The Bioscan 19(2) S2: 137-142, 2024 www.thebioscan.com

Congo Red-based assay to quantify the modified Glucagon Like Peptide-1 Aggregation by Spectrophotometric Scanning Method

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DOI: https://doi.org/10.63001/tbs.2024.v19.i02.S2.pp137-142

KEYWORDS
Congo red, mGLP, Fibrils, Aggregation, Spectral Scan

Received on:
21-06-2024

Accepted on:
01-10-2024

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ABSTRACT

Aggregation of peptides that are of therapeutic importance when administered to patients leads to cytotoxic effect. Recently Glucagon-like peptide-1-based drug formulations are being prescribed to help alleviate Type-2 diabetes. Few studies reported this peptide tends to aggregate and form fibrils. Congo red is a histological stain known for its strong binding to β-sheet-rich fibrillar proteins, which results in a shift in its absorbance spectrum when it attaches to amyloid structures. This characteristic has been utilized in earlier research to investigate interactions with proteins such as insulin. In this study, we have developed a spectrophotometric assay specifically designed for quantifying aggregated forms of modified GLP-1 (mGLP) peptide. This method is straightforward and efficient, offering rapid results without the need for specialized equipment or costly reagents. Here CR–mGLP Spectrophotometric assay provides precise and cost-effective quantification by directly measuring aggregated mGLP in absolute terms, facilitating investigation of peptide aggregation and its implications in various biological and therapeutic applications.

INTRODUCTION

Versatility and specificity of small peptides make them valuable tools in developing novel therapies for addressing challenges such as antimicrobial resistance and many complex diseases. It is well known that the anti-diabetic molecule, Glucogan-like peptide-1 peptide in modified form is used as a drug substance during the preparation of liraglutide. Modified GLP-1 (mGLP) is known to aggregate in the absence of stabilizers and this was confirmed earlier by stability studies done at various time points using scanning electron microscopy(Brichtová et al., 2023; Kulkarni R, 2023). Aggregated drug substances pose serious health risks by creating cytotoxicity(Liu et al., 2024). During the preparation of the liraglutide drug product, mGLP peptide is synthesized by chemical synthesis method or by recombinant DNA technology and later conjugated to fatty acid linkers to improve bioavailability. During the conjugation process, aggregated peptides get masked and such hindrance makes conjugation ineffective thereby most of the peptides are left unconjugated and subsequently eliminated during bioprocessing. In this way, aggregation poses a serious loss to manufacturers.

The amyloidogenic peptide, mGLP forms fibrillar aggregates even in optimally set buffers in the absence of stabilizers. Here this peptide has strong aggregation-prone regions (APRs) that are responsible for the formation of fibrillar aggregates.(Ulamec, Brockwell, & Radford, 2020). Peptide aggregation is influenced by a variety of factors including sequence, concentration, temperature, pH, ionic strength, and solvent conditions.(Zapadka, Becher, Gomes Dos Santos, & Jackson, 2017). The current study might aid researchers in developing strategies to quantify aggregation caused by sequence modifications, chemical modifications, formulation conditions, and stabilizing additives.(Frokjaer & Otzen, 2005). Typically, research on peptide aggregation is conducted using techniques such as Circular Dichroism, Fluorescence Spectroscopy, Transmission Electron Microscopy, Atomic Force Microscopy, Dynamic Light Scattering, and Size-Exclusion Chromatography.(Etienne et al., 2007; Rupsi & Kumar, 2022). In the current study, we have used a simple spectrophotometer-based method to carry out the preliminary analysis. This does not involve separation of the dye-peptide complex from the free peptide and quantitative analysis can be done easily. Congo Red's (CR) ability to bind to beta-sheet structures and its characteristic absorbance change can be leveraged for studying and quantifying peptide aggregates in solutions of modified GLP peptide.

MATERIAL & METHODS

Congo Red preparation

To prepare a 500 mM stock solution of CR (Sigma), equivalent to approximately 0.34 mg/ml, of Congo red solid was solubilized in 10 ml of Ultrapure Water and was filtered using glass fiber filters (Sterilite GF-75). The concentration of the stock solution was calculated from absorbance data measured at the wavelength of 505 nm by taking an aliquot of the filtrate after the removal of the solid and using the extinction coefficient 5.93×10^4. This made it possible to determine the correct concentration of Congo red in the stock

solution for subsequent experiments with a reasonable degree of precision.

mGLP aggregate preparation and estimation

The mGLP peptide was initially dissolved in Ultrapure Water to achieve a final concentration of 100 µM (338 µg/ml), resulting in a uniform solution.(Girych et al., 2016). This solution was then stirred magnetically vigorously for 48 hours at a cold temperature. Following this, aggregated mGLP peptide was collected by centrifugation at 28,000g for 15 minutes, with the aggregation level found to be 99% leaving behind unaggregated peptide to 1%. Throughout the experiment, the aggregated mGLP stock solutions were continuously shaken to maintain consistency until aliquots were withdrawn for binding assays. To dissolve the aggregated protein, 100 µL of fibrils were mixed with 100 µL of 99% formic acid. The protein concentration in this solution, as well as in the initial supernatant, was determined by measuring absorbance at 280 nm and adjusting for the peptide's specific molar extinction coefficient of 6990 M^{-1} cm^{-1}. For the standard curve, mGLP was reconstituted in 50% formic acid, with standards ranging from 0 to 100 µg/ml prepared such that the final formic acid concentration was 5% (1.34 M) and ultrapure water made up the remaining 95%, totalling 1000 µL. The spectrophotometer was calibrated with ultrapure water, and absorbance measurements of the standards were taken at 280 nm.

Spectral Analysis of CR-mGLP complex

To determine the binding between Congo red and mGLP fibrils, the method described by Klunk was used (Klunk, Pettegrew, & Abraham, 1989a). For mGLP and CR Binding studies were analyzed with a UV-Vis spectrophotometer (Shimadzu). Spectral analysis of the binding assay of all samples was conducted in wavelength scan mode from 350–600 nm on the instrument blanked with ultrapure water. Spectra of the CR-mGLP complex were analyzed to find the peak absorbance at a particular wavelength and to locate an isosbestic point that remained consistent across all concentrations of both CR and CR-mGLP. In the preliminary analysis, various concentrations of CR alone—ranging from 0.7 µM to 5.6 µM in increments of 0.7 µM—were used to examine the spectra. In the second round total of 0.3 µM CR was incubated with different concentrations of mGLP ranging from 0.3 to 5.6µM as separate aliquots to know the binding profile by incubating at room temperature for 30 min before the spectral studies(Klunk et al., 1989a). Prior to it to identify the minimal ratio of CR-mGLP complex needed was tested by taking 22.4µM mGLP was incubated with CR at varying concentrations of 0.3 to 5.6 µM and 10, 20, 40, and 80 µM to ensure that CR molecules were binding strongly and specifically to the mGLP fibrils to reach saturation at the microscale in third round. Furthermore, the primary light scattering resulting from mGLP peptide fibrils alone was captured using absorbance spectra of mGLP fibril concentrations ranging from 0 to 30 µg/ml. All these rounds of scans made it possible to calculate the quantity of CR attached to mGLP and enabled us to derive an equation to quantify CR bound to mGLP.

RESULTS & DISCUSSION

Spectral analysis of CR-mGLP complex

Congo Red, is used as a histological marker to study amyloid beta-peptides in brain tissues during postmortem examinations. Its effectiveness in detecting amyloid in pathological samples was well-established early on, due to its strong and specific binding affinity for amyloid proteins which are mediated by the secondary structure of amyloid which comprises of ß-pleated sheet (Rancati et al., 2018). CR dye undergoes only a minor colour change, which correlates with a change of the characteristic absorbance spectrum of the dye, upon interaction with fibrillar modified glucagon-like peptide-1 (mGLP)(Howie, Brewer, Howell, & Jones, 2008). The absorbance spectra of CR alone recorded between 350 and 600 nm on a spectrophotometer, demonstrated a distinct concentration-dependent variation. At lower concentrations, specifically around 0.7µM, the absorbance was relatively modest, with the spectra showing a characteristic peak around 499 nm, indicative of CR absorption maximum. As the concentration increased to 5.6µM, the absorbance intensity significantly heightened, displaying a pronounced and sharper peak at the same wavelength. This increase in absorbance with concentration reflects a typical Beer-Lambert behaviour, confirming that CR absorbance is directly proportional to its concentration within the tested range. An isobestic point in CR spectra typically indicates a wavelength where absorbance remains constant despite changes in concentration. For CR, this point is observed around 393 nm which can be seen in **Figure 1. a**. At this wavelength, the absorbance remains stable even as the concentration or other conditions vary, which can provide insights into the stability and behaviour of the dye under different experimental conditions.(Borate SR, 2023).

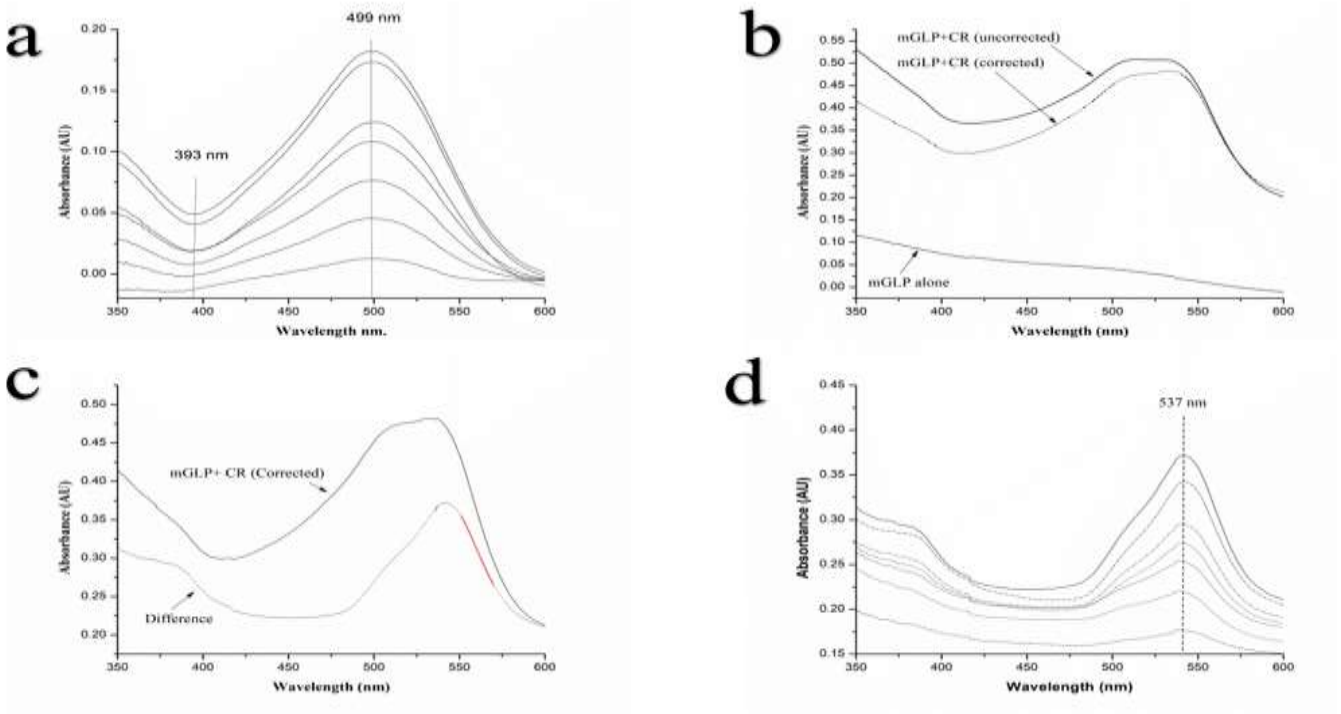

Figure 1: a) Absorbance spectra of only CR in deionized water at concentrations ranging from 0.7 to 5.6 µM, 393 and 499 nm are isosbestic points across t he various concentrations. **b)** Absorbance spectra of suspension of fibrillar mGLP with 22.4 µM concertation in the presence of 3.5 µM of CR. mGLP + CR (corrected) results from the substruction of mGLP scattering mGLP + CR (uncorrected). **c)** The difference spectrum is obtained by deducting absorbance spectra of 3.5 µM CR alone from the mGLP+CR complex spectra. **d)** Difference spectra taken at various CR concentrations, with a constant mGLP concentration of 22.4 µM, revealed that 537 nm consistently represented the point of maximum difference for all CR concentrations.

The type of spectral property alterations in CR has also been demonstrated when it forms complexes with other amyloid fibrillar proteins including insulin and amyloid beta(Turnell & Finch, 1992). The findings of the present study suggest that the selectivity of amyloid fibrils to alter the CR spectrum may be a function of the state of protein aggregation(Klunk, Pettegrew, & Abraham, 1989b). Firstly, CR spectra get shifted to a different wavelength and secondly, amyloid fibrils intrinsically exhibit light scattering(Kozan & Nahi, 2023). The deconvoluted and overlaid mGLP fibrillar absorption spectra and CR dye are shown in **Figure 1. b**. The uncorrected absorbance spectrum is higher than the corrected spectral curve as a function of the wavelength, rising most sharply at shorter wavelengths due to the increased scattering of mGLP. The subtraction of the mGLP alone spectrum from the uncorrected mGLP:CR results in corrected absorbance by mGLP:CR complex which can be seen in **Figure 1. c**. Bound/free CR molar absorptivity at 393 nm is equal to that of isosbestic point suggesting that bound and free CR have the same molar absorptivity value. In the comprehensive spectral analysis of the mGLP:CR complex, the greatest spectral difference was observed at 537 nm after subtracting the spectrum of CR alone which can be seen in **Figure 1.d**. Previous models suggest that the ß-sheet conformation of amyloid is crucial for Congo Red binding, as reflected in the current ß-sheet confirmation model. Proteins that had no or very low content of ß-sheet secondary structure organization did not have CR staining(Dec, Babenko, & Dzwolak, 2016).

A key aspect shared by all these models was interaction between CR and amyloid fibrils is both stoichiometric and saturable. This interaction caused a change in the dye's absorption properties, although the underlying reasons were not fully understood at the time. Nevertheless, this stoichiometric and saturable nature of the interaction led to a shift in the spectral readings, which allowed for the precise quantification of the CR–amyloid complex concentration in earlier research.(Yamaki et al., 2005). Other pros of the CR-aggregated peptide technique are the following: no need to use expensive and specifically designed tools; versatility; no need to use radioactive or other expensive materials; the technique is fast; and is rather easy. Most importantly, this technique had the distinct advantage of being able to measure dye–amyloid interactions in an absolute manner.(Yaneva & Georgieva, 2012). Previously, this method was intended for measuring unknown concentrations of amyloid by comparing the results with standard values. At that time, it was challenging to maintain identical attenuation levels in both the sample and reference cells containing amyloid fibrils. Fortunately, this issue was less critical because the light scattering by amyloid fibrils was linearly dependent on their concentration, and there was a direct correlation between the extent of light scattering and the wavelengths used.(Murphy & Pallitto, 2000). The light scattering spectral scan conducted at varying concentrations of mGLP demonstrated a linear incremental increase in scattering intensity at both 537 nm and 393 nm. As the concentration of mGLP was progressively increased, the scattering intensity at 393 nm exhibited a consistent rise, reflecting that absorbance at various wavelengths of mGLP has characteristics of aggregate or particle nature to scatter light more effectively. The light scattering at 393 nm was found to linearly increase, and the intensity was generally higher compared to 537 nm as can be seen in **Figure 2. a**.

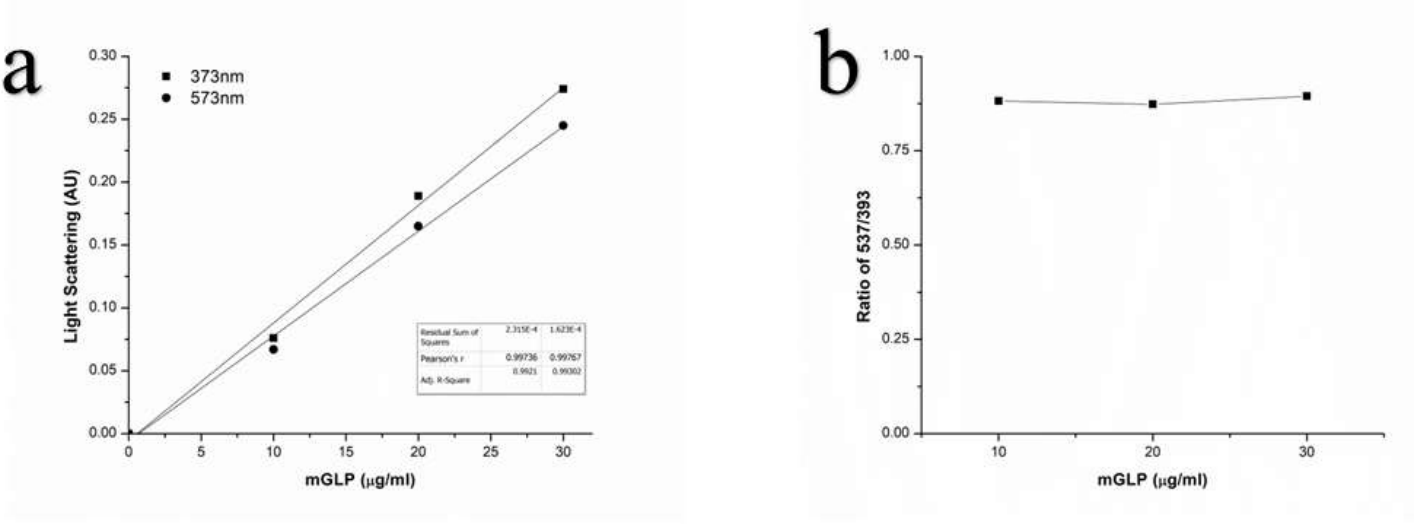

Figure 2: a) The light scattering effect of mGLP fibrils at 393 nm and 537 nm was analyzed across different mGLP concentrations. The scattering exhibited a linear relationship with mGLP concentration at both wavelengths, with a Pearson correlation coefficient of 0.99. **b)** The ratio of light scattering at 537 nm to 393 nm remained fairly consistent across all tested mGLP concentrations, resulting in a ratio of 0.88.

The light scattering of amyloid-beta mGLP fibrils at 393 nm and 537 nm is directly proportional, and there is a constant ratio between the behaviors at the two wavelengths. This phenomenon enables the determination of light scattering factor at 393 nm ($^{393}S_{mGLP}$) and 537 nm ($^{537}S_{mGLP}$) that could be used for the normalization of ratios for light scattering in subsequent experiments. The constant Ratio calculated from **Figure 2. b** i.e. 0.88 is referred to as 'r'. This linear relationship between concentration and scattering intensity at both wavelengths suggests a proportional correlation, indicative of stable and predictable aggregation behavior of mGLP in solution. It was noted that light scattering was linearly proportional to the concentration of amyloid fibrils, and a strong correlation existed between light scattering intensity at various wavelengths. This relationship helped mitigate issues related to varying concentrations in the sample and reference cells. In general, some compounds and concentrations could influence the results, while the connection between light scattering and wavelength can be employed to quantify unknown amyloid concentrations even though there may be limitations.

It was analyzed during the spectral study to undermine the relationship between the variable concentration of mGLP fibrils with constant CR concentration to establish the CR to mGLP ratio by which optimal saturation of binding sites of fibrils with dye. Examining the spectral scan profile, we found ratio of more than 1:5 is a good fitting optimal ratio for analyzing the CR-mGLP complex to

quantifying fibrils that can be studied confidently. From **Figure 3.a** it was found that the absorbance at 537 nm reached its highest value with a moderate concentration of mGLP fibrils and increased only slightly with additional fibrils, causing a minimal change in light scattering at lower wavelengths.These results underscore the reliability of light scattering measurements for quantifying peptide concentration and understanding aggregation dynamics. The spectra of the difference are presented in **Figure 3.b** for the various concentrations of CR with constant mGLP concentration. Of interest, both the isosbestic point and maximum difference point do not change their positions across the wide CR concentration gradient. This indicates that the binding interactions between CR and the fibrils are unaltered at varying concentrations of CR which signifies saturation reaches and reaction between CR and mGLP is irreversible. Since it is known that the number of binding sites is proportional to the fibrils and the fact that each fibril has a certain number of binding sites, then the amount of CR that occupies the binding sites can be calculated which enable for quatificaiton of aggreagation levels of mGLP.

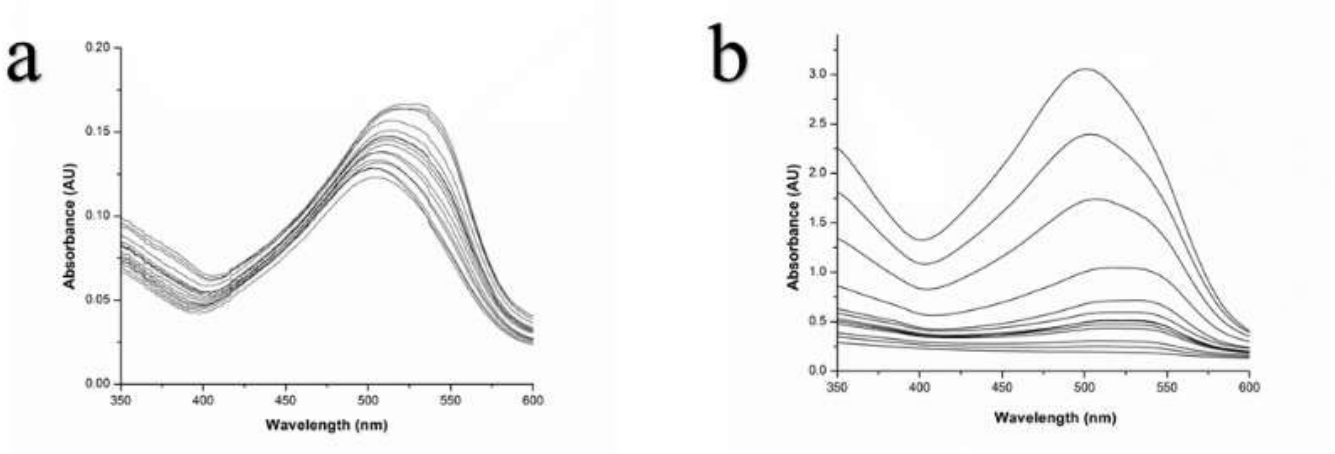

Figure 3 a) Absorbance spectra of different concentrations of mGLP ranging from 0.3μM – 5.7μM with 0.3μM concentration of CR. **b)**Absorbance spectra of different concentrations of CR ranging from 0.7μM – 7.0 μM and 10μM,20μM,40μM and 80μM with 22.4μM concentrations of mGLP fibrils

This method was developed to measure some indicators of mGLP aggregation with the help of the equations that were used in the past CR–insulin method (Klunk et al., 1989a). There was perhaps a presumption that other circumstances being favourable, the errors could be rendered small enough to admit the use of the technique for estimating differences in degree of aggregation.

Derivatization of CR-mGLP complex

The interaction between CR dye and mGLP fibrils results in observable spectral changes within the visible spectrum. By applying the Beer-Lambert law, it is possible to estimate the concentrations of both bound and unbound CR. The total absorbance at any wavelength ($^{w}A_t$) is the combination of absorbance by the CR that is bound to mGLP fibrils and the absorbance by free CR. Thus, by determining the absorbance wavelengths of CR bound to mGLP fibrils ($^{w}A_b$) and free CR in the solution ($^{w}A_f$), a system of simultaneous equations can be set up and solved to calculate the concentrations of bound (c_b) and free CR(c_f) respectively.

$$^{w}A_t = {}^{w}A_b + {}^{w}A_f = {}^{w}a_b c_b + {}^{w}a_f c_f \quad [1]$$

Furthermore, the determination of the 393 nm absorbance of the mGLP: CR complex along with the absorbance of CR alone helps in the calculation of $^{393}S_{mGLP}$ which in turn provides an approximation of the directly measured values. In summary, the spectral signals of CR enable the assessment of the degree to which it binds to amyloid fibrils.

The path length (b) is typically kept constant at 1 cm. By measuring the total absorbance at two different wavelengths, w1 and w2, two equations with two unknowns (c_b and c_f) can be obtained.

$$^{w1}A_t = {}^{w1}a_b c_b + {}^{w1}a_f c_f \quad [2]$$

$$^{w2}A_t = {}^{w2}a_b c_b + w2a_f c_f \quad [3]$$

Solving for cf at both wavelengths gives

$$c_f = ({}^{w1}A_t / {}^{w1}a_f) - ({}^{w1}a_b / {}^{w1}a_f)c_b \quad [4]$$

$$c_f = ({}^{w2}A_t / {}^{w2}a_f) - ({}^{w2}a_b / {}^{w2}a_f)c_b \quad [5]$$

By setting Equation [4] equal to Equation [5] and solving for cb, you obtain:

$$c_b = ({}^{w1}A_t / {}^{w1}a_f) - ({}^{w2}A_t / {}^{w2}a_f) \;/\; ({}^{w1}a_b / {}^{w1}a_f) - ({}^{w2}a_b / {}^{w2}a_f) \quad [6]$$

In the spectrum of a mixture with both bound and free CR, the intensity peaks at 537 nm, which corresponds to the maximum difference in the spectrum. This value is particularly useful for accurate calculations, especially at low ratios of bound to free CR. Furthermore, if w_2 is an isosbestic point, which is inherently true, then the ratio of absorbance for bound to free CR at w_2 is consistently equal to one, meaning $^{w2}a_b = {}^{w2}a_f$ and $({}^{w2}a_b / {}^{w2}a_f) = 1$. From spectral analysis, the isosbestic point was identified to be 393 nm. Using these parameters and substituting [CR–mGLP] for c_b, Equation [6] can be modified to produce:

$$(\text{CR–mGLP}) = ({}^{537}A_t / {}^{537}a_f) - ({}^{393}A_t / {}^{393}a_f) \;/\; ({}^{537}a_b / {}^{537}a_f) - 1 \quad [7]$$

From the above experiment, the molar absorptivity of CR was calculated at both bound and free state with a constant concentration of aggregated mGLP tabulated in Table 1. When the molar absorptivity of the complex, CR–mGLP, had been determined, the concentration of the complex could be calculated from the absorbance at 537 nm, and an appropriate isosbestic wavelength. Absorptivity values for the free ligand can be readily determined using standard methods with CR solutions that do not contain mGLP fibrils. The molar absorptivity of saturated bound ligand was measured i.e., when cf = 0(Macii & Biver, 2021). This was made possible through the application of a stock concentration of 100 μM mGLP fibrils.

Table 1: Molar absorptivity of complex and free dye

Wavelength(nm)	Bound CR-mGLP	Free CR	Difference (%)
537	15300	14100	108.5
393	13410	13400	0.01

By inserting the molar absorptivity values from Table 1 into Equation [7], the resulting calculation is:

$$(CR\text{–}mGLP)=(^{537}A_t/14100)-(^{393}A_t/13400)/(15300/14100)\ –\ 1$$

This can be simplified to

$$(CR\text{–}mGLP) = (^{537}A_t/1200) - (^{393}A_t/1140) \quad [8]$$

A mathematical correction for scattering that had to be introduced to allow for quantitating mGLP aggregation was presented.By using the following equation of subtracting the scattering from $^{393}S_{mGLP}$ with the total scattering, the correction for scattering was feasible. This correction helped in obtaining the actual quantity of mGLP as the scattering interfered with the measurements.

$$(CR\text{–}mGLP) = [(^{537}A_t - {}^{537}S_{mGLP})/1200]\ –\ [(^{393}A_t - {}^{393}S_{mGLP}/1140] \quad [9]$$

where $^{393}S_{mGLP}$ and $^{537}S_{mGLP}$ indicate the light scattering effects caused by mGLP fibrils at the specific wavelengths of 393 nm and 537 nm, respectively. These terms represent how the presence of mGLP fibrils influences the scattering of light at these wavelengths.

As $^{537}S_{mGLP} = r * {}^{393}S_{mGP}$ (where $r = {}^{541}S_{mGLP}/{}^{393}S_{mGLP}$)

$$[CR\text{–}mGLP] = [(^{537}A_t\ –\ (^{393}S_{Ab}* r)/1200]\ –[\ (^{393}A_t - {}^{393}S_{mGLP}/1140] \quad [10]$$

Knowing that $^{393}S_{mGLP} = {}^{393}A_t - {}^{393}A_{CR}$ (where $^{393}A_{CR}$ signifies the absorbance of CR measured specifically at its isosbestic point of 393 nm). This is the wavelength where the absorbance of CR remains constant regardless of the binding state or concentration of the complex being studied.

$$[CR\text{–}mGLP] = (^{537}A_t/1200) - [(^{393}A_t - {}^{393}A_{CR}*r/1200]\ –\ [^{393}A_t\ –\ (^{393}A_t - {}^{393}A_{CR})]/1140$$

Which reduces to

$$[CR\text{–}mGLP] = (^{537}A_t/1200)\ –\ [^{393}A_t/1200/r)]\ +{}^{393}A_{CR}[\ r/1200)\ –\ (1/1140)] \quad [11]$$

To calculate $^{393}A_{CR}$ which can be done in a couple of methods.
In the first method, the total absorbance of CR at 393 nm was measured in samples containing only CR. This approach was effective in standard aggregation experiments where the concentration of CR was fixed, but the scattering from different mGLP concentration varied. The second method for determining $^{393}A_{CR}$ was applicable when the intensity of mGLP fibrils remained constant while the concentration of CR changed. Under these conditions, $^{393}S_{Ab}$ was held steady, and $^{393}A_{CR}$ was calculated as the difference between $^{393}A_t$ and $^{393}S_{Ab}$. This technique was useful for determining the exact concentration of CR in individual samples. However, it was not typically used in standard mGLP aggregation experiments and was reserved for analysing the Reaction Rates and Proportionality of CR binding in samples with known mGLP fibril content.
It can be observed that $^{393}A_{CR}$ is given by the product of $^{393}a_{CR}$ and the total concentration of CR in the assay. Specifically, $^{393}a_{CR}$ is the molar absorptivity of Congo Red at 393 nm, which is 13,410 L/(mol·cm). Thus, $^{393}A_{CR}$ is calculated as 13,410 multiplied by [CR], where [CR] denotes the concentration of Congo Red added to the assay. When this expression for $^{393}A_{CR}$ is substituted into Equation [11], it allows for the calculation or adjustment of various parameters within the equation based on the known values.

$$[CR\text{–}mGLP] = (^{537}A_t/1200)\text{–}[^{393}A_t/(1200/r)] + (13410)([CR])[r/1200)- 1/1140)]$$

Which reduces to

$$[CR\text{–}mGLP]=(^{537}A_t/1200)- [^{393}A_t /1200/r)] + ([CR])[(r/0.089) - 0.085] \quad [12]$$

This equation is effective for assessing mGLP aggregation when CR is present at a constant concentration. By substituting r = 0.88 into the equation, it simplifies the Equation [11], making it easier to work. This substitution streamlines the calculations and provides a more straightforward expression for analyzing the data, thus facilitating the evaluation of mGLP aggregation in the context of the CR concentration used in the experiment.

$$[CR\text{-}mGLP]=[^{393}A_t/1200]-[^{393}A_{CR}/1364] - [^{393}A_{CR}/6802] \quad [13]$$

And Eq [12] to

$$[CR\text{-}mGLP]= [^{537}A_t/1200]-[^{393}A_{CR}/1364] +9.88[CR] \quad [14]$$

To handle unknown levels of mGLP aggregation, the absorbance of the CR-alone control at 393 nm ($^{393}A_{CR}$) is used to account for dilution effects from concentrated CR solutions, thereby reducing potential inaccuracies from measuring high concentrations of CR. It should be recognized that if the focus had been on the concentration of bound CR, one of Equations 11 through 14 would have been the appropriate choice. But when the aim was to quantitate mGLP aggregation, then Equation 14 reframed to Equation 15 which need to be employed.

$$mGLP_{fib}=(^{537}A_t/1200)-(^{393}A_t/1364)+9.88[CR] \quad [15]$$

Equations 15 provide the concentration of mGLP fibrils in mg/ml, which could be used to define the CR to mGLP ratio and vice versa. Describing suspensions of fibrillar aggregates with unspecified sizes in mol/L was impractical, so mass/L units were used instead, offering a more general measure without requiring specific details about aggregate size.

CONCLUSION

Since this absolute quantitation of mGLP was essential for identifying the impact of peptide change on aggregation, it was critical to demonstrate its applicability. Measuring aggregation also was necessary for assessing proposed drugs to be used in treatment to reduce the likelihood of mGLP fibril formation. The CR–mGLP assay seemed to have the possibility to quantify the protein amyloidosis propensity for different mGLP preparations. It could have potentially contributed to the methodological standardization of several significantly challenging and variable mGLP protein amyloidosis assays. In summary, the CR-mGLP method did not involve the use of any sophisticated equipment, allowed for accurate quantitative analysis of mGLP aggregation, and had the potential for drug discovery and standardization of amyloidosis assays by providing a means to quantify accurately mGLP fibril formation from various preparations.

ACKNOWLEDGMENT

We thank Dr.Volaty Srinivas, Principal Scientist at the Centre for Cellular & Molecular Biology for providing us insightful thoughts during the study and also we are grateful to Ms Lekha Arrolla & Ms. Sanjana Sutram for assisting in experimental trials.

CONFLICT OF INTERESTS

All authors have seen and approved the manuscript being submitted. Hence, on behalf of all authors, the corresponding author states that there is no conflict of interest.

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