the different SLS concentrations used. The excitation and emission slit widths were kept constant at 2.5 nm in all measurements. Myoglobin concentration was fixed at 0.2 mg ml^{-1} in all samples. Before RLS measurements, all samples were incubated overnight at room temperature. The different concentrations of SLS alone without any protein were also measured as control.

2.4. ThT binding assay

The SLS-induced amyloid fibril formation of myoglobin at different conditions was analyzed using ThT fluorescence assay. ThT (at 5.0 μM concentration) was added to all pre-treated samples of myoglobin alone or with 0.05, 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 mM of SLS. All samples were then incubated in the dark for 30 min. The fluorescence emission spectra of ThT incubated samples were recorded at room temperature, and emission spectra were scanned in the range of 470–600 nm after excitation at 440 nm. The excitation and emission slits width were at 5.0 nm. Due to their hydrophobic nature, ThT will bind to SLS. To minimize its possible effect on the results, ThT fluorescence was measured in samples containing different concentrations of SLS with no protein present at respective pH conditions.

2.5. Circular dichroism spectroscopy

Far-UV CD spectropolarimeter was used to measure the changes in secondary structure of myoglobin. Applied Photophysics, Chirascan-Plus UK spectropolarimeter, was used to scan the far-UV CD spectral region of myoglobin in the presence or absence of SLS. A Far-UV CD spectrum was collected in the range of 200–260 nm (1.0 nm steps) using Quartz cuvette with 0.1 cm path length. The spectra of myoglobin protein (at a concentration of 0.2 mg ml^{-1}) in the presence of varying SLS concentrations (0, 0.05, 0.1, 0.5, 0.7, 1.0, 1.5 mM) at pH 3.5 and 7.4 were recorded. CD spectra of all treated and non-treated samples were averaged from three scans. The far-UV CD results were expressed as mean residual ellipticity (MRE) and defined as;

$$MRE = \theta obs(mdeg/10x \ n \ x \ Cp \ x \ l$$

where θobs is the CD in millidegrees, n is the number of amino acid of the myoglobin, l is the path length of cuvette in centimeters and Cp is the molar fraction of myoglobin. The percent secondary structure of myoglobin was calculated by an online sever "K2D2".

3. Results and discussion

3.1. Light scattering measurements

Rayleigh light scattering (RLS) at 350 nm is a widely used probe to monitor protein aggregation in solutions as light scattering signals increase drastically at these wavelengths as proteins start aggregating (Khan et al., 2016a,b). As myoglobin gets exposed to an increasing concentration of SLS, aggregation was seen only at acidic pH conditions (Fig. 2). The light scattering signal of myoglobin starts increasing drastically at SLS concentrations of 0.5
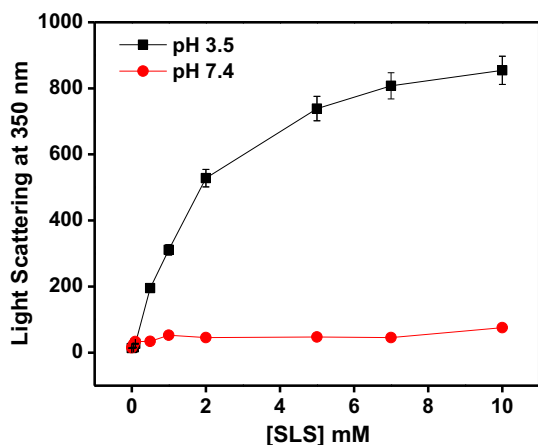
Fig. 2. Rayleigh light scattering of myoglobin (0.2 mg ml^{-1}) in the presence and absence of SLS was examined by taking scattering at 350 nm after excitation at 350 nm wavelength. Myoglobin was treated with different concentrations of SLS (0.0–10.0 mM) at pH 3.5 (-■-) and at pH 7.4 (-●-) was plotted. All the treated and untreated samples were incubated overnight prior to measurements at room temperature.
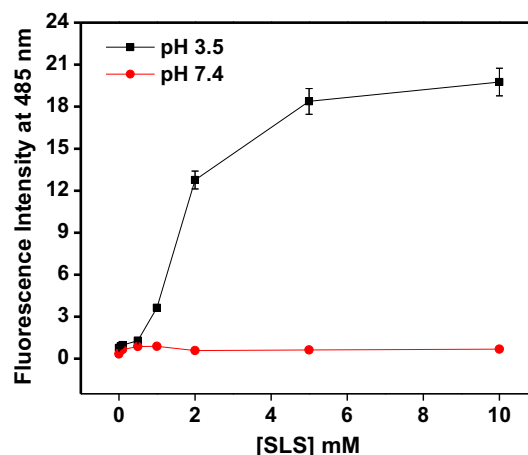
Table 1
Spectroscopic data at pH 3.5 of SLS without protein added.

S. no.	[SLS] mM alone	F.I. at 485 nm	Light scattering at 350 nm
1	0	0.77	13.77
2	0.05	0.63	13.87
3	0.1	0.54	14.75
4	1.0	0.69	13.78
5	2.0	0.78	16.67
6	5.0	0.84	19.87
7	10.0	0.98	18.98

mM to 10.0 mM at pH 3.5. Maximum light scattering was found to be at 10 mM. On the other hand, at lower concentrations of SLS (i.e. 0.05 and 0.1 mM) at similar acidic conditions, light scattering was found to be minimum and close to baseline. At neutral pH conditions (pH 7.4), light scattering of all samples containing myoglobin in the presence of SLS (0.05–10.0 mM) did not change and remained close to baseline. RLS was also measured for samples containing different concentrations of SLS with no protein present at pH 3.5. Light scattering was found to be negligible, indicating that SLS by itself it's not forming any aggregates (data shown in Table 1).

3.1.1. ThT binding assay

ThT is a yellow colored dye that fluoresces when bound to amyloid fibril (Suganthy et al., 2016). As amyloid fibrils start forming, more hydrophobic patches from the protein get exposed. As a result, an increase in the fluorescence signal of ThT would be detected. Proteins with minimum exposure of hydrophobic patches within their structures would not be bound by ThT, resulting in minimum or no increase in fluorescence signal intensity (Kumar et al., 2014).

Results from ThT fluorescence intensity (485 nm) when plotted against SLS concentration (Fig. 3) were in general agreement with RLS results discussed above. Conformational alteration in myoglobin were only detected when the protein was mixed with SLS at acidic conditions, however, higher SLS concentrations (1 mM) were necessary to induce fluorescence signal increase. Myoglobin did not go through any conformational alterations when mixed with SLS at concentrations of 0.5 mM and lower in acidic conditions (pH 3.5) and at all SLS concentrations tested at neutral condi-

Fig. 3. Morphology of myoglobin (0.2 mg ml^{-1}) aggregates was determined by ThT fluorescence. ThT (5.0 μM) fluorescence intensity at 485 nm was plotted against SLS concentration at pH 3.5 (-■-) and 7.4 (-●-) after excitation at 440 nm.

tions (pH 7.4). All spectroscopic data obtained at every condition tested are shown in Table 2.

3.2. Circular dichroism (CD) measurements

The effect of increasing concentrations of SLS on the secondary structure of myoglobin was investigated by far-UV circular dichroism (far-UV CD). Far-UV CD spectrum of myoglobin at both pH conditions tested exhibited two minima, first at 208 nm and second at 222 nm as expected from an alpha helical rich protein (Fig. 4) (Khan et al., 2015; Rub et al., 2017a,b,c; Kumar et al., 2017; Dave et al., 2010). At pH 7.4, increasing SLS surfactant concentration did not influence conformational changes on the protein tested. However, at pH 3.5, conformational changes on the protein occurred at high SLS concentrations (0.5–1.5 mM). The change in secondary structure of myoglobin at different conditions was calculated by "K2D2" software and results are shown in Table 3. We note from the table that α-helical content of myoglobin only reduced in the presence of high SLS concentration at acidic conditions and that β-sheet content increased at these SLS concentrations as well, reflecting an aggregation event. Overall, far-UV CD results come in agreement with RLS and ThT binding assay data supporting SLS-induced myoglobin aggregation.

4. Discussion

Sodium lauroyl sarcosinate (SLS) is a widely used surfactant especially during protein production. Evaluating the effect of SLS on proteins under different environmental conditions would not only help scientists to choose better conditions during protein production stages, but also would shed some light into the nature of interactions between SLS and proteins in general. Using multiple biophysical techniques like Rayleigh light scattering, ThT binding assay, and circular dischroism, different trends in terms of myoglobin aggregation and conformational stability were seen in the two conditions tested (pH 3.5 and 7.4). Myoglobin tended to loss its conformational stability and aggregate faster when incubated with high SLS concentrations in acidic conditions, however, in basic conditions under all SLS concentrations tested, negligible effects were seen in terms of aggregation induction and conformational instability were observed. The drastic effect of the presence of SLS in relatively high concentrations on myoglobin under acidic conditions could be explained by the induced electrostatic interactions between the negatively charged SLS head and positive myo-

Table 2
Spectroscopic data under various experimental conditions including control.

| S. no.	Condition	pH 3.5		pH 7.4		pH 10		pH 11	
		F.I. at 485 nm	Light scattering at 350 nm	F.I. at 485 nm	Light scattering at 350 nm	F.I. at 485 nm	Light scattering at 350 nm	F.I. at 485 nm	Light scattering at 350 nm
1	Myoglobin alone	0.77	13.77	0.34	14.65	0.32	13.15	0.29	13.01
2	Myoglobin + 0.05 mM SLS	0.88	15.38	0.65	24.87	0.41	12.73	0.33	13.17
3	Myoglobin + 0.1 mM SLS	0.98	26.95	0.65	33.53	0.55	13.43	0.50	14.87
4	Myoglobin + 0.5 mM SLS	1.29	195.19	0.87	33.97	0.61	13.54	0.56	16.32
5	Myoglobin + 1.0 mM SLS	3.64	310.98	0.89	52.96	0.54	12.79	0.58	15.98
6	Myoglobin + 2.0 mM SLS	12.76	528.25	0.59	45.75	0.60	15.85	0.65	16.15
7	Myoglobin + 5.0 mM SLS	18.38	738.8	0.63	47.54	0.42	17.50	0.62	15.90
8	Myoglobin + 7.0 mM SLS	18.98	807.65	0.67	45.65	0.56	14.31	0.59	13.89
9	Myoglobin + 10.0 mM SLS	19.76	854.76	0.69	75.76	0.73	15.12	0.87	14.76
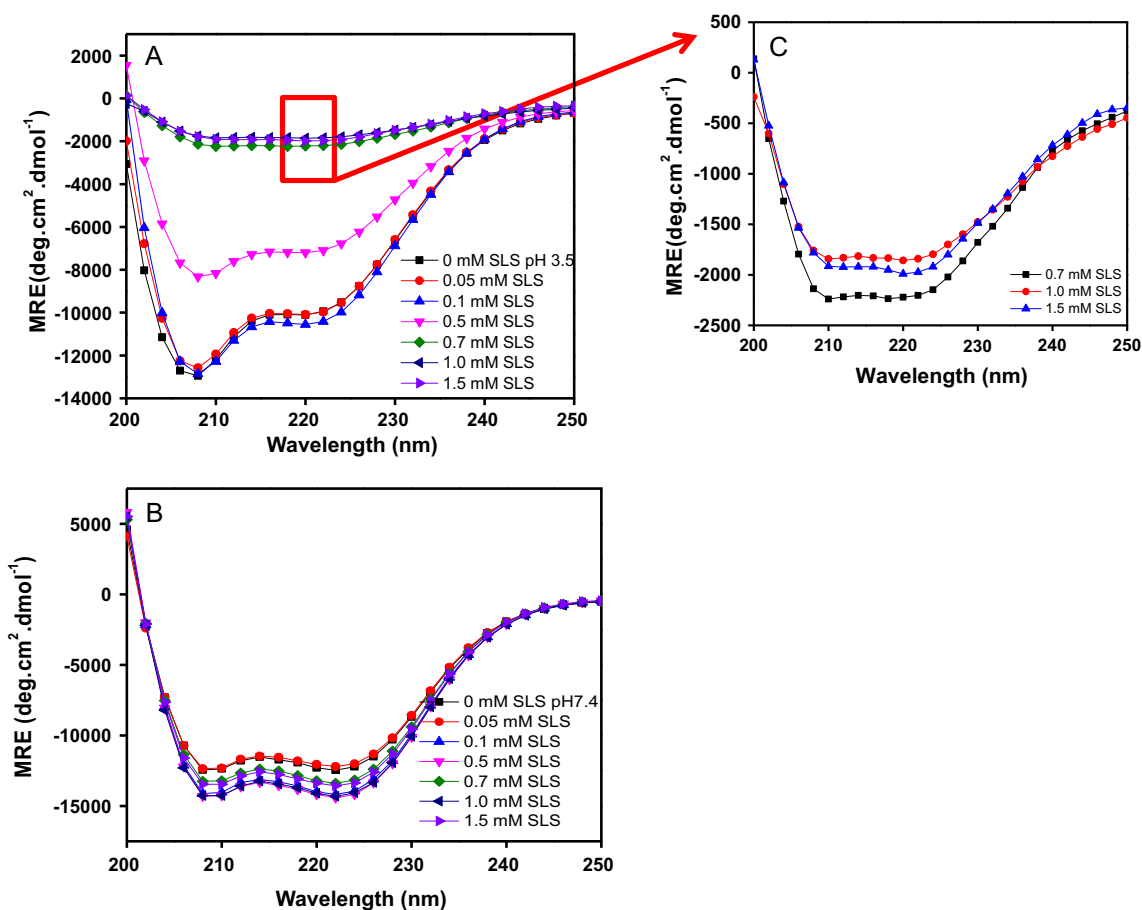
Fig. 4. Secondary structural change was evaluated in the presence different concentrations of SLS at pH 3.5 (Panel A) and pH 7.4 (Panel B). Far-UV CD spectra of myoglobin (0.2 mg ml^{-1}) in the absence (-■-) and presence 0.05 mM (-●-), 0.1 mM (-▲-), 0.7 mM (-▼-), 1.0 mM (-♦-) and 1.5 mM (-◄-) of SLS at pH 3.5. (Panel C) showed the aggregated spectra of myoglobin for more clarity.

Table 3
Percentage of secondary structure change of myoglobin estimated at different conditions by K2D2 software.

S. no.	Conditions	pH 3.5		pH 7.4	
		% α-helix	% β-sheet	% α-helix	% β-sheet
1	Myoglobin	74.41	2.43	78.32	1.97
2	Myoglobin + 0.05 mM SLS	74.41	2.43	78.39	1.98
3	Myoglobin + 0.1 mM SLS	73.53	2.43	79.78	1.12
4	Myoglobin + 0.7 mM SLS	43.65	8.43	81.32	1.42
5	Myoglobin + 1.0 mM SLS	37.54	10.45	82.98	1.21
6	Myoglobin + 1.5 mM SLS	50.17	12.43	82.34	1.41

globin (resulting from protonated Lysines, Arginines, and Histidines). Such interactions would facilitate other interactions between the two to happen. Hydrophobic interactions, for example, would occur between SLS tail and myoglobin resulting in the disruption of the water layer covering the protein surface and exacerbate aggregation propensity. Similar type of behavior was discussed before using five different variants of serum albumin protein (human, bovine, porcine, sheep and rabbit) (Khan et al., 2014a,b). In this paper, these variants were incubated at pH 3.5 with three different types of negatively charged surfactants (sodium dodecyl sulphate (SDS), sodium dodecylbenzene sulfonate (SDBS) and sodium bis-(2-ethyl-1-hexyl) sulfosuccinate (AOT)). Light scattering was assessed and similar conclusions to ours were found. In another paper using human serum albumin, ThT binding assay was used to assess the influence of quaternary amine of rosin diethylaminoethyl ester (QRMAE) (Ishtikhar et al., 2015). The results concluded conformational destabilization with increasing concentration of QRMAE, similar to results presented in this paper.

5. Conclusion

In this study, we tried to assess the effect of increasing concentrations of sodium lauroyl sarcosinate on the colloidal and conformational stability of myoglobin using different biophysical techniques. Results were in agreement with each other and factors responsible for protein instability were primarily pH and SLS concentration. Acidic conditions were found to be strong inducers for conformational alterations and aggregation, whereas, at neutral conditions, increasing SLS concentration did not have any detectable effect on the protein. The driving forces for conformational alterations and aggregation were found to be electrostatic and hydrophobic in nature. Initial interactions through electrostatic forces are believed to be the rate-limiting step for the protein to aggregate.

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