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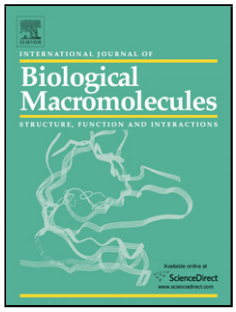

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PII: S0141-8130(16)31979-1
DOI: http://dx.doi.org/doi:10.1016/j.ijbiomac.2017.01.097
Reference: BIOMAC 7004

To appear in: *International Journal of Biological Macromolecules*

Received date: 13-10-2016
Revised date: 27-12-2016
Accepted date: 23-1-2017

Please cite this article as: Nasser Abdulatif Al-Shabib, Javed Masood Khan, Mohd Shahnawaz Khan, Mohd Sajid Ali, Abdulrahman M.Al-Senaidy, Mohammad A.Alsenaidy, Fohad Mabood Husain, Hamad A.Al-Lohedan, Synthetic Food Additive Dye "Tartrazine" Triggers Amorphous Aggregation in Cationic Myoglobin, International Journal of Biological Macromolecules http://dx.doi.org/10.1016/j.ijbiomac.2017.01.097

Synthetic Food Additive Dye "Tartrazine" Triggers Amorphous Aggregation in Cationic Myoglobin

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Abstract

Protein aggregation, a characteristic of several neurodegenerative diseases, displays vast conformational diversity from amorphous to amyloid-like aggregates. In this study, we have explored the interaction of tartrazine with myoglobin protein at two different pHs (7.4 and 2.0). We have utilized various spectroscopic techniques (turbidity, Rayleigh light scattering (RLS), intrinsic fluorescence, Congo Red and far-UV CD) along with microscopy techniques i.e. atomic force microscopy (AFM) and transmission electron microscopy (TEM) to characterize the tartrazine-induced aggregation in myoglobin. The results showed that higher concentrations of tartrazine (2.0-10.0 mM) induced amorphous aggregation in myoglobin at pH 2.0 via electrostatic interactions. However, tartrazine was not able to induce aggregation in myoglobin at pH 7.4; because of strong electrostatic repulsion between myoglobin and tartrazine at this pH. The tartrazine-induced amorphous aggregation process is kinetically very fast, and aggregation occurred without the formation of a nucleus. These results proposed that the electrostatic interaction is responsible for tartrazine-induced amorphous aggregation. This study may help in the understanding of mechanistic insight of aggregation by tartrazine.

Abbreviations: Congo Red-CR and Atomic Force Microscopy-AFM

Keywords: Tartrazine, Food Additive dye, Myoglobin, Protein Aggregation, Amorphous Aggregates, pH and Congo Red.

1. Introduction

Food additive dyes (natural or synthetic) are important because they improve appearance, flavor, color of various foodstuffs and are also known to enrich the high demand for consumers. Apart from natural dyes, synthetic dyes have found much more attention because of high use and less sensitive to light, pH, microbial contamination and reasonably low production cost [1]. Tartrazine is a water soluble, yellow colored, synthetic azo dye widely used as a coloring agent in various foodstuffs. Tartrazine is an anionic dye, contains two sulphonic, one azo (N=N) and one carboxylic functional group shown in **Fig. 1A.** Tartrazine is extensively used in various food products (ice cream, pastries, hard candy, cake, soft drink, alcoholic beverage, sports drink, corn chips, potato chips, chewing gum, and jam), cosmetics (lipstick, face powder, eyeshadow, and foundation) and drugs (capsule, pill and gel). Tartrazine is known to cause allergic reactions in atopic individuals and hyperactivity in children. A large number of other side effects of tartrazine are also known, for example, lethal asthma, nettle rash, hives, DNA damage, tumors of the thyroid and attention deficit hyperactivity disorder (ADHD). Regarding DNA damage, contradictory reports are available as in one report it was stated that tartrazine is damaging the DNA, while in other reports it was suggested that it is safe for DNA [2,3]. Furthermore, it was also seen that tartrazine cause neuronal behavior, diseases in female rats [4]. Till date, an enormous amount of a toxicological study of tartrazine has been done, though, most of the studies were related to its effect on DNA and not to on the proteins. It will be fascinating to investigate the interaction of tartrazine with essential proteins to ascertain its effect on protein conformation. In the same context, it is extensively reported that small molecules like metals, surfactants, natural food additive dye (curcumin), peptidoglycan and lipids are inducing aggregation in various

proteins. The aggregates are formed by these molecules are toxic in nature [5]. It is important to check the role of tartrazine in the amyloid fibrillation process in protein. For this, we have chosen one of the most extensively studied model proteins in the area of protein folding field i.e., myoglobin. Myoglobin is a heme-containing muscle protein and widely used for conformational stability studies. Form the X-ray crystallography, it was found that the myoglobin protein possesses almost 75 percent alpha-helical secondary structure and is made up of 8 separate right-handed alpha helices shown in Fig. 1B [6]. Myoglobin protein is extensively utilized to understand the conformational stability under *in vitro* conditions [7]. A variety of factors are employed to test the conformational stability of proteins under *in-vitro* conditions, and these factors are temperature, pH, urea, guanidium hydrochloride, metals, cationic and anionic surfactants [8,9]. These factors also have a high ability to misfold the proteins under *in-vitro* conditions [10]. Monomeric myoglobin protein is released into circulation due to muscle deface by several factors, such as vigorous exercises, therefore released myoglobin may come in contact with tartrazine and undergo aggregation [11]. It is also seen that the myoglobin tended to form aggregates at different sets of conditions. In one report, it was found that the hydrophobicity of tryptophan residue is not only the factor which causes amyloid fibril, but it is also having a tendency to establish favorable interactions with other residues of the proteins [12]. The misfolding of proteins leads to almost 30 types of neurodegenerative diseases, such as Parkinson's, Alzheimer's, Dialysis-related amyloidosis, Creutzfeldt–Jacob, Type II diabetes and so on [13]. Various proteins (α-synuclein, amyloid beta, prion protein and β2M) are recognized, which are directly linked to neurodegenerative diseases and causing toxicity [14]. It is also observed that other proteins which are not related

to any neurodegenerative diseases also form amyloid-like structure under *in-vitro* conditions and have some cytotoxic effects [15].

Tartrazine is an anionic dye that mimics to the sodium dodecyl sulphate (SDS) in some asspects because both molecules have a sulphate group at one end. Earlier, we have studied SDS-induced amyloid fibril formation of various mesophilic proteins [16]. Therefore, it will be fascinating to check the role of tartrazine in myoglobin aggregation. At present, there are no reports exist regarding tartrazine-induced protein aggregation. Only a few reports are avilabe regarding tartrazine-interaction with serum albumin. Tartrazine has a binding affinity in the subdomain IIA (a site I) of human serum albumin and at the interface of sub-domain IIA and IIIA of bovine serum albumin [17]. The tartrazine binding affinity is found higher in BSA compared to HSA calculated by isothermal titration measurements at 25 °C which also increased the thermal stability of both the albumins [18]. Tartrazine has a better binding affinity to HSA compared to other available natural dyes confirmed by a docking and atomistic molecular dynamics simulation method [19]. Therefore, it is important to study the aggregation induction of tartrazine towards myoglobin to get detailed insight into the mechanism of tartrazine-induced aggregation of myoglobin.

For this, we have investigated how the negatively charged tartrazine interacts with positively and negatively charged myoglobin and what is the possible interaction which facilitates to myoglobin to form aggregates. Therefore, a bunch of spectroscopic as well as microscopic techniques has been used to characterize the molecular binding mechanism of tartrazine with myoglobin and to investigate the mechanism of tartrazine induced protein aggregation. To the best of our knowledge, it is the first report about synthetic dye induced protein aggregation.

2. Materials & Methods

2.1. Materials

Myoglobin from equine muscle, tartrazine, Tris-HCl, Congo Red (CR) and glycine-HCl was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals and reagents used in this study were of a high-quality analytical grade. Milli-Q water was used throughout this study.

2.2. Stocks of myoglobin solution preparation

The myoglobin protein stock solution was made in 20 mM Tris-HCl buffer of pH 7.4. Myoglobin was not further purified because it is in pure form, a single band is found in SDS-PAGE electrsphorasis data are not shown. Myoglobin concentration was quantified by taking absorbance at 409 nm with the use of the molar extinction coefficient of 157000 M^{-1} cm^{-1} [20].

2.3. pH measurements

pH of every solution was measured by the Mettler Toledo pH meter. Before use, all the buffer solutions were filtered through PVDF 0.25 µm syringe filter from Millipore Milex-HV.

2.4. Rayleigh light scattering measurements (RLS)

RLS study was performed to characterize the mode of interaction between tartrazine and myoglobin at two different pHs (7.4 and 2.0). This experiment was carried out on a Jasco FP-750 spectrofluorometers at 25 °C in 1.0 cm path length cuvette. Myoglobin was incubated with and without tartrazine at two different pH, 7.4 and 2.0 for 12 hrs. Both pHs samples were excited at 650 nm and emission was taken at 650 nm. Both excitation and emission slit width were fixed at 5.0 nm for the measurements. For this study, the myoglobin

concentration was fixed (2.84 μM) and the tartrazine concentrations were varied from 0.0 to 10.0 mM. Low concentrations of myoglobin were taken in Rayleigh light scattering because of the instrument limitation. The fluorescence intensity beyond 1000 can not be detected.

2.5. Turbidity measurements

Perkin-Elmer double beam UV-Vis spectrophotometer was used to check the turbidity of the samples by taking the absorbance at 650 nm. Myoglobin (11.3 μM) was incubated with and without tartrazine (0-10 mM) at two different pHs (7.4 and 2.0). The strength of the buffer was kept constant 20 mM for both buffers (Tris-HCl and Glycine-HCl). All the samples which contained myoglobin with and without tartrazine were incubated for 12 hrs before measurements.

2.6. Kinetics of Tartrazine induced aggregates

Kinetics of tartrazine-induced aggregation were measured on Hitachi F7000 spectrofluorometer at 25°C in the 3 ml cuvette in a 1 cm path length. The myoglobin samples were treated with different concentrations of (0.5, 2.0, 3.0 and 5.0 mM) tartrazine were excited at 650 nm and emission was taken at the same wavelength with time in seconds. The excitation and emission slit widths were chosen 2.5 nm and myoglobin concentration was also fixed (11.3 μM) in all the samples.

2.7. Steady-state fluorescence measurements

Before steady-state fluorescence measurements, all the samples containing myoglobin and tartrazine were centrifuged several times and washed with respective buffers. Washing was done to remove the dye. The intrinsic and extrinsic fluorescence studies were performed in the absence and presence of tartrazine at pH 2.0 on Jasco FP-750 spectrofluorometer. Measurements were done at fixed temperature of 25 °C in a 1 cm path length cuvette. For

intrinsic fluorescence measurements, the samples were excited at 295 nm and emissions was taken in the range of 300 to 400 nm while in extrinsic fluorescence measurements, the samples were excited at 380 nm and emission was taken in the range of 400-600 nm. The myoglobin concentration (11.3 μM) was set in all intrinsic and extrinsic fluorescence measurements. The excitation and emission slit width were set 5 nm in intrinsic as well as extrinsic fluorescence measurements. Before the intrinsic as well as extrinsic fluorescence measurements, all the samples were incubated for 12 hrs.

2.8. Congo Red (CR) binding

Fresh CR was dissolved in Milli-Q water and filtered through 0.2 μ Millipore syringe filter. After filtration, the concentration of CR was calculated by spectrophotometrically by taking extinction coefficient 45000 M^{-1} cm^{-1} at 498 nm. Aggregated samples which were induced by tartrazine were centrifuged three times at 5000 RPM for the duration of 10 minutes, and every times precipitates were washed with respective buffers. The precipitate was collected and resuspended in the desired buffer. The aggregated (myoglobin (11.3 μM) + tartrazine (0.0- 10.0 mM)) and non-aggregated (myoglobin (11.3 μM) alone) samples were incubated with CR (5.0 μM) for 30 minutes. The absorbances of aggregated and non-aggregated samples were taken in the range of 400-600 nm on Perkin-Elmer double beam UV-Vis spectrophotometer. The spectra of aggregated samples without Congo red was subtracted with the spectra of aggregated samples with Congo red. This substraction is done to nullified the chance of tartrazine and Congo red interaction.

2.9. Far-UV CD measurements

The Far-UV CD measurements were done on an Applied Photophysics, Chirascan-Plus, UK spectropolarimeter attached with Peltier and water circulator. The myoglobin (11.3 μM) was

incubated with different concentrations (0.0 to 10.0 mM) of tartrazine for 12 hrs. The incubated samples were centrifuged at 5000 RPM for 10 minutes to remove the tartrazine dye by washing with desired buffers. The Far-UV CD spectra of aggregated and non-aggregated samples were scanned in the range of 200–250 nm in a cuvette of 0.1 cm path length. Every sample was scanned three times, and average spectra were plotted. The averaged spectra were smoothed by the Savitzky–Golay method with a convolution width of 2.

The far-UV CD results were demonstrated as mean residual ellipticity defined as

$$MRE = \theta_{obs}\ (mdeg)/10 \times n \times Cp \times l$$

Where θ_{obs} is the CD in millidegrees, n is the number of amino acid residues of myoglobin, and l is the path length of the cell in centimeters and Cp is the molar fraction of myoglobin. The percent secondary structure of myoglobin at different conditions was calculated by online server i.e. K2D2 method.

2.10. Atomic force microscopy study

Myoglobin (11.3 μM) was incubated with two different concentrations (2.0 mM and 5.0 mM) of tartrazine at pH 2.0 for 12 hours. The incubated samples were further diluted two times and then placed on the freshly prepared mica surfaces. After 15 minutes, mica was rinsed with Milli-Q water and then left for 3 hours of dryness. After three hours, nitrogen was flushed to remove residual moisture. AFM image was captured by using Bioscope Catlyst AFM (Bruker, USA) in the tapping mode. The obtained images were further processed for publication by using Nanoscope Analysis v.1.4.

2.11. Transmission electron microscopy (TEM)

TEM images were captured by using JEOL transmission electron microscope operating at an accelerating voltage of 200 kV. The morphology of myoglobin aggregates was examined by

applying 6 mL of a sample containing myoglobin (11.3 μM), 2.0 and 5.0 mM of tartrazine on a 200-mesh, copper grid covered by the carbon-stabilized formvar film. Extra, fluid was removed after 2 minutes and the grids were then negatively stained with 2% (w/v) uranyl acetate. Images were viewed at 20000 X magnification. Before taking the image grid was dried in a desiccator for overnight.

3. Results

3.1. RLS study

RLS study was conducted to access the effect of tartrazine on myoglobin aggregation at pH 2.0 and 7.4. RLS technique is used to quantify the number and size of aggregates in liquid samples. The RLS measurements were performed in all tartrazine-induced aggregates samples at pH 2.0 by taking fluorescence intensity at 650 nm. The fluorescence intensity of all samples was plotted against different concentrations of tartrazine at 650 nm. The RLS data reflected that at low concentrations (0.1-1.0 mM) of tartrazine, scattering was negligible while at higher concentrations from 1.5 to 10.0 mM, a gradual increase in scattering was noticed as shown in Fig. 2A. The samples containing myoglobin and 1.5 to 10.0 mM of tartrazine have shown high fluorescence intensity compared to low concentrations of tartrazine. The fluorescence intensity of samples, which contains myoglobin and different concentrations of tartrazine at pH 7.4 is insignificant and shown in Fig. 2B. The RLS results are suggesting that the higher concentrations of negatively charged tartrazine are encouraging the formation of bigger size aggregates in myoglobin at pH 2.0 and not to at pH 7.4.

3.2. Turbidity

Turbidity measurement is a highly utilized method to characterize insoluble aggregates in the liquid samples by taking optical density at 650 nm. In this study, we have measured turbidity

at 650 nm because tartrazine is a colored dye showing some absorption below 600 nm so shown in supplementary fig. S1. Turbidity is used to quantify the magnitude of aggregation in liquid samples. This method is extensively employed to identify the change in the number and size of the protein due to oligomerization of monomer proteins, protein aggregates, protein-ligand and protein-protein interaction [21,22]. Turbidity at 650 nm was carried out to evaluate the interaction of tartrazine with myoglobin protein at pH 7.4 and 2.0. Tartrazine binding was ineffective at very low concentrations (0.1-1.5 mM) while at higher concentrations (1.5-10.0 mM) continuous increase in turbidity was recorded as shown in Fig. 2C. Moreover, we also inferred the interaction of tartrazine with myoglobin at pH 7.4; the turbidity was not seen at any tartrazine concentrations (0.0 to 10.0 mM) which are shown in Fig. 2D. The turbidity results are also suggesting that higher concentration of tartrazine is stimulating myoglobin aggregation at pH 2.0. The quantity of aggregates is dependent on tartrazine concentrations and pH of the solution. The overall RLS and turbidity data are presented in table 1.

These two quantitative measurements suggest that high concentrations of tartrazine are provoking myoglobin to form bigger size aggregates at pH 2.0. The possible cause of tartrazine induced aggregation is electrostatic as well as hydrophobic interaction because tartrazine is negatively charged due to the presence of two sulphate groups while myoglobin is positively charged due to protonation of positively charged amino acids at pH 2.0. The negatively charged sulphate group of tartrazine interacted with protonated positively charged amino acids and consequently neutralized all the surface charges resulting into the formation of aggregates. Due to the interaction of tartrazine with myoglobin, the solvent-myoglobin

interaction was disturbed, and tartrazine-myoglobin interaction increased which ultimately caused aggregation in myoglobin.

3.3. Kinetics of Tartrazine induced-aggregation

To elucidate the role of tartrazine in the aggregation of myoglobin protein at two pHs (7.4 and 2.0). We executed the RLS measurements in the presence of low and high concentrations of tartrazine with a function of time. Fig. 3A, showed the changes in fluorescence intensity at 650 nm with respect to time in seconds. The fluorescence intensity was almost negligible in the absence and presence of 0.5 mM of tartrazine at both pH until 110 seconds. However, the addition of higher concentrations (2.0, 3.0 and 5.0 mM) of tartrazine at pH 2.0, the fluorescence intensity was continuously increased to 30 seconds and after that saturation achived. The fluorescence intrensity was found higher in the presence of high concentrations of tartrazine. These results suggest that the higher concentrations of tartrazine induce bigger size aggregates compared to low concentrations. The interesting thing which we have noticed that the tartrazine-induced aggregation process is following nucleus independent manner. The fluorescence intensity continuously increased just after mixing and got saturated very early even at 30 seconds of the scan. The kinetics of tartrazine induced aggregation did not follow the sigmoidal transition; it started with elongation phase, where lots of monomers joined and formed oligomer structure. After some time, the elongation phase disappeared and reached a plateau where all the monomers had reached equilibrium. The kinetics results showed that the higher concentrations of tartrazine is stimulating aggregation in myoglobin at pH 2.0.

3.4. Changes in tertiary structural organization of protein

Intrinsic fluorescence measurements were extensively employed to characterize the ligand induced conformational change in tertiary structures of protein. Tryptophan fluorescence measurements examined the change in the tertiary structure of myoglobin upon tartrazine dye binding. The change in wavelength maximum and fluorescence intensity was occurring due to changes in polarity of aromatic amino acids and solvation of protein core. The shifts in wavelength peak were revealing the positioning of tryptophan residues in protein. A blue shift in wavelength maximum indicates that the tryptophan residues are buried in the core of proteins while the red shift gives information about the exposure of tryptophan residues in proteins. Fig. 3C showed the fluorescence emission spectra of myoglobin at pH 7.4 without the addition of tartrazine, and its maximum intensity was noticed around 333 nm which is in consistent with other published reports [23]. After incubation at pH 2.0, the fluorescence emission maximum was red-shifted around 15 nm with a massive increase in fluorescence intensity as shown in Fig. 3C. The wavelength maximum of myoglobin at pH 2.0, was found around 348 nm which signified that the myoglobin tertiary structure was disrupted, and tryptophan is exposed to the non-polar environment. With the addition of tartrazine, the fluorescence intensity was found to drop with a 3-5 nm blue shift in wavelength maximum shown in Fig. 3B. This change in wavelength maximum and the reduction in fluorescence intensity affirms that the myoglobin is transformed into aggregated structures. The tratrazine alone was also excited at 295 nm and emission was taken in the range of 300-400 nm but tratrazine was not showing any fluorescence properties in this reason shown in Fig. 3C as a controll. We further plotted the wavelength maximum against the different concentration of tartrazine at pH 2.0. Fig. 3D showed that the wavelength maximum was found to be 333 nm at pH 7.4 and 348 nm at pH 2.0 and after addition of tartrazine the wavelength maximum was

blue shifted. The obtained intrinsic fluorescence results suggest that the myoglobin becomes unfolded at low pH, and aggregated in the presence of higher concentrations of tartrazine. The change in wavelength maximum at different concentrations of tartrazine are shown in table 1.

Tryptophan exposure was further confirmed by ANS binding study, displayed in Fig S2. ANS is largely used to monitor the changes involving in the hydrophobic core of proteins. The ANS fluorescence intensity of native myoglobin was found very less confirming that hydrophobic patches of myoglobin are buried inside the core. On the other hand, we observed a huge increase in fluorescence intensity with a blue shift in wavelength maximum in a sample which was incubated at pH 2.0 ascribing that the tryptophan residue of myoglobin is exposed to solvent due to unfolding of myoglobin. Similarly, ANS binding study was also done in a sample which was incubated with tatrazine at pH 2.0. The fluorescence intensity of ANS was found high in tartrzine incubated samples confirmed that tartrazine is inducing aggregation in myoglobin protein. Hence, the noticed intensity increase describing that tratrzine-induced aggregates has generated new hydrophobic patches due to myoglobin aggregation.

3.5. Congo Red (CR) Binding Assay

We have also conducted a CR dye binding assay to confirm the architecture of tartrazine-induced aggregates [24]. We were also interested to know whether tartrazine induces amyloid or amorphous-like aggregates in myoglobin protein. Congo red is an azo dye, widely used for the identification of amyloid and amorphous structures in aggregated solutions or tissues [25]. CR binds or intercalates to the cross-β and β-sheets of amyloid fibrils and gives pronounced red shift in the absorbance maximum. The CR absorbance spectra of myoglobin

at pH 7.4 and aggregated samples are shown in Fig. 4A. The absorbance spectrum of myoglobin in the absence of tartrazine has shown maximum around 488 nm. While in the presence of tartrazine dye the absorbance maximum was red-shifted almost 10-12 nm. The change in absorbance maximum indicated that the tartrazine-induced aggregates in myoglobin have amorphous like architecture.

3.6. Far-UV CD measurements

Far-UV CD spectroscopy is employed to measure the tartrazine-induced secondary structural change. Far-UV CD is a very sensitive spectroscopic technique, utilized to analyze the changes in secondary structures of proteins in solutions. The far-UV CD spectra of native myoglobin exhibited two characteristic minima, one at 208 and the second one at 222 nm, at pH 7.4, indicative of α-helical structure shown in Fig. 4B (filled square) [26]. The far-UV CD spectra of myoglobin at pH 2.0, showed a single minimum at 200 nm, suggesting that the secondary structure of myoglobin is lost due to unfolding, shown in the figure as an empty square. Furthermore, the far-UV CD spectra of myoglobin in the presence of different concentrations of tartrazine 2.0 mM (filled circle), 3.0 mM (filled triangle) and 5.0 mM (empty circles) are drastically reduced, but the pattern of spectra is same as native spectra. These kinds of far-UV CD spectral changes signify that the protein was transformed into the aggregated structure. This is an interesting observation in the aggregation point of view. It is not always true that the aggregated proteins have a high content of β-sheet structure. The β2M protein acquires alpha helical structure when it forms amyloid-like aggregate in the presence of TFE at pH 2.0 [27]. K2D2 software was used to calculate the percent secondary structure content of myoglobin alone and in the presence of tartrazine and the obtained data were expressed in table 2. The far-UV CD results permit us to summarize the secondary

structural transition data. At low pH, myoglobin secondary structure is lost due to unfolding while in the presence of tartrazine, myoglobin retained some residual alpha helix structure with reduced ellipticity which is due to aggregation.

3.7. Atomic Force Microscopy

Atomic force microscopy (AFM) is most commonly used microscopy to visualize the morphology of protein aggregates [28]. Different kinds of amyloid fibril shapes were visualized by AFM [29]. From the dye binding assay and CD measurements, it is confirmed that the tartrazine is inducing amorphous like aggregates in myoglobin at pH 2.0. Therefore, AFM image of tartrazine-induced aggregates was taken after 12 hrs incubation at room temperature which is shown in Fig. 5 (panel I). The myoglobin forms amorphous like aggregates in the presence of 3.0 and 5.0 mM of tartrazine at pH 2.0. No specific topology of tartrazine-induced aggregates were found in both tested concentrations of tartrazine. From the AFM and Congo Red results, it is established that the tartrazine-induced myoglobin aggregates have an amorphous structure. Same kind of morphology was also found in lysozyme protein when it was incubated (10 mg ml^{-1}) for 2 hours at 65 °C with a continuous string at1200 RPM [30].

3.8. Transmission electron microscopy (TEM measurements

Another microscopy i.e. TEM was used to checked the morphology of tartrazine-induced aggregation of myoglobin. TEM has highly used microscopic techniques to characterize the types of aggregates are formed (fibrillar or amorphous) in solutions [31]. The myoglobin protein at neutral (7.4) and acidic (2.0) pH did not show any aggregate structure, whereas, in the presence of 2.0 and 5.0 mM of tartrazine at pH 2.0, amorphous aggregates were formed

as shown in Fig. 5 (panel II). The TEM and AFM images are attributed that tartrazine-induced aggregates, is having amorphous morphology.

4. Discussion

Protein misfolding is a booming research area, and enormous research groups are involved in answering the mechanism of protein misfolding [32]. It is vital to understand the protein misfolding mechanism because protein misfolding has a direct link to the various human diseases. Understanding the protein misfolding mechanism helps in the designing of molecules for the treatments of misfolding diseases. Partially unfolded states of proteins are more prone to form protein aggregates or amyloid as compared to the fully folded states [33]. These partially unfolded states in proteins generate due to various in-vitro and in-vivo factors, i.e. temperature, pH, organic solvents, additives like surfactant, lipid, guanidine hydrochloride and urea [34]. It will be good to conduct an experiment under in-vitro conditions and decipher the mechanism of protein aggregation. It is widely reported that the small molecules are rapidly promoting protein aggregation in various proteins under *in-vitro* conditions [35]. A huge number of reports have already published regarding amyloid fibril formation and inhibition under in-vitro condition [36]. In this context, we have taken a step to see the role of synthetic azo dye (tartrazine) into protein aggregation induction. Till date, no study has reported about aggregation induction by tartrazine dyes.

In this study, we have done a series of spectroscopic and microscopic experiments to identify the binding mechanism of tartrazine with myoglobin proteins at low pH. From the results, it was found that tartrazine is stimulating amorphous aggregation in myoglobin at pH 2.0 and not at pH 7.4. The turbidity and RLS study indicate that myoglobin forms bigger size

aggregates in the presence of 2.0 to 10.0 mM of tartrazine at pH 2.0 while at low concentrations no aggregation was seen in both measurements. Serum albumin showed high scattering in the presence of 60 % methyl cyanide at pH 7.4, suggesting serum albumins from bigger size aggregates [22].

Turbidity and RLS measurements were also executed at pH 7.4, for the investigation of the mode of interaction between negatively charged myoglobin and tartrazine. The turbidity and scattering results at pH 7.4 reveals that no aggregation was found because both the molecules have the same sign of charges. The possible interaction which is responsible for tartrazine-induced amorphous aggregation at low pH is electrostatic interaction because at low pH, myoglobin is positively charged due to protonation of Arginine, Lysine and Histidine and the total charges on myoglobin at pH 2.0 is 33.0 (calculated by PROTEIN CALCULATOR v3.3) which enables the protein to interact with the negatively charged sulphate group of tartrazine. Conversely, at higher pH (7.4) both myoglobin and tartrazine possess a same negative charge that results into electrostatic repulsion between both the molecules, consequently, no aggregation was taking place. Previously, we have reported that the cationic state of lysozyme formed amyloid fibril in the presence of SDS and extent of aggregation is dependent on the total positive charge of lysozyme [37]. Furthermore, we have carried out kinetics experiments to know the rate of tartrazine-induced aggregation. Kinetics results showed that the tartrazine-induced aggregation is nucleus independent which means the lag phase was absent. This kind of aggregation kinetics was also found in serum albumin proteins when these were exposed to anionic surfactants (SDS, SDBS, and AOT) at pH 3.5 [38]. Nucleus-independent pathways are starting from extension phases, not to nucleation phase. The results suggest that myoglobin starts forming aggregates just after contacting with

tartrazine electrostatically. It was seen that the aggregation process follows nucleation pathway. But tartrazine-induced aggregation is avoiding nucleation route due to very strong electrostatic interaction between sulphate group of tartrazine and positively charged residues of myoglobin protein. The rate of tartrazine-induced aggregation was independent on tartrazine concentration though the fluorescence intensity increased on increasing the tartrazine concentration probably due to the formation of larger aggregates. Similar kind of kinetics results was also seen in human serum albumin (HSA), when the "f" form of the HSA was treated with SDS, SDBS and AOT followed by the continuous increase in scattering [38]. Intrinsic fluorescence study results exhibited a red shift in fluorescence spectra when incubated at pH 2.0 indicating that myoglobin is unfolded at this pH. After addition of tartrazine at the same pH, the wavelength maximum of myoglobin was blue shifted ascribed to the change in the tryptophan microenvironment towards nonpolar environment due to aggregation of proteins. The intrinsic fluorescence spectra of aggregated Con A were also blue-shifted in the presence of low cationic surfactant at physiological pH [39]. Furthermore, tryptophan residue exposure of myoglobin was also detected by ANS binding. The ANS fluorescence intensity at pH 2.0 was found very high compared to at pH 7.4. The fluorescence intensity was incersenig with incersing concentrations of tartrazine at pH 2.0. The ANS results indicated that the tartrazine-induced aggregates has generated new hydrophobic clusters. Congo Red dye binding assay was also performed to characterize the aggregate structures, whether it contains amorphous or amyloid fibril architecture. CR results shown positive binding with myoglobin aggregates. From the CR results it was confirmed that tartrazine is stimulating amorphous aggregation of myoglobin. It is believed that CR binds only cross β-structure of amyloid and native β-sheet structure of proteins, but it was

also found that CR bind α-helical proteins also [40]. This reference is supporting our findings that CR can bind amyloid as well as amorphous aggregates too. Far-UV CD measurements were done to characterize the tartrazine-induced secondary structural transitions. Myoglobin is alpha helical proteins, and its helicity is maintained in the presence of all tartrazine concentrations, but the only change was found in its ellipticity. In a case of aggregation, ellipticity is reduced compared to native protein. The secondary structure of myoglobin was transformed into a random coil structure in the absence of tartrazine at pH 2.0. CD results demonstrated that tartrazine-induced aggregates have an amorphous structure. The percent secondary structural changes in myoglobin were calculated at different conditions are shown in table 2. We further performed AFM and TEM measurements to confirm the morphology of tartrazine-induced aggregates, whether it induces amorphous or amyloid-like aggregates. AFM and TEM results exhibited that tartrazine is promoting amorphous aggregates in myoglobin at pH 2.0. From these observations, it can be concluded that the tartrazine is promoting amorphous aggregation in myoglobin protein only at pH 2.0 and at pH 7.4 it was ineffective to promote any aggregation. Based on the observations at two different pHs at which myoglobin has different charges, it can be concluded that electrostatic interaction is playing a dominant role in myoglobin aggregation. All the spectroscopic data are shown in table 1. The details mechanism of tartrazine-induced amorphous aggregation is given in the next paragraph.

The overall data of this study are summarized in Fig. 6 as a schematic diagaram. Myoglobin is a basic protein containing total negative charges on the surface at neutral pH because its isoelectric point (pI) is 6.8. At neutral pH, the glutamic and aspartic amino acid residues liberated its H^+ in solution and myoglobin become negatively charged. Similarly, arginine,

lysine, and the histidine residue become positively charged at pH 2.0 because of protonation of the side chain amino group of positively charged amino acids. The negatively charged sulphate groups of tartrazine interact electrostatically with positively charged amino acids, particularly arginine, lysine and histidine at pH 2.0 leading to the amorphous aggregation. While at neutral pH, the strong electrostatic repulsion was taking place between negatively charged tartrazine and negatively charged myoglobin resulting in no aggregation.

5. Conclusions

In this study, we have seen the mode of interaction between negatively charged tartrazine and cationic as well as an anionic form of myoglobin. From all spectroscopic and microscopic measurements, it can be concluded that tartrazine was accelerating amorphous aggregation in myoglobin when it was incubated at acidic pH. The anionic sulphate group of tartrazine interacted electrostatically to a cationic amino acid residue of myoglobin leads to amorphous aggregation. The electrostatic interaction is playing an important role in amorphous aggregation. The aggregation propensity of myoglobin is entirely dependent on the cationic state of myoglobin. Tartrazine was unable to induce aggregation in myoglobin when it was incubated at neutral pH. This study strongly, suggests that tartrazine can cause aggregation in any protein when protein was cationic in nature. These results help us to understand the mechanism associated with protein aggregation via food additive dye.

Acknowledgements

The authors wish to thanks the Agricultural Research Center, the College of Food and Agricultural Sciences and Deanship of Scientific Research, King Saud University, Saudi Arabia, for supporting this work.

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Figures and legends:

Fig. 1A: Food additive dye tartrazine molecular structure. **(1B),** Crystal structure of myoglobin from PDB as 1mbn.pdb file and highlighting the position of heme group and cationic amino acids (arginine, lysine and histidine) in different color.

Fig. 2: RLS measurements of myoglobin in the absence and presence negatively charged tartrazine was done by taking fluorescence intensity at 650 nm after excitation at 650 nm. In **Fig. 2A,** Myoglobin (2.84 μM) was exposed to different concentrations of negatively charged tartrazine from 0.0 to 10.0 mM at 20 mM Glycine-HCl pH 2.0 buffer. In **Fig. (2B),** myoglobin was incubated with similar concentrations of tartrazine at pH 7.4 as a control.

Turbidity measurements were carried out on samples to detect the quantity of aggregates at 650 nm. The myoglobin (11.3 μM) was incubated with and without different concentrations (0.0-10.0 mM) of tartrazine at pH 2.0 **(Fig. 2C)** and pH 7.4 **(Fig. 2D).** All the samples were incubated for 12 hrs before measurements and measurements were carried out at room temperature.

Fig. 3A: Kinetics of tartrazine-induced aggregation of myoglobin. The Myoglobin was incubated at pH 7.4 (-■-) alone and in the presence of different concentrations of 0.0 (-□-) 0.5 (-Δ-), 2.0 (-●-), 3.0 (-▲-) and 5.0 (-○-) mM tartrazine at pH 2.0.

Intrinsic fluorescence emission spectra of myoglobin alone and treated with different concentrations of 2.0 mM (-●-), 3.0 mM (-▲-) and 5.0 mM (-○-) of tartrazine are shown in **(Fig. 3B).** The intrinsic fluorescence spectra of myoglobin of native (-■-), low pH (-□-) and tartrazine alone (-♦-) are shown in **Fig. 3C**. The wavelength maximum of intrinsic fluorescence was plotted at different concentrations of tartrazine at pH 7.4, and 2.0 shown in **Fig 3D.** The myoglobin concentration (11.3 μM) was kept constant in both kinetic and intrinsic fluorescence measurements.

Fig. 4A: The UV-Visbile spectra of tartrazine-induced aggregates of myoglobin were plotted in the presence of Congo Red (CR). The absorbance spectra of native myoglobin (-■-) and aggregated samples (presence of 2.0 mM (-●-) and 5.0 mM (-○-) were shown in **Fig. 4A.** CR measurements were performed at room temperature.

Fig. 4B, Secondary structural conformational change was seen by far-UV CD measurements. Far-UV CD spectra of myoglobin at pH 7.4 (-■-), pH 2.0 (-□-) and with 2.0 (-●-), 3.0 (-▲-), 5.0 (-○-) mM of tartrazine was plotted. Before CD measurements the tartrazine-induced aggregated samples were centrifuged and washed to avoid interference of dye in the far-UV range. The concentration of myoglobin was fixed 11.3 μM in all Congo Red and CD measurements. The CD was also done at room temperature.

Fig. 5: AFM (panel I) and TEM (panel II) was done to identify the morphology of tartrazine-induced aggregates of myoglobin. The myoglobin (11.3 μM) was treated with 2.0 mM and 5.0 mM of tartrazine at pH 2.0. The imaging was done overnight incubated samples..

Fig. 6: Diagrammatic illustration of tartrazine interaction with myoglobin at two different pH 7.4 and 2.0.

Figures:

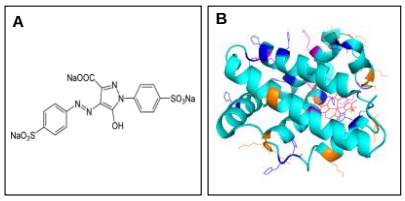

Fig. 1

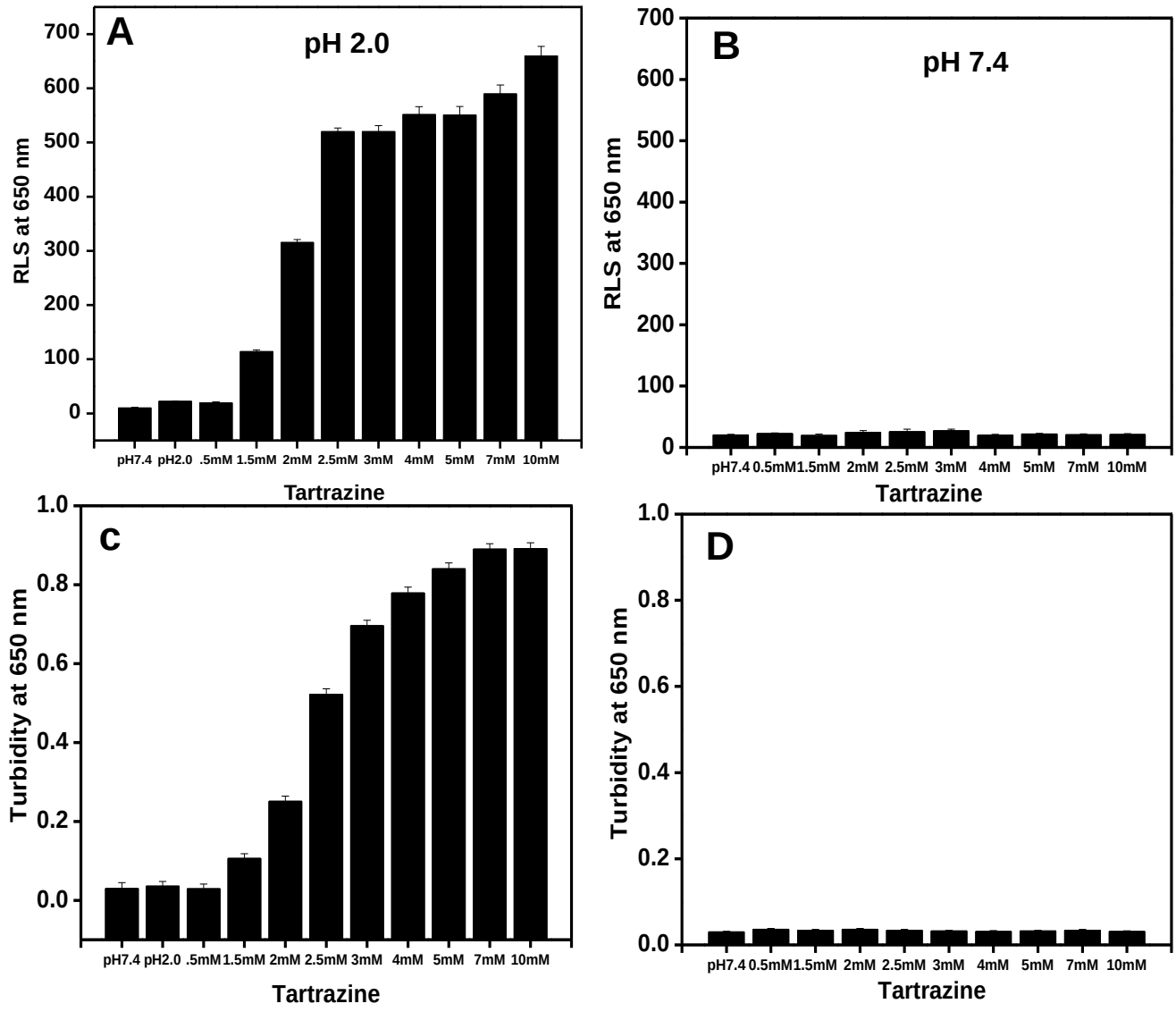

Fig. 2

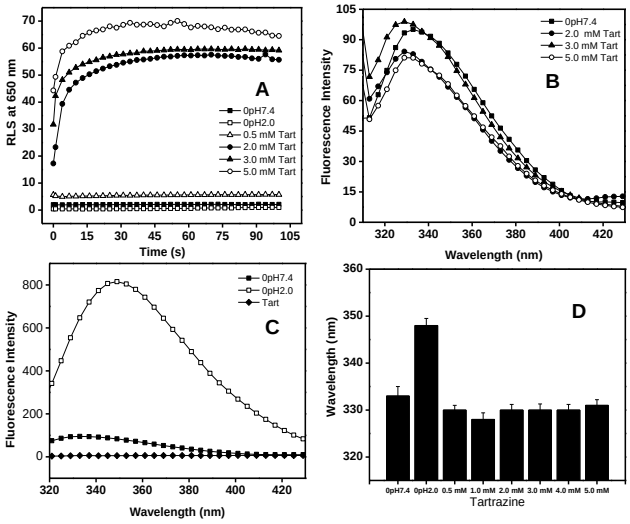

Fig. 3

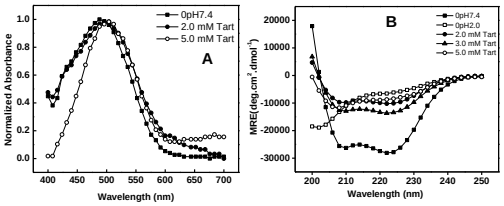

Fig. 4

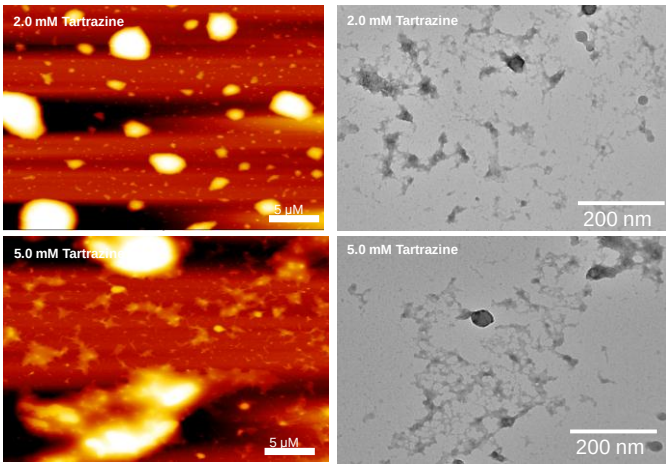

Fig. 5

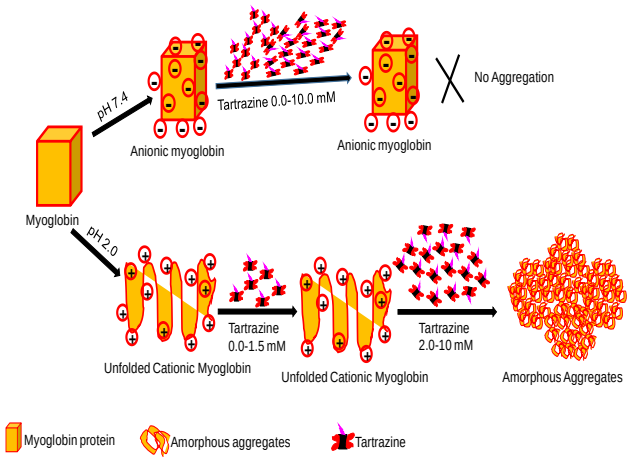

Fig. 6

Table 1: Spectroscopic data of myoglobin at different in-vitro conditions

S. No.	Conditions	Fluorescence Intensity at 650 nm	Turbidity at 650 nm	λ_{max} at 295 nm
1	Myoglobin at pH 7.4	20.234± 1.1	0.03±0.012	333±2
2	Myoglobin at pH 2.0	22.409±1.5	0.036±0.011	348±1.5
3	Myoglobin at pH 2.0+2.0 mM Tartrazine	315.846±5.3	0.251±0.014	330±1
4	Myoglobin at pH 2.0+2.5 mM Tartrazine	520.34±5.7	0.522±0.013	330±1.4
5	Myoglobin at pH 2.0+3.0 mM Tartrazine	520.101±10.2	0.696±0.014	330±1.2
6	Myoglobin at pH 2.0+4.0 mM Tartrazine	551.651±14.0	0.779±0.015	331±1.3
7	Myoglobin at pH 2.0+5.0 mM Tartrazine	550.715±16.2	0.84±0.014	330±1.2
8	Myoglobin at pH 2.0+7.0 mM Tartrazine	590.117±16.1	0.89±0.013	330±1.2
9	Myoglobin at pH 2.0+10mM Tartrazine	600.095±16.3	0.891±0.014	330±1.3

Table 2: Percent secondary structure of myoglobin was calculated in the absence and presence of tartrazine by K2D2 online software.

S. No.	Conditions	% α-Helix	% β-Sheet
1	Myoglobin at pH 7.4	69.46	1.8
2	Myoglobin at pH 2.0	21.57	28.13
3	Myoglobin at pH 2.0+2.0 mM Tartrazine	23.7	26.2
4	Myoglobin at pH 2.0+3.0 mM Tartrazine	25.78	24.71
5	Myoglobin at pH 2.0+5.0 mM Tartrazine	23.21	23.85

Error is under 0.4.