Low molecular weight nucleoside gelator: A novel approach for protein and peptide aggregation inhibition

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DECLARATION

I hereby declare that the work presented in this thesis is a bonafide and genuine research work carried out by myself under the supervision of Dr. Maria Marlow, Dr. Mischa Zelzer, Prof. Stephanie Allen at the University of Nottingham, United Kingdom in support of my application for the degree of Master of Research. No part of this work has been submitted for any other degree at the University of Nottingham or any other institutions.

05/09/18 Litty Johnson

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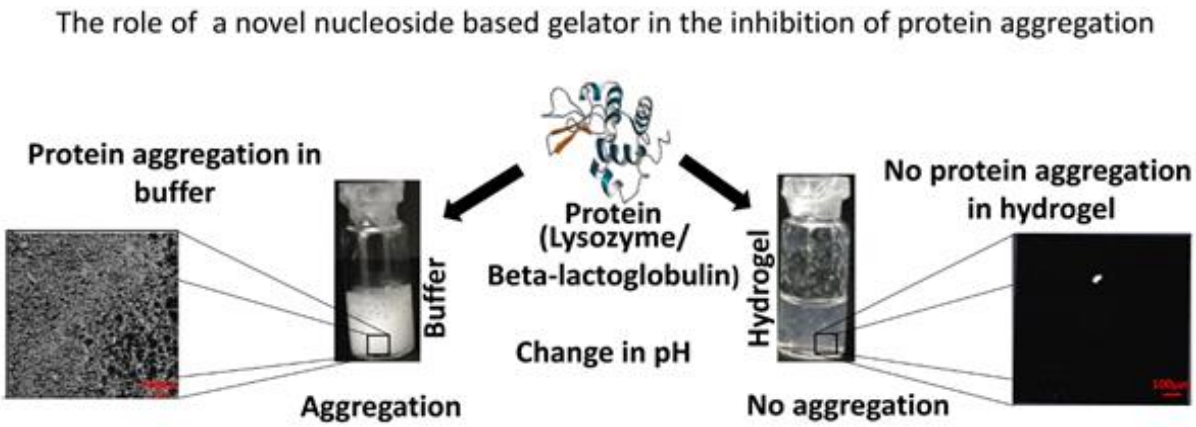
ABSTRACT

Protein and peptide based therapeutics have become one of the most rapidly growing class of therapeutics that have potential to treat human disease. However, their inherent instability limits their application, which in turn minimises their therapeutic potential. Among all the physical and chemical instabilities, protein aggregation has become the major important cause of concern in the biopharmaceutical industry. Several additives are currently employed to prevent this. However, these additives possess several drawbacks such as their ability to denature proteins. Thus, there is a need to develop a novel method for the prevention of aggregation that could extend the shelf life of these biotherapeutics.

Low molecular weight nucleoside gelators have been widely used as a candidate for the delivery of biologics due to their excellent biocompatibility. However, their influence on the inhibition of protein aggregation has not been studied. Herein, we are reporting the ability of the nucleoside gelator, *N*4-octanoyl-2'-deoxycytidine to inhibit protein aggregation.

Using turbidimetric, spectroscopic and microscopy methods, we have demonstrated that inhibition of protein aggregation is dependent on gelator concentration. Moreover the model proteins were found to be functionally active in the hydrogel system. All our results revealed that this gelator exhibited the ability to suppress aggregation of proteins with a broad range of isoelectric points. Thus, the 2' deoxycytidine based hydrogel has potential in tackling one of the most important challenges in the formulation of biologics i.e.protein aggregation.

GRAPHICAL ABSTRACT



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ABBREVIATIONS

% w/v	Percentage weight by volume
µl	Microlitre
µm	Micrometer
µM	Micromolar
1D	One-dimensional
3D	Three-dimensional
AFM	Atomic force microscopy
ANOVA	Analysis of variance
ANS	8-Anilino-1-napthalene sulfonic acid
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
hr	Hour
KDa	Kilodalton
LC-MS	Liquid chromatography-mass spectrometry
LMWG	Low molecular weight gelator
M	Molar
mg/ml	Milligram per millilitre
mg/ml	Milligram per millilitre
min	Minutes
mM	Millimolar
NaCl	Sodium Chloride
nm	nanometer
rpm	Revolutions per minute
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UV-Vis	Ultraviolet-visible

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Protein and peptide pharmaceuticals

Protein and peptide pharmaceuticals are considered as one of the most rapidly growing class of clinical therapeutics.[1] The recent advancements in the field of biotechnology and gene technology has contributed enormously to the increased development of these pharmaceuticals. These exogenous proteins, generally categorized as enzymes, hormones, antibodies, globulins etc. [2] and peptides [1] exhibit a great influence on the regulation and integration of several life processes.[3]

1.1.1 Application of protein and peptide pharmaceuticals

Most of these protein and peptide therapeutics (about 75%) are administered through the parenteral route. However short half-lives and hence frequent administration of these therapeutics have reduced patient compliance.[2,4] Thus, alternative non-invasive routes like oral, pulmonary, transdermal and nasal routes are employed to overcome these limitations. Though with these routes of administration, there are further challenges such as the instability of biologicals in low pH, enzymatic degradation, mucociliary clearance etc.[2,5,6]

Despite these limitations, protein and peptide drugs are widely used for the treatment of several chronic diseases such as leukaemia, diabetes, rheumatoid arthritis, cancer, hepatitis, short bowel syndrome, infectious diseases, immunological diseases, haematological disorders, hormonal disorders, genetic disorders, respiratory disorders, and eye disorders etc.[7,8] Susan *et al* in their review commented about the promising approach of treating cancer by peptide therapeutics.[9] Here we listed an estimate of the

number of protein and peptide therapeutics in the market and in clinical trials in Table 1.

Table1: Estimated number of protein and peptide therapeutics as of March 2017. Adapted from Reference [10]

Protein/Peptides	**Number**
Approved	60
In clinical trials	155
Withdrawn (previously approved but no longer in market now)	8
Discontinued (terminated prior to approval)	261
Total	484

The advancement of biotechnology and gene technology will definitely prompt the discovery of novel biotherapeutics with a wide range of applications. Thus there is a need for a cost effective drug delivery systems for these therapeutics.

1.1.2 Challenges of protein and peptide therapeutics

Despite their importance, the applications of protein and peptides are restricted by several challenges that limit their potential as therapeutics. Some of the main challenges of protein and peptide drugs included the following.

a) Protein and peptide instability

Proteins molecules are distinguished by their three dimensional structure and this represents the functionality of the molecule. These native three dimensional structures are maintained by a balance of certain interactions such as hydrogen bonding, covalent linkages, hydrophobic forces etc. Any change to this balanced environment can trigger protein instability which finally results in protein degradation and inactivation.[11] Some of the triggering factors include pH, temperature, agitation forces, chemical interactions, and ionic strength.[12] These

instabilities are found to occur during manufacturing, storage, and transportation.

The protein instabilities are categorized as either physical or chemical in nature. In physical instability the chemical composition of proteins remains unaltered but the physical state of the protein changes. Protein aggregation, denaturation, precipitation are some of these instabilities. While in case of chemical instability, new chemical entities are generated by making or breaking of covalent bonds. This further leads to the alteration of the chemical composition of the protein. Some of the chemical instabilities involve oxidation, hydrolysis, deamidation of protein molecules.[13] These instabilities in protein and peptide formulation decrease the therapeutic efficacy and can create problems of immunogenicity. Thus this is considered as one of the most important challenges faced by the pharmaceutical industry in designing an efficacious drug delivery system for protein and peptides.[14]

b) Enzymatic degradation

Protein and peptide drugs display a tendency for enzymatic degradation when administered through different routes. Proteins and peptides delivered by the oral route is degraded by the proteolytic enzymes present in the GIT. Similarly they are susceptible to enzymatic degradation even through other routes of administration. [15] The degradation impairs the therapeutic efficacy of these biologicals [16]

c) Immunogenicity

Protein therapeutics administered to patients, have a potential to elicit undesirable immune responses. This directly affects the safety, efficacy and potency of the therapy.[17] These immunological responses occasionally cause anaphylaxis and lead to life threatening autoimmunity.[18] This has been a concern in the recent years and the regulatory bodies are seeking an immunogenicity risk assessment for the approval of protein therapeutics.[19]

d) Short half-life and low bioavailability

Delivery of most of the protein and peptide therapeutics in the pharmaceutical market are limited due to the short circulatory half-life

and lower bioavailability. This difficulty results in the frequent administration of the therapeutics which further increases the cost of treatment and lowers patient compliance.[20]

If we can overcome some of these challenges, we can effectively design a safe and efficacious drug delivery system for protein and peptide therapeutics. Hence, the main focus of this study has involved finding a solution to the important stability challenge of biopharmaceuticals i.e. protein aggregation.

1.2 Protein and peptide aggregation

Protein aggregation can be defined as a biological phenomenon in which misfolded protein molecules self-assemble into a structure other than that of the native quaternary structure of protein. These structures formed as a result of aggregation are biophysically and biochemically different from the native structure of protein.[21]. Protein therapeutics tend to aggregate in almost all stages of production starting from manufacture to administration of the therapeutic to the patient.[22] Thus to control this phenomenon, a good knowledge and understanding about the different types of aggregates, factors triggering the process and the mechanism of aggregation is vital.

Protein aggregates can be independently classified into five categories:-.
Size Based on size, they can be classified as visible aggregates (> 100 µm), sub-visible aggregates (1-100 µm), sub-micrometre soluble aggregates (100-1000 µm) and nanometre aggregates/oligomers (<100 nm).
Reversibility Whilst based on reversibility, they can be classified as reversible (aggregates formed by weak non-covalent interaction and existing in equilibrium with native monomers) and irreversible.
***Chemica*l** Also, they can be classified by chemical modification as chemically modified or chemically non-modified.
Conformation By conformation they can be classified as native (conformation resemblance with non-aggregated native protein), partially unfolded (conformation change is detectable and some native structures were

retained), unfolded, misfolded (conformation totally different from native structure) and amyloid.

Morphology Finally based on morphology they can be classified as intrinsic and extrinsic.[23]

All of these aggregates formed by different processes are triggered by numerous factors which included change in temperature, ionic strength, pH, interfacial exposure, addition of external chemical agents etc.[24] Alkaline pH and solvents like guanidine hydrochloride stimulated the aggregation of lysozyme[25] whereas neutral pH was enough to aggregate bovine serum albumin.[26]

Numerous studies have proved that the process of aggregation is driven by certain forces that transforms the monomeric state of proteins to the aggregated state. This includes Van der Waals force and hydrophobic forces, that results in an interaction between the side chain and backbone of proteins. In addition to these forces, interactions like hydrogen bonding, electrostatic attraction, intermolecular interaction, chain entropy was found to be maximum during aggregation.[27] The mechanism of formation of protein aggregates under the influence of these driving forces are discussed below.

a) Aggregation of monomeric proteins by self-assembly

   In this mechanism, the native protein that has complementary structure facilitates intermolecular interactions and forms reversible oligomers naturally. With time these reversible oligomers grow larger in size and are transformed to irreversible aggregates (Figure 1.1 a).[28] An example for this mechanism is the formation of irreversible aggregates of insulin at neutral pH.[29]

b) Aggregation of altered monomeric protein

   This mechanism involves the change in conformational structure of native protein by triggering factors such as pH and temperature. These conformationally altered monomers, collectively referred as misfolded proteins form protein aggregates by polymerization (Figure 1.1 b). This type of aggregation is inhibited by stabilizers that can maintain the native monomeric structure of the protein. An example for this

mechanism is the formation of aggregates of human interferon-γ in the presence of 0.9 M guanidine hydrochloride.[30]

c) Aggregation by nucleation and seeding mechanism

Visible aggregates are formed by a nucleation and seeding mechanism. In this mechanism, the native monomeric proteins form small or moderate size oligomers at relative low concentrations. These oligomers of critical size act as a critical nucleus for aggregation. Thus several monomers are combined in this nucleus and form large species which include visible and sub-visible aggregates (Figure 1.1 c). The nucleation mechanism can be categorized as heterogeneous (where a contaminant like silica from glass acts as the seed), homogeneous (where an oligomer acted as the critical nucleus).[31] An example of this mechanism is the formation of amyloid fibrils at neutral pH.[32]

d) Surface induced aggregation

This mechanism of aggregation involved the binding of the native monomer protein to a surface by hydrophobic or electrostatic interaction. This interaction caused a change in confirmation of protein that finally leads to aggregation of proteins (Figure 1.1 d).[28]

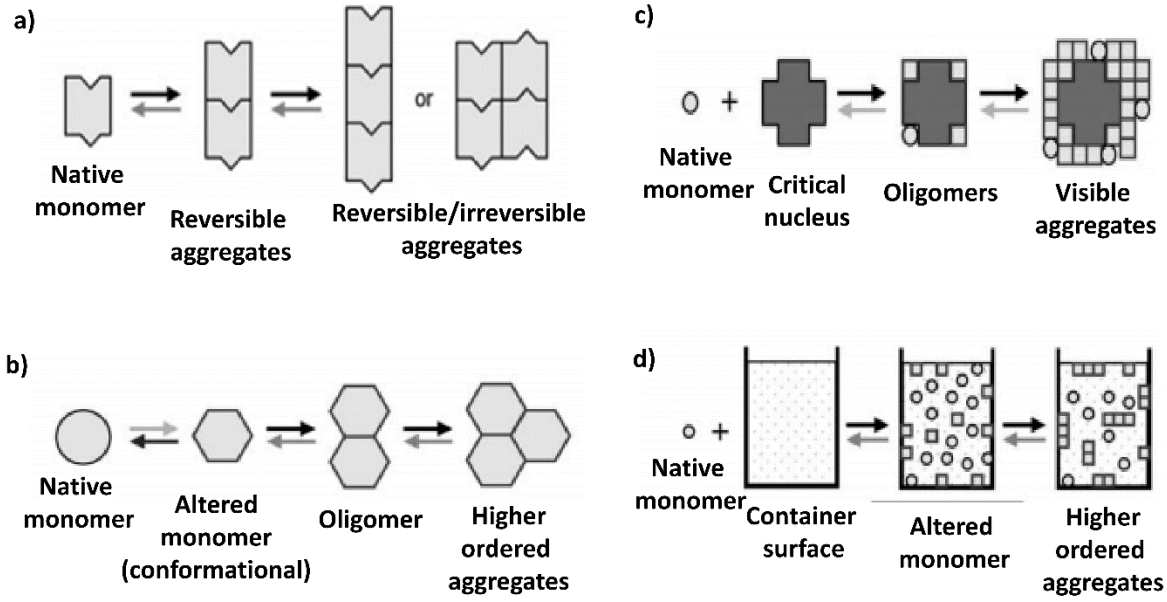

Figure 1.1: Schematic illustration of the different mechanisms of aggregation of protein a) aggregation by self-assembly, b) aggregation of altered protein c) aggregation by nucleation and seeding d) surface induced aggregation. Adapted from Reference [32]

The process and mechanism of peptide aggregation is similar to that of proteins as discussed above.

1.2.1 Impact of protein and peptide aggregation

The aggregates formed by all these mechanisms affect the quality of the biopharmaceutical product both positively and negatively. In some cases, the reversible aggregates were shown to benefit product quality as it improved the stability and solubility of protein when compared to the native form. However, in most cases these were shown to impair the product quality to a large extent. The quality of the product was impaired mainly by the change in the physiochemical and biological properties of the biologicals. This included changes to the solubility of the protein in the formulation which in turn interfered with the dosing of the therapeutic and affected patient compliance.[33] Moreover it changed the solution viscosity in some proteins and this exerted a huge impact on intramuscular, subcutaneous or intravenous drug delivery. In addition to this, presence of the aggregates in patient's blood stream evoked immune responses. This further lead to the progression of autoimmune diseases or anaphylaxis. [34] Thus there is a need to find ways to inhibit protein and peptide aggregation.

1.3 Inhibition of peptide and protein aggregation

Protein aggregation remains as a key challenge in the biopharmaceutical industry and has become the main focus for researchers in this field. In this work we are investigating whether a novel low molecular weight nucleoside gelator can inhibit peptide and protein aggregation. In order to test this hypothesis, it was necessary to know about the current strategies employed in inhibiting protein aggregation with their limitations and the main techniques by which the inhibition in aggregation is monitored. In this section a brief overview of these areas are presented.

1.3.1 Strategies for protein/peptide aggregation inhibition

To combat protein aggregation, a large number of combinations of therapeutics and excipients have been extensively studied. These excipients include polyphenols, sugars, salts and buffers, surfactants, and metal chelators. These excipients with their limitations are described in detail below.

a) Polyphenols

These are small aromatic molecules composed of one or more phenol rings. One of the most extensively studied polyphenolic compounds for inhibiting protein and peptide aggregation is curcumin (a major component of turmeric). Many studies has supported the ability of curcumin to inhibit aggregation of amyloid beta peptide.[35] Curcumin was found to increase the reconfiguration rate of the protein α-synuclein which in turn slowed down aggregation. [36] In addition to this, curcumin aided the down regulation of an endosomal sorting complex, whose upregulation was found to be linked to increases aggregation by polyglutamine.[37] Resveratol is another polyphenolic excipient (mainly found in grapes) that prevents aggregation in beta amyloid peptide by decreasing the amyloidogenic cleavage of the amyloid precursor.[38,39] Amyloid fibrillisation of hIAPP (Human islet amyloid polypeptide) [40] and lysozyme [41] was also reported to be diminished by this molecule. There is a long list of compounds under this group such as epigallocate, epicatechin, myricetin, ferulic acid which have been studied extensively as potential inhibitors of aggregation of proteins and peptides.[42] Even though all these studies gave strong evidence of polyphenols as potential inhibitors, there are several contradictory studies that have limited the use of these molecules.[43-45] In a study conducted by Thapa *et al* curcumin was only found to regulate beta amyloid aggregation rather than inhibition.[43]

b) Sugars

Sugars are small organic osmolytes that prevents the changes in the conformation of proteins that eventually lead to protein aggregation. These molecules are able to stabilize the native monomeric state of proteins by preferential exclusion.[46] The most common excipient

studied in this category is sucrose. It exhibited a strong ability to maintain the structural and colloidal state of lysozyme at extreme conditions of aggregation (high temperature and extreme pH).[47] Trehalose can stabilise proteins under stress conditions and has also shown wide applicability.[48] Other excipients such as mannitol and sorbitol are reported to increase the stability of proteins for example, human serum albumin.[49] The main limitation associated with this excipient was its inability to stabilise the aggregation induced by the surface as it leaves the protein-surface interfacial tension unaltered.[50]

c) Salts

Salts at low concentration stabilise proteins and prevent aggregation. The mechanism of stabilisation involves their strong ability to bind to the protein molecule by electrostatic interaction.[51] This interaction leads to cross linking of charged residues which prevent the protein from aggregating. This was exemplified by the inhibition of heat induced aggregation of lysozyme by ammonium salts reported in a study conducted by Hirano *et al*.[52] Salts like sodium chloride, magnesium chloride, and calcium chloride inhibited aggregation by this same mechanism. The main limitations of these excipients are their ability to denature protein at high concentration and their tendency to destabilise the aggregation prone protein by altering the solubility.[53]

d) Surfactants

Surfactants stabilise proteins by their preferential adsorption and association with proteins. The most commonly studied agents for suppressing protein aggregation in this category are polysorbates and poloxamers. These non-ionic surfactants suppress aggregation triggered by agitation, shaking, freeze drying, and thawing.[54] The two most commonly employed polysorbates are polysorbate 20 and 80. Polysorbate 80 was reported to suppress aggregation of a monoclonal antibody by its preferential adsorption to the protein without inducing any structural changes.[55] Poloxamers (also known by the trade name pluronics) such as poloxamer 188 and tetronic 1107 are claimed to completely suppress the heat induced aggregation of bovine serum albumin and lysozyme by their association with these proteins.[56] Sadly,

the ability of these excipients to oxidise the protein by forming peroxides limits their use.[57] In addition to this, they have a dual effect at different concentrations i.e. a stabilizing and destabilizing effect. For example, polysorbate 20 was described to suppress agitation induced aggregation of pegylated granulocyte colony stimulating factor, but during the long term storage these proteins were found to be in the aggregated state.[58]

e) Others

Arginine is another additive known for its stabilization of protein formulations. It prevents aggregation by increasing the solubility of aggregation prone regions in protein molecule. It was claimed to be successful in preventing aggregation of proteins and peptides, for example amyloid beta[59], insulin, and carbonic anhydrase[60], Despite this potential, arginine induced aggregation in α-lactalbumin by the formation of supramolecular structure of non-fibrillar species which reduced its suitability .[61].

The role of vitamins has also been studied from as early as 1976 when they first reported platelet aggregation inhibition with vitamin E. [62] The mechanisms of inhibition by these substances were not well established. The inhibition was considered to be due to the antioxidant nature of vitamins. Folic acid has been shown to prevent aggregation of amylin [63], and vitamin k3 was reported to prevent lysozyme and beta amyloid aggregation. [64]

Certain co-solvents such as glycerol inhibit protein folding and stabilise proteins by developing a strong interaction with the hydrophobic surface region of the proteins.[65] The aggregation of proteins like ovotransferin, insulin and α-lactalbumin were found to be inhibited by glycerol.[66]

From the review of all these excipients and their limitations, we can conclude that the action of a particular stabilizer or excipient depends on the nature of target protein, and concentration of stabilizer. Thus, there is a need for an effective strategy/stabiliser that can inhibit aggregation for a wide range of proteins at a lower concentration. Hence, herein we are investigating the ability

of novel low molecular weight nucleoside gelator to prevent protein/peptide aggregation. This gelator can be used as a hydrogel formulation for peptide and protein drugs as will be described later in section 1.4.3.

1.3.2 Characterization of protein aggregates

In order to understand the kinetics and mechanism of aggregation and to design an effective strategy or method to prevent protein aggregation, the characterisation of protein aggregates was necessary. Certain efficient analytical methods alone and in combination helped in achieving this objective and are discussed in the following section.

a) Visual inspection

   For visual inspection, the clarity and opalescence of the aggregated protein in solution can be assessed. It is an easy technique for visualisation of aggregates of sizes greater than 50 µm and hence visible to the naked eye.[67] The main drawback of this method is its subjective nature i.e. it does not give a quantitative assessment of aggregation. Thus, this method was used in combination with others.

b) Turbidimetry

   Protein aggregates in solution display a colloidal nature and scatter light in the visible region. Thus, turbidity measurements were used as an effective tool in characterising protein aggregates. Zhao *et al* gave an account of the measurement of turbidity as a cost effective method of analysing amyloid fibrils.[68] In addition to this Shmueli *et al* adapted this method to monitor the aggregation propensity of single purified proteins.[69] By this method the loss in the relative intensity of scattered light at the wavelength of maximum absorption was measured and it was considered to be proportional to the amount of aggregates in the solution.[70] The main drawback of this method is that the results vary with different sizes and concentrations of protein.

c) Optical microscopy

   This technique aided the visualization of the aggregated particles of sizes varying from 1 µm to mm. However optical microscopy, does not

allow explicit differentiation of aggregates from other particles or foreign matter in samples.[67]

d) Fluorescence spectroscopy

Fluorescence spectroscopy is used as a powerful tool in the study of interactions of proteins, their folding mechanism, mechanism of amyloidogenesis etc. This method was found to be broadly applicable to almost all the proteins and were found to be highly sensitive (analyses small quantity of samples in small concentration) and fast. This method involves the use of two types of fluorophores i.e. intrinsic fluorophores (tryptophan, tyrosine) and extrinsic flurophores.[71] Extrinsic fluorophores like ANS, thioflavin T, and congo red are the most extensively used ones in protein aggregation studies. Aggregates like protofibrils, oligomers and amorphous aggregates were characterised by using ANS dye [72] whereas thioflavin T was used for amyloid fibrils.[73] The use of ANS dye for the characterization of lysozyme aggregates is detailed by Ravi *et al*.[74] He found an increased fluorescence intensity with the presence of amorphous aggregates in the solution. Similarly Carrotta *et al* outlined the use of dyes like thioflavin T and ANS for characterising heat induced aggregation of beta-lactoglobulin by this technique.[75] These hydrophobic dyes were established to bind to the exposed hydrophobic regions of the protein aggregates by forming a non-covalent bond (by hydrophobic or electrostatic interactions).[76] This non-covalent interaction with the protein finally leads to an increase in the fluorescence and thus is used as an evaluation method for aggregation.

e) Fluorescence microscopy

In this technique, the samples of protein aggregates are stained by hydrophobic dyes like nile red, congo red, ANS, and thioflavin T. The principle of this method is similar to that of fluorescence spectroscopy. The only difference is that the high fluorescence exhibited is characterised and visualised by a fluorescence microscope. This method gives information on the particle size and shape of the aggregates. Moreover, this is also a highly sensitive and importantly selective technique for the characterization of protein aggregates.[77,78]

f) Atomic force microscopy

Atomic force microscopy, a high resolution technique is employed to provide both qualitative and quantitative details of the protein aggregates. This method is able to determine the morphology and conformation of the formed aggregates accurately even in the nanometer size range.[79] The principle of this technique involved the topographical scanning of the sample by a cantilever fitted with a sharp tip.[80] The deflection in cantilever on raising and lowering is monitored and at a constant laser position an accurate image is being generated.[81]

g) Electron microscopy

The size and morphology of protein aggregates are characterised from nm to mm size range by this technique. This includes high resolution scanning electron microscopy (SEM) and transmission electron microscopy (TEM). By this method protofibrils, amyloid fibrils and amorphous aggregates can be imaged and thus it is considered highly sensitive. [67]

h) Other techniques

Circular dichroism spectroscopy, dynamic light scattering, SDS-page are other techniques that are used to evaluate the protein aggregates.

1.4 Low molecular weight gelators (LMWG)

Low molecular weight gelators also termed as supramolecular gelators are molecules that have a molecular mass of less than 3000 Dalton and exhibit the ability to gel in water and organic solvents at relative low concentrations. The diverse growth of the low molecular weight gelators was initiated by its initial discovery in the 19th century, when scientists E. Goldmann and E. Barmann synthesised dibenzoyl-L- cysteine and found that it formed stable gel in solvents such as alcohol. From then on this became an intensively studied topic of research. Numerous applications of these molecules came into existence such as delivery of drugs, tissue engineering, electronics, sensors, and actuators.[82] These molecules may have further highly promising undiscovered applications e.g inhibition of protein/peptide aggregation. To explore their ability to inhibit protein and peptide aggregation, it is essential to

understand the mechanism of gelation and know the gelators reported in the literature. Thus in the following sections they are discussed.

1.4.1 Types of supramolecular gelators (LMWG) and mechanism of gel formation

Gelators that are used to create gels for drug delivery and tissue engineering applications can be categorised as described in the review by Skilling *et al* [83] and are summarised below:-

a) Gelators of organic solvents

   These are gelator molecules that form a stable gel in organic solvents. These includes derivatives of ALS (compounds consisting of an aromatic group A connected through functionalised linker L through a steroidal moiety S), fatty acid, steroids, sugars, amino acids, nucleosides and urea containing molecules.

b) Hydrogelators

   These are gelators that form a stable gel in water. These includes derivatives of amino acids and dipeptides, carbohydrates, nucleosides and gemini surfactants.[83,84]

The process of gel formation by all of these supramolecular gelators is relatively simple and similar. The process of gelation is initiated by the dissolution of the gelator molecule in a suitable solvent like water/organic solvent. Various triggering factors aided the dissolution process. The triggering factors included physical methods such as the modulation of temperature/ultrasound, ionic strength and chemical factors including pH changes and photochemical reactions. Among all these stimuli the most commonly employed method is temperature using a heating/cooling cycle where a solution is formed upon dissolution and on cooling a supersaturated solution then forms a gel. When this phenomenon is considered at the molecular level, it can be explained as a process which involves certain forces or interactions. A gel is formed by the influence of various non-covalent interactions between the gelator molecules. The most prominent ones are interactions such as π-π stacking, london dispersion forces, ionic interaction,

hydrogen bonding, Van der Waals forces, and solvophobic forces. The process of cooling of the dissolved gelator molecules leads to the formation of a primary nanostructure (one dimensional) by the self-assembly of gelator molecules. These self-assembled structures form fibres. These fibre structures then further entangled and cross-link to form a three dimensional network that can immobilise solvent molecules through capillary forces. Thus a gel is formed at the macroscopic level (Figure 1.2).[84-86]

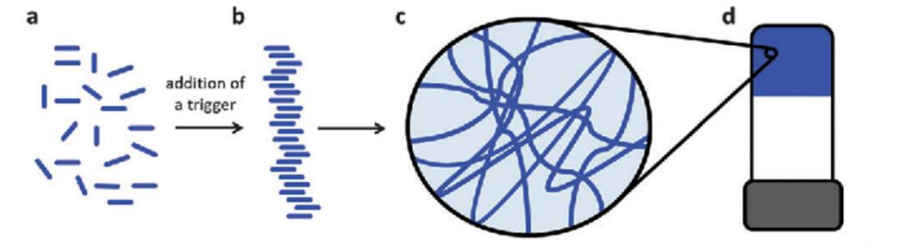

Figure 1.2: Schematic representation of gel formation which includes a) dissolution of LMWG in a solvent b) self- assembly of LMWG c) cross linking to form a 3D gel network d) gel at macroscopic level. Adapted from Reference[86]

1.4.2 Low molecular weight nucleoside gelators

There are a number of supramolecular gels derived from nucleobases. Presently this class of gelators are receiving considerate attention. [87]

All these nucleoside gelators possessed a nitrogen containing heterocyclic ring in its structure and are generally categorised as purine and pyrimidine containing gelators. The strong ability of these molecules to form intermolecular interactions between base pairs in water makes them an ideal candidates for the self-assembly process and gelation. The main intermolecular force that governs the self-assembly process is through non-covalent bonding. Some of these non-covalent bonds are formed by strong hydrogen bonding interactions that take place between the gelator molecules due to their bicyclic structure and the abundance of hydrogen donor and acceptor groups.[87] Similarly, the aromatic nature of these molecules leads to π-π stacking, an additional non-covalent interaction. These interactions can

result in heterodimer formation. Also, the availability of expanded surfaces for π-π stacking and edges for hydrogen bonding leads to the formation of homodimers. Both the formation of homodimer and heterodimer results in the evolution of the three dimensional network of fibres which constitutes the gel.

The first report of cytidine based gelator was by Andersen and co-workers. Their description of the gelator did not disclose the gelation mechanism or properties. [88] Further in 2010 David *et al* investigated the gelation properties of aryl substituted 5-triazolylcytidine.[89] From his studies he concluded that his synthesized molecule were able to form gels in water at a concentration of 0.3% w/v. In addition to this no other cytidine based gelators were reported except the ones synthesised by our group.

The focus of this thesis has been to study hydrogels formed from a nucleoside derivatised with a functional group that is hydrophobic in nature.[87] Many nucleoside based gelators has been developed similarly by derivatisation .For example, alkyl chains are added to the nucleoside to induce hydrophobicity. For example Moreau *et al* and his co-workers synthesized a uridine based gelator (uridine phosphocholine amphiphiles) by the addition of saturated and unsaturated acyl chains to the uridine molecule (Figure 1.3 A). This molecule formed a stable gel in water.[90]Seela *et al* reported a stable gel formation at low concentrations in 8-aza-7-deazaadenosine conjugate synthesized by the cycloaddition of benzyl azide to the nucleoside analogue.[91] Whilst Pan *et al* described the self-assembly process of amino nucleoside phospholipids which formed hydrogels at a concentration of 6% w/v. This gelator was found to bind to DNA molecules and thus has great potential in gene delivery.[92] A 2' deoxyadenosine gelator synthesised by the addition of octyl hydrocarbon chain to the nucleobase (Figure 1.3 C) exhibited molecular self-assembly in water using ultrasound as trigger factor.[93] Kim *et al* reported the synthesis of a nucleoside gelator with uracil base by modifying its structure with the addition of an alkylbenzyl triazole group (Figure 1.3 B). This compound formed a metastable gel at concentrations over 2.5% w/v.[94]. Skilling *et al* and colleagues have developed a cytidine based gelators by introducing acyl chains to the N4 position of the cytosine ring (Figure 1.3 D).[95].

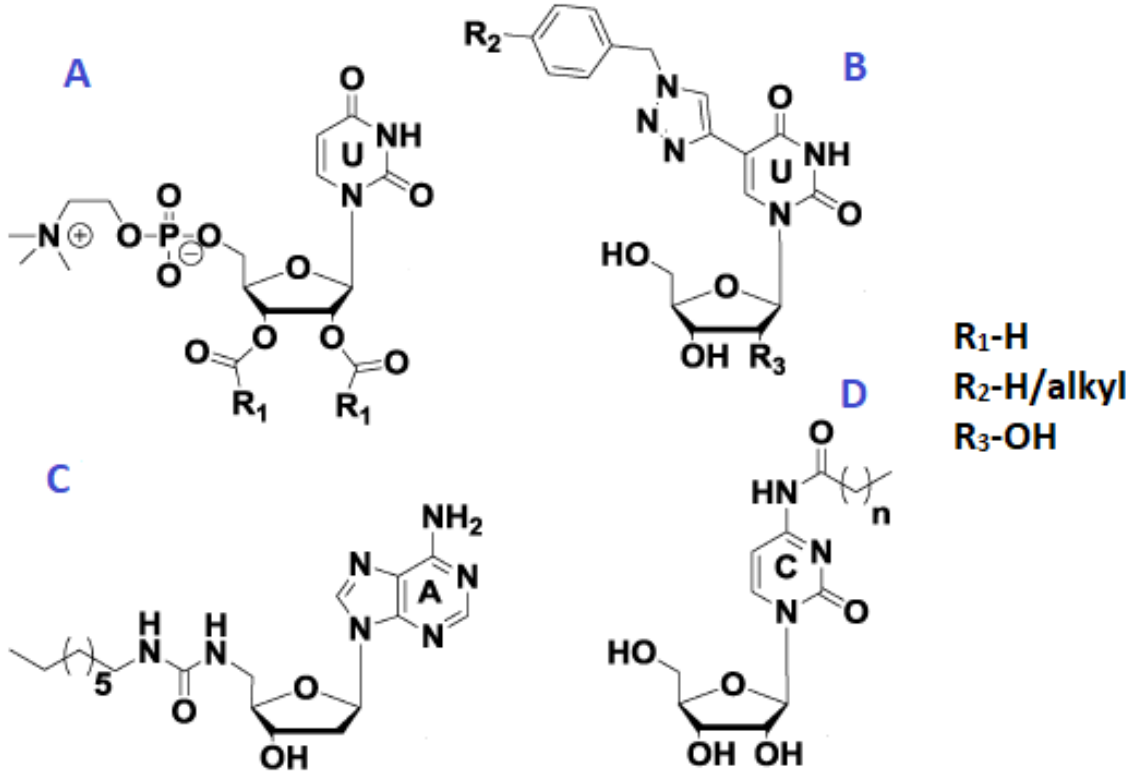

Figure 1.3: Examples of derivatised nucleoside gelators that form a stable hydrogels **A)** diacylated uridinophosphocholine derivative **B)** 2' deoxyadenosine and ribonucleoside derivative **C)** 2' deoxyadenosine derived gelator **D)** Cytidine based hydrogelator. Adapted from Reference[87].

These nucleoside gelators have found promising applications in various biological and biomedical fields. Some of these include drug delivery and tissue engineering as illustrated by the following examples. Sreenivaschary *et al* reported a hydrogel derived from guanosine (guanosine-5' hydrazide) with acyclovir, vitamin c or vancomycin incorporated in the three dimensional fibrillar network formed by the gel; which gave controlled drug release. [96] A supramolecular photoactivable hydrogel synthesised by the derivatization of guanosine nucleoside was found to exhibit good potential as an anticancer agent as it showed selective toxicity for cancerous cells.[97] Data for our group reported by Skilling *et al* demonstrates the suitability of a low molecular mass nucleoside gelator derived from cytidine for targeted intra-tumoural delivery. In addition to this we have synthesized nucleoside gelators derived from

gemcitabine and lamivudine and reported their potential applicability as a localised drug delivery systems.[98] The potential application of a guanosine based nucleoside for tissue engineering scaffold was established by Lauren *et al*. This gelator (8 methoxy 2', 3', 5' tri-o-acetyl guanosine) formed a stable gel in aqueous solution at relative low concentrations and in the presence of a salt.[99]

As described above, nucleoside based gelators have not been extensively explored for drug delivery applications and also the literature reports of cytidine derived gelators are scarce. Thus our group is investigating the inherent potential of cytidine based nucleoside gelators as drug delivery systems for protein and peptide delivery.

1.4.3 Low molecular weight gelator for protein and peptide delivery

The properties of low molecular weight gelators such as the inherent biocompatibility, proteolytic stability, and ease of injection makes them ideal candidates for the delivery of biologics. Currently, several research groups are focusing on designing hydrogels for protein delivery using supramolecular gelators.[100] Several successful studies have showed the potential of these gelator molecules for protein and peptide delivery. Nagai *et al* demonstrated that protein molecules can be incorporated into the self-assembled network of fibres formed by the gelation of a peptide gelator.[101] Also a peptide based nanofibrous hydrogel was found to be effective for delivering HIV DNA vaccines.[102] Similarly an injectable two layered peptide hydrogel system was described as an approach for delivering therapeutic antibodies.[103] A novel self-assembling peptide nanofiber hydrogel scaffold formulated from acetyl-(Arg-Ala-Asp-Ala)$_4$-CONH$_2$ [Ac-(RADA)$_4$-CONH$_2$] was also reported to be used as a carrier system for therapeutic proteins with different physical and chemical properties.[104] Further investigations lead to the development of a subcutaneous hydrogel drug delivery system for insulin from Puramatrix^{TM} (a peptide based gelator). This system delivered insulin in a controlled manner and increased the bioavailability thereby increasing the hypoglycaemic activity of insulin.[105] A self- assembling peptide developed by Guo *et al* also gave to

controlled release of vascular endothelial growth factor (VEGF). The self-assembled network formed by this gelator showed good capacity to incorporate proteins effectively.[106]

All these reported studies have established the utilisation of low molecular weight nucleoside gelator for the controlled delivery of macromolecules like proteins and peptides but none of these researchers have focused on the ability of these gelators to inhibit protein and peptide aggregation. Hence, for the first time we are investigating the ability of our novel supramolecular nucleoside gelator in inhibiting protein aggregation.

1.5 *N*4-octanoyl-2'-deoxycytidine as a platform for protein and peptide drug delivery

*N*4-octanoyl-2'-deoxycytidine is the supramolecular nucleoside gelator developed by our group (Marlow) in the University of Nottingham from the nucleoside cytidine (Figure 1.4).

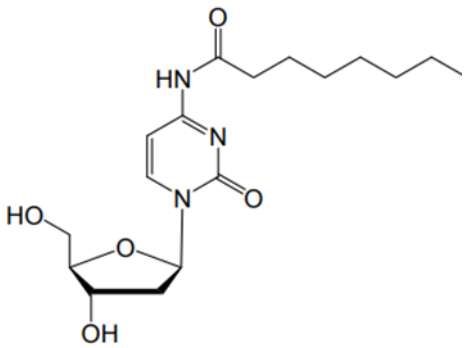

Figure 1.4: Structure of *N4-octanoyl-2'-deoxycytidine*

This gelator molecule was synthesized by the addition of an acyl chain at the 4N position of cytosine ring by the addition of a fatty acid. This molecule formed a stable gel in water and solvents such as ethanol at low concentration i.e. 0.15-5% w/v.

The mechanism of gel formation for this deoxycytidine gelator is similar to that of the low molecular weight gelators in general i.e. by a nucleation mechanism.

The gelator molecule self-assembly under the influence of non-covalent interactions lsuch as π-π stacking, hydrogen bonding, Van der Waals force, and solvophobic forces. The cytosine nucleobase of the gelator was the source for hydrogen donor and acceptor groups and thus initiated hydrogen bonding whereas the acyl chains introduced, brought hydrophobicity to the molecule and thus initiated solvophobic forces. [95] Further these interactions lead to the self-assembly of the gelator molecule into oligomeric structures which then formed a cylindrical structure i.e a fibre. This cylindrical structure consisted of aliphatic chains that faced the core and hydrophilic sugars which were directed towards outside (Figure 1.5). The formed cylindrical structure was further stabilized by hydrogen bonding.[107]

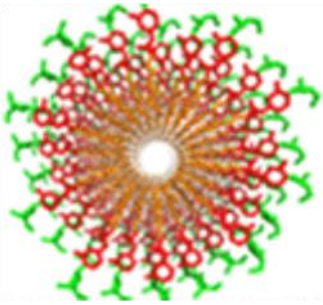

Figure 1.5: The structural arrangement of crosslinked fibre network of the gelator where the aliphatic chains (red) are facing the core and hydrophilic sugars (green) towards the external side. Adapted from Reference[107].

The *N*4-octanoyl-2'-deoxycytidine displayed certain properties that made it ideal for the design of an efficient drug delivery system for proteins and peptides. These included:

a) Excellent biodegradability and biocompatibility

   The hydrogel formed by this gelator has excellent biodegradability and biocompatibility due to its high water content and natural occurrence of the building blocks. These properties make it less toxic to the human body. The biocompatiblity of the gelator was assessed by performing *in vitro* cell toxicity studies. The results showed that the gelator and the cytidine parent compound showed very low and comparable GI_{50} values.[95]

b) Self-healing ability and injectability

The hydrogel also possesses the ability to reform independently after application of sheer stress which is termed as the self-healing ability, whereby the structure and the mechanical properties are recovered by the formation of new bonds.[108] Thus the hydrogel can be effectively injected through a syringe and then reform as a hydrogel depot *in situ*. This property of the hydrogel makes it an ideal candidate for the delivery of biologics.

c) Drug loading capacity and controlled release property

The hydrogel also exhibits the ability to load both small and high molecular weight compounds in the microscopic cavities of the fibrillar network. This was studied by Skilling *et al* where they incorporated both low molecular weight compounds (fluorescein) and high molecular compounds (FTIC labelled dextrans). In the release studies this high molecular weight compound incorporated showed controlled release with no change to the macrostructure of gel after 24 hours.[95,109] The kinetics of release was controlled by the increased nanofiber density[104] and this property enables it to be used as a depot.

d) Non-toxic nature

From the toxicity studies conducted on MCF-7 and HCT 116 cell lines. this gelator molecule was found to be non-toxic and showed no adverse effect on the viability and proliferation of cells.[109].

1.6 Aim and objectives

Aim and hypothesis

The overall aim of this work was to test the hypothesis whether the low molecular weight nucleoside gelator, *N*4-octanoyl-2'-deoxycytidine can inhibit aggregation of proteins and peptides.

Objectives

To achieve this aim the objectives of the thesis are as follows:-

1. To assess stability of the nucleoside based hydrogel alone and with proteins incorporated (lysozyme) using visual observations and LCMS and define a period of testing for peptide and protein aggregation studies (Chapter 3).
2. To evaluate whether the nucleoside based gelator can inhibit aggregation of a peptide- amyloid beta peptide using fluorescent microscopy (Chapter 4)
3. To evaluate whether the nucleoside based gelator can inhibit aggregation of two proteins (lysozyme and beta-lactoglobulin) using turbidimetry, optical microscopy, fluorescence spectroscopy and microscopy (Chapter 5).

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Glassware

Table 2: List of Glassware and its manufacturer

Glassware	Manufacturer
Clear glass vials 6 mm-2 ml	Sigma-Aldrich
96 flat costar flat transparent bottom well plates	Corning, NY 14831, USA
Microscope slides	Thermo Scientific
Cover glasses	Marienfeld GmbH and Co KG, Germany
96 Nunclon delta surface black flat bottom well plates	Thermo Fischer Scientific
Cell view cell culture slides- glass bottom	Greiner Bio-One Ltd, UK
HPLC glass vials 9 mm	Sigma-Aldrich
Screw cap 9 mm with white silicone septa with slits	Chromatography Direct
250µl conical glass insert 6 mm	Chromatography Direct

2.1.2 Gelator, proteins, peptide, reagents, chemicals and solvents

Table 3: List of raw materials with its manufacturer

Materials	Manufacturer and Lot Number
*N*4-octanoyl-2'-deoxycytidine	Synthesised as previously reported in our laboratory[109]
Amyloid beta peptide	Bachem Ltd, Lot:1074452, P Code:401447
Lysozyme from chicken egg white	Sigma-Aldrich, Lot: SLBT5160, P Code:1002453347
Beta-lactoglobulin from bovine milk ≥90%	Sigma-Aldrich, Lot: SLBP8394V, P Code: 1002486531

Dulbeccos phosphate buffered saline	Sigma-Aldrich, Lot: RNBG2431, P Code: D8537
Sodium hydroxide	VWR Chemicals, Lot: 13K210004, P Code:28244295
Monobasic sodium phosphate	Sigma-Aldrich, Lot: BCBS4419V, P Code:101048208
Sodium chloride ≥99.5%	Sigma-Aldrich, Lot: SZBB3550V, P Code: S9625
Hydrochloric acid	Sigma-Aldrich, Lot: SZBG0260V, P Code:30721
8-anilino-1-napthalene sulfonic acid	Sigma-Aldrich, Lot: SLBG0546V, P Code:1002134440
Milli Q water	18.2 MΩ·cm
Thioflavin T	Sigma-Aldrich, Lot: MKCF7580, P Code:1002631713
LCMS grade methanol	Chromasolv TM, Lot: 102414, P Code: 34966
Micrococcus lysodeikticus cells	Sigma-Aldrich, Lot: SLBR1182V, P Code:1002518405

2.2 EXPERIMENTAL METHODS

2.2.1 Stability study of gelator in hydrogels and hydrogels with protein

2.2.1.1 Formulation of hydrogels of lysozyme in sodium phosphate buffered saline (pH 7.4)

To formulate hydrogels, the required concentration (1% w/v) of the gelator-*N*4-octanoyl-2'-deoxycytidine (1% w/v) was accurately weighed into 2 ml glass vials and 450 µl of sodium phosphate buffered saline (pH 7.4) was added to these vials. The mixture was then heated to 90 ºC on a heated magnetic stirrer at 700 rpm until the gelator was completely dissolved (approximately 1 hr). For formulating hydrogels with lysozyme, 50 µl protein (lysozyme) stock solution was mixed into the solution at 37 ºC for 1 min. Finally, the solution was allowed to cool at room temperature for 10-15 min.

These samples were then stored in the following conditions for 1 month.

a) In refrigerated condition at 4 ºC.
b) At room temperature, 25 ºC.
c) In the stability oven at 37 ºC.

2.2.1.2 Visual observation of samples

The samples of gels and gels with proteins were visually compared to see if there was any difference in the physical appearance of the gel samples with time.

2.2.1.3 Preparation of working samples for LC-MS

The gel samples were dissolved in LC-MS grade methanol, vortexed for 2 min, filtered with 0.22 µm Millipore filter and diluted suitably in such a way that the gelator concentration was 28 µM. From the final dilutions, 200 µl of each samples were pipetted into glass inserts in HPLC vials.

2.2.1.4 LC-MS analysis

The stability samples were analysed by LC-MS. LC-MS was performed by using an Accela UHPLC system (Thermo Fischer Scientific) coupled to a high-resolution extractive orbital-trap mass spectrometer (Thermo Fischer Scientific). The chromatographic separation was done using a ZIC-pHILIC column (4.6 x 150 mm, 5 µm particle size) maintaining a temperature of 45 ºC and a flow rate of 300 µl/min. The mobile phase used was methanol and PBS (pH 7.4). The run time was about 15 min and the injection volume was 5 µl and samples were maintained at 4 ºC during the analysis. Mass spectrometry was performed in simultaneous ESI+ and ESI- full scan mode with a spray voltage of 40(ESI+) and 30 (ESI-). The data collected had a gain control of 1 x 10^6. Each measurements were carried out in triplicate with different samples and the data reported are the averages of these measurements.

2.2.2 Aggregation / fibrillisation study of amyloid beta peptide

2.2.2.1 Aggregation / fibrillisation of amyloid beta peptide

Aggregation in amyloid beta peptide was induced at neutral pH (pH 7.4) at 37 ºC.[110-112]

A stock solution of beta amyloid peptide ($A\beta_{1-42}$) was prepared by dissolving the lyophilised powder in Millipore water. This was then sonicated for 5 to 10 min in a bath sonicator. The solution was then filtered through a 0.22 µm Millipore filter to remove the undissolved peptide and impurities. The concentration of beta amyloid peptide was confirmed as 24 µM by the Nanodrop 2000C.

To prepare the aggregates of amyloid beta peptide, the stock solution was diluted with 10 mM sodium phosphate buffer (pH 7.4) to make the final concentration of peptide to 12 µM. This was then incubated at 37 ºC in a stability oven for 24 hours.

2.2.2.2 Formulation and evaluation of hydrogels of amyloid beta peptide

To formulate a hydrogel system of amyloid beta peptide, different concentrations of gelator (0.5% w/v, 1% w/v) were accurately weighed in 2 ml glass vials and 50 µl of sodium phosphate buffer (pH 7.4) was added to these vials. The mixture was then heated to 90 ºC with a heating magnetic stirrer at an rpm of 700 until the gelator had completely dissolved (approx. 1 hour). For formulating hydrogels with amyloid beta peptide, 50µl of peptide stock solution was added into the completely dissolved gelator solution after 1 minute by maintaining the temperature of 37 ºC. Finally the solution was allowed to cool at room temperature for 10-15 min.

The gel formation was confirmed by vial inversion test as previously reported.[113-115] Vial inversion test commonly termed as table top rheology technique is the most commonly used test to confirm gelation. In this method, the vial containing sample is turned upside down and the flow of sample is

checked. The sample which possesses a good yield stress i.e. a gel will not flow whereas ones which are viscous i.e. solution will flow.[116]

2.2.2.3 Evaluation of the aggregates/ fibrils of amyloid beta peptide in the solution and in the nucleoside based hydrogel

2.2.2.3.1 Thioflavin T binding assay

To evaluate the ability of the gelator to inhibit peptide aggregation, the aggregates in the samples have to be evaluated. To accomplish this, the aggregates were evaluated by thioflavin T assay as reported previously.[112,117-119] 90 µl samples of amyloid aggregates/fibrils were incubated for the required time were pipetted into a 96 well Nunclon delta surface black flat bottom well plates. A stock solution of thioflavin T was prepared by dissolving it in 10 mM sodium phosphate buffer (pH 7.4), sonicating in a bath sonicator and then filtering through a 0.45 µm Millipore filter. The filtered stock solution was diluted further and 10 µl of thioflavin T solution was added into the well plate containing samples in such a way that the final $A\beta_{1-42}$ and thioflavin T concentrations were 11 µM and 2.4 µM respectively. Similarly gel samples with peptide (G+P1, G+P2), gel samples without any peptide (G) and peptide samples in non-aggregated state (P) were pipetted into the well plates with a similar quantity of thioflavin T. The fluorescence intensity of the samples were measured after 1 hour using the TECAN spark 10M plate reader by exciting the samples at 440 nm and recording the emission at 482 nm. The measurements were carried out in triplicate and the average of the recorded values were calculated and then the background fluorescent measurement of the buffer subtracted.

2.2.3 Aggregation study of lysozyme and beta-lactoglobulin

To demonstrate the broad applicability of the gelator, further studies were carried out with the model proteins, lysozyme and beta-lactoglobulin.

2.2.3.1 Aggregation of lysozyme

Aggregation in lysozyme was induced by using alkaline pH (pH 12.2) at room temperature.[74,120-122]

100 mM sodium phosphate solution (pH 12.2) was prepared with sodium hydroxide and monobasic sodium phosphate. The prepared solutions were filtered using 0.45 µm Millipore filter to ensure the absence of any particles or impurities. Successively, fresh stock solution of 300 mg/ml lysozyme was prepared in Milli-Q water. To induce the aggregation process, 450 µl of filtered sodium phosphate solution, pH 12.2 was added to 50 µl of protein stock solution in such a way that the final solution contained 30 mg/ml (3% w/v) lysozyme in the solution. To compare the aggregation, 450 µl 0.01M phosphate buffered saline pH 7.4 (control) was added similarly to 50 µl of protein solution to give the same final protein concentration (3% w/v).

2.2.3.2 Aggregation of beta-lactoglobulin

Aggregation in beta-lactoglobulin was induced by using acidic pH (pH 4.6) at room temperature.[123,124]

The stock solution of beta-lactoglobulin (300 mg/ml) was prepared in 1M NaCl solution by stirring for about 2 hr on a magnetic stirrer. The solution was then filtered by using 0.45 µm filters. The pH of the solution was adjusted to 9 with 0.1M NaOH. To induce aggregation, the pH was brought down to 4.6 with 1M HCl. Thus the final solution contained 30 mg/ml (3% w/v) beta-lactoglobulin. All the solutions used were filtered prior to use. The aggregation of beta-lactoglobulin in pH 4.6 was compared with 50 µl of protein (5% w/v) in 450 µl phosphate buffered saline (pH 7.4)

2.2.3.3 Formulation of hydrogels

2.2.3.3.1 Formulation of hydrogels of lysozyme and beta-lactoglobulin in sodium phosphate buffered saline (pH 7.4)

Hydrogels of lysozyme and beta-lactoglobulin in sodium phosphate buffered saline was formulated as per the procedure described in section 2.2.1.1.1.The gel formation in the system was confirmed by vial inversion.

2.2.3.3.2 Formulation of hydrogels with and without lysozyme in sodium phosphate solution (pH 12.2)

Different concentrations of hydrogels (0.3-1.25% w/v) with and without lysozyme were prepared by a process similar to that in 2.2.3.1. The only change was in the solvent system. Instead of phosphate buffered saline (pH 7.4), sodium phosphate solution (pH 12.2) was used. All other conditions and methods were similar and for the hydrogel with protein, 50 µl lysozyme stock solution was mixed into the solution of gelator such that the protein concentration of 3% w/v was maintained. Again, gel formation was confirmed by vial inversion.

2.2.3.3.3 Formulation of hydrogels with and without beta-lactoglobulin in sodium chloride solution (pH 4.6)

Different concentrations of hydrogels (0.15-1% w/v) with and without beta-lactoglobulin were formulated by weighing the accurate quantity of gelator into glass vials and 450 µl 1M NaCl was added to these vials. The pH of the solution was adjusted to 9 with 0.1 M NaOH. The mixture was then heated to 90 ºC on a heating magnetic stirrer, set at 700 rpm, until the gelator had completely dissolved (approximately. 1 hr). For formulating hydrogels with beta-lactoglobulin, 50 µl protein stock solution was mixed into the gel at 37 ºC for 1 min. Finally, the gel was allowed to cool at room temperature for 10 min. After cooling, 1M HCl was added to bring down the pH to 4.6. The gel formation was confirmed by vial inversion.

2.2.3.4 Evaluation of the protein aggregates of lysozyme and beta-lactoglobulin

2.2.3.4.1 Visual observation

The presence of protein aggregates gives a white cloudiness to the solution. The cloudiness is clearly evident when the glass vial containing the aggregates are observed against a black background. Hence, different concentrations of gelator containing lysozyme and beta-lactoglobulin were visually observed and compared against appropriate controls to detect changes in protein aggregation.

2.2.3.4.2 Turbidimetry

100 µl solutions of protein aggregates (lysozyme and beta-lactoglobulin) in respective solution (pH 12.2, 4.6) and 100 µl of proteins formulated in different concentrations of gelator (0.3 to 1.25% w/v) were pipetted into 96 well Costar corning microplates. The UV-Vis absorbance of these samples were measured at 500 nm at 26 ºC, employing the absorbance detection function of Tecan Spark 10M microplate reader. The turbidity values of the sample and controls were compared to determine the gelator's ability to inhibit protein aggregation. To confirm reproducibility of the measurements they were carried out at least three times.

2.2.3.4.3 Optical/Light microscopy

Sample solutions (1 µl) of the protein aggregates and proteins in different concentrations of gelator (0.3-1.25% w/v) was pipetted onto a clean glass slide and this was then covered with a glass cover slip to prevent drying. The microscopic images of the samples were captured by utilizing the microscopic function of Zeta Profilometer (Zeta instruments, USA) at a magnification of 20X. The microscopic images of protein aggregates were then compared with the protein in different concentrations of gelator to determine the ability of gelator to inhibit protein aggregation.

2.2.3.4.4 ANS fluorescence spectroscopy

The hydrophobicity of aggregated protein was monitored by using the fluorescent probe 8-Anilino-1-napthalene sulfonic acid. The samples and controls (90 µl of each) were prepared in 96 well Nunclon microplates. The samples were gelator and protein at pH 12.2 and pH 7.4, whilst the controls used were sodium phosphate solution, protein solution, gelator solution, protein aggregates, gelator with prefomed protein aggregates at pH 12. A stock solution of ANS was prepared in water and 10 µl was added to each 90 µl of sample or control. The concentration of ANS was 10 molar in excess of that the protein. The fluorescence intensities were then measured after 5 hr using the fluorescence intensity scan detection function of Tecan Spark 10 M microplate reader. The excitation wavelength was set at 370 nm and the emission wavelength was scanned from 450 to 550 nm. The Z position was fixed at 17923. The fluorescence intensity at 476 nm for samples and controls were compared to evaluate the ability of gelator molecule to inhibit protein aggregation. To confirm reproducibility of the measurements they were carried out at least three times. The data were compared using one way ANOVA followed by post hoc Tukey's multiple comparison test.

2.2.3.4.5 Fluorescence Microscopy

The sample and controls (90 µl) were prepared in glass bottom cell view cell culture slides. The stock solution of ANS was prepared in water and 10 µl of this stock solution was added to each well of the slide containing the samples and controls. Concentration of ANS used was 10 molar in excess of that the protein. The images were captured using Zeiss Elyra PS1 confocal (780 scan head) microscope using the 63x water immersion objective lens. The samples were excited with 405 nm laser and the emission was collected at 446-553 nm. To analyse large areas, tile scan mode was applied, images were switched using Zen Black, and exported as tif files. Samples having only proteins in solution were scanned by finding the reflection of the laser on the coverslip and the focus was set 4-6 microns deep in the sample. The

microscopic images of the samples and controls were compared using the same settings on the histogram in Zen Black to draw the conclusions.

.2.2.3.5 Activity assay of protein

2.2.3.5.1 Lysozyme enzymatic activity assay

The activity of lysozyme in the hydrogel was confirmed by measuring the change/decrease in the optical density of the suspension of lyophilized *Micrococcus lysodeikticus* cells by turbidimetry. The cell suspension was prepared by adding 10 mg of lyophilized cells to 20 ml of phosphate buffered saline (pH 7.4). Hydrogels with proteins of concentration 1% w/v were prepared as described before in 2.2.3.1. The concentration of lysozyme was maintained at 3% w/v in both the gels. To 150 µl of the hydrogel samples containing lysozyme, 300 µl of the suspension of cells were added and the decrease in optical density was measured using Agilent UV-Vis spectrometer at the wavelength of 460 nm at different time intervals.
Similarly the gel without any protein and lysozyme without any gels were analysed as the control samples. The measurements were only carried out once as the results were in agreement with the results previously reported.

CHAPTER 3

STABILITY OF LOW MOLECULAR WEIGHT GELS

In this chapter, the stability of *N4*-octanoyl-2'-deoxycytidine (LMWG) with and without proteins are investigated to define the time frame for the evaluation of the inhibition of aggregation described in Chapters 4 and 5.

3.1 Introduction

Stability of a formulation assures the purity, potency and identity of a product.[125] A product is said to be stable if it remained within its physical, chemical and microbiological specification for a required time.[126] Before starting the aggregation experiments it was necessary to determine the time frame up to which the gel and gel with proteins are stable. Hence, we investigated the stability of supramolecular low molecular weight gels of *N*4-octanoyl-2'-deoxycytidine for a period of 1 month. The physical stability of samples were estimated by simple visual observation whereas the chemical stability was established by LC-MS techniques.

The storage conditions chosen for the study were 4 ºC where the protein formulations are commonly stored; 25 ºC which is nominally room temperature and 37 ºC which is the temperature for forced degradation. The first step in the LC-MS study involved the development and validation of a method to determine *N*4-octanoyl-2'-deoxycytidine in the gel samples.[127] The possible degradation products were used as the internal standards.
The stability studies were performed as described in experimental methods 2.2.1

3.2 Results and discussion

3.2.1 Visual observation of samples

The gel samples with and without proteins were visually compared after different time periods at different storage conditions to detect any physical change in the gel formulation with time. From the observations, it was clear that there was precipitation in all the gel samples when stored for more than 3 days. In case of gel samples (with and without proteins) stored at 37 ºC, precipitation was observed in the form of a large white solid mass which increased with time. Similarly samples stored at room temperature (25 ºC) showed the appearance of a large white solid mass which also increased with time but their appearance was less when compared to that of samples at 37 ºC. However the samples stored at 4 ºC showed the least appearance of this solid mass (Figure 3.1). This white solid mass formation may be due to the increased precipitation of gelator due to increase in temperatures. Xu *et al* has previously reported the phenomenon of precipitation of gelator molecules at high temperature in a peptide based hydrogel system.[128] High temperature storage (here 37 ºC and 25 ºC) may be causing the gelator to be in solution which further increases the intermolecular interactions leading to nucleation/crystallisation with time. This phenomenon results in precipitation of the gelator molecule in water as previously reported .[85,129]

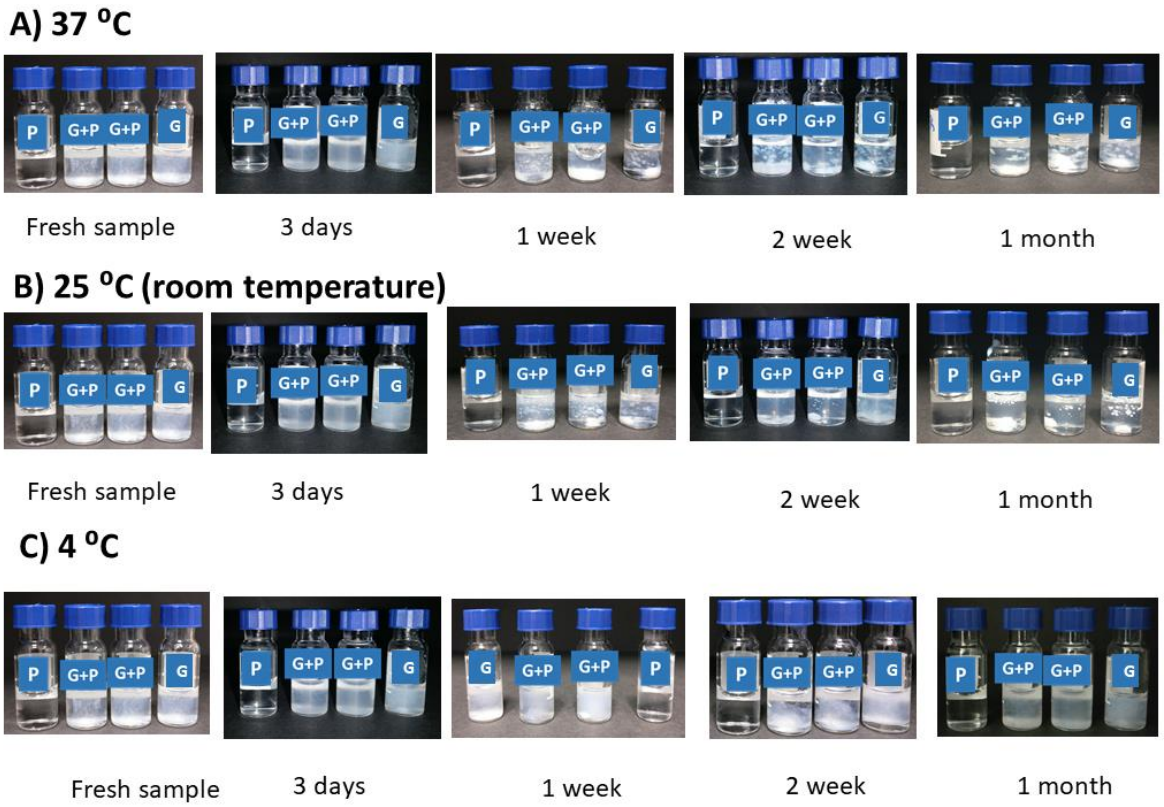

Figure 3.1: Visual observation of stability samples of gels -1% w/v (G), gel with protein (G+P), protein (P) stored at 3 different storage conditions A) 37 ºC, B) 25 ºC (room temperature) and C) 4 ºC for a time period of 0 days(fresh sample), 3 days, 1 week, 2 week and 1 month respectively. Fresh sample denotes the samples formulated on the same day.

3.2.2 LC-MS analysis

From the visual observations, we were able to observe that there was precipitation of gelator with time. Further we needed to confirm whether this precipitation was due to chemical degradation of the gelator. To test this chemical analysis was carried out by LC-MS.

From the results, it was observed that all the gel samples stored at different temperatures had at least 95% of the initial gelator concentration even after storing them for a period of 30 days. Thus, all the gel samples exhibited good chemical stability. The gelator with protein samples stored at 4 ºC and 25 ºC exhibited more than 95% gelator concentration whereas samples stored at 37

ºC showed a slight decrease in the amount of gelator i.e. the gelator concentration was about 84% (Figure 3.2A).

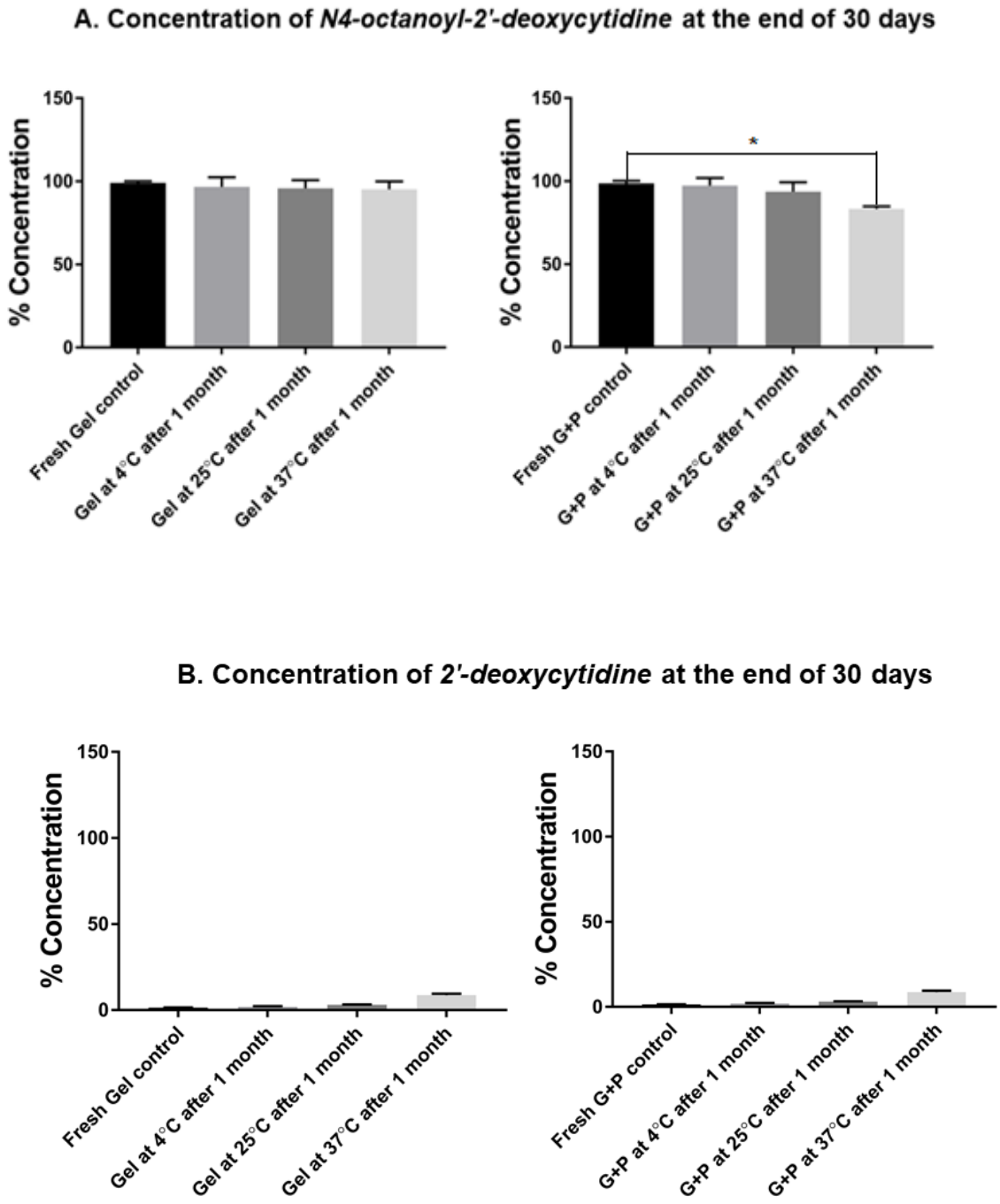

Figure 3.2: Bar graph showing the concentrations of **A)** gelator-*N*4-octanoyl-2'deoxycytidine **B)** degradation product -2'deoxycytidine in the samples of gel and gel with proteins (G+P) at the end of 30 days. Fresh gel control and fresh G+P denotes the gel and gel with protein samples formulated on the same day of analysis. * indicates significant difference with P-value of 0.0006 by one way

ANOVA followed by post hoc Tukey's multiple comparison test. Data shown are mean± SD, n is 3 in all cases.

To further ensure the stability of samples, possible degradation products of gelators were also analysed. The most prevalent degradation products were 2' deoxycytidine, cytosine, octanoic acid and 2-deoxy-D-ribose. The concentration of these degradation products in the samples were measured. From these results, it was observed that negligible quantities (nearly zero) of cytosine, octanoic acid and 2-deoxy-D-ribose were present in the sample of gels and gels with protein whereas trace amounts of 2' deoxycytidine were detected. The samples stored at 4 ºC and 25 ºC showed only a minor concentration of 2' deoxycytidine. The generation of these small concentration of 2' deoxycytidine may be due to the ionisation of gelator molecule occuring during the LC-MS technique. In case of samples (both gel and gel with protein) stored at 37 ºC the amount of 2' deoxycytidine was slightly higher than the other samples. This may be considered due to chemical degradation (Figure 3.2 B).

3.3 Conclusion

All samples exhibited high physical instability in the form of precipitates in all the storage conditions when stored for longer than 3 days. Thus, further studies were carried out by formulating hydrogels freshly and carrying out the evaluation within a 3 day time frame where the gel was found to be stable. The gelator in the hydrogel system and hydrogel system with proteins stored at 4 ºC, 25 ºC and 37 ºC may be considered chemically stable throughout the period of study as significant chemical degradations were not observed.

CHAPTER 4

PEPTIDE AGGREGATION INHIBITION BY LMWG

In this chapter, the ability of *N*4-octanoyl-2'-deoxycytidine (LMWG) to inhibit peptide aggregation is evaluated.

4.1 Introduction

The advancement of biotechnology has resulted in the development of protein and peptide therapeutics. Currently more than 140 peptide therapeutics have been discovered and these are receiving considerable attention among pharmaceutical scientists.[130] Their intrinsic properties such as an excellent pharmacological profile, and low production costs make them ideal as therapeutics. Despite this potential their poor chemical and physical instability and short circulatory half-life limits their applicability.[1] Among these, peptide aggregation is the most important instability that is considered seriously due to its impact on the product quality and efficacy.

The peptide aggregation generally termed as peptide fibrillisation involves the formation of dimers and small oligomers of peptide which further grow into protofibrils and fibrils.[131] This fibrils formed can accumulate in the human body in certain tissues or neurons and can stimulate the occurrence of several events like neurotoxicity, alteration in intracellular signalling pathway, abnormal calcium fluxes [132], pathogenic pores in the neurons [133,134], oxidative stress and inflammation. [135,136]

4.1.1 Amyloid beta peptide aggregation

Amyloid beta peptide is a 42-residue amyloid beta protein fragment predominately found in the brains of people with Alzheimer's disease and Down's syndrome. It has a molecular formula of $C_{203}H_{311}N_{55}O_{60}S$, molecular

weight of 4514.08 Daltons and isoelectric point of 4.73. Amyloid beta peptide was extensively reported to aggregate at several conditions such as neutral pH (pH 7.4) [112] and in Tris-HCl buffer[137].

In this study, we are using neutral pH (pH 7.4) for aggregating the amyloid peptide as it was the condition that is extensively reported. At this pH the native secondary structure (α-helix,β sheet structure) of peptide was found to be altered and there was formation of a cross beta sheet.[138] This conformational change leads to the formation of amyloid fibres (aggregates).[139] The fibrillisation process of amyloid beta peptide follows a nucleation dependant mechanism of aggregation and involves three phases i.e. lag phase, exponential growth (elongation) phase and stationary phase (Figure 4.1).[127,140] The initial stage is the lag phase where the nucleus formation is initiated.[141,142] The native amyloid peptide at pH 7.4 and on incubation at 37 ºC undergoes a conformational transition and forms structures such as dimers.[143] This acts as a nucleus and is considered as the rate-determining step in the fibrillisation process. This stage was followed by the accelerated fibrillary growth phase. In this phase the native monomeric proteins accrete to the nucleus and form higher ordered aggregates such as trimers, tetramers (oligomers) with time[144,145]. When a certain amount of monomeric peptides are absorbed, the growth phase slows down to a stationary or steady phase. This steady phase is characterised by the maturation of protofibrils[146,147] to mature amyloid fibrils and then fibrillar growth stops.[146,148,149]

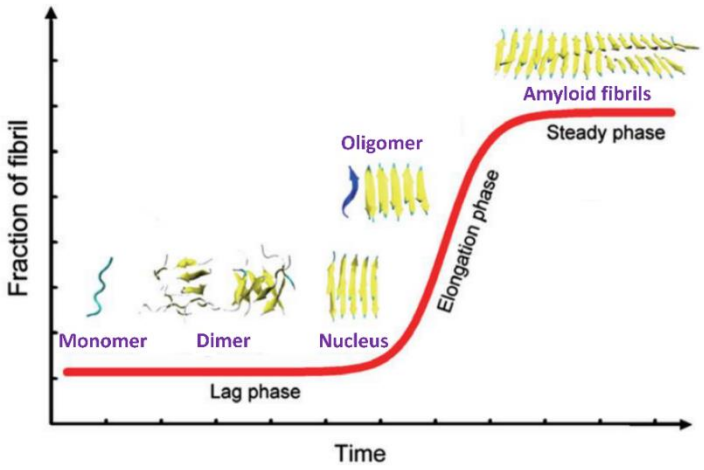

Figure 4.1: Illustration of the nucleation dependant aggregation of amyloid beta peptide at pH 7.4. Adapted from Reference[127]

Thus in this chapter, we investigated whether peptide aggregation could be prevented through weak non-covalent interactions between the peptide and the gelator in a hydrogel formulation. To investigate this hypothesis we chose the model peptide amyloid beta peptide, which is well known to aggregate at neutral pH.

To investigate inhibition of *in vitro* aggregation, the peptide aggregation was induced by incubating the peptide at 37 ºC in phosphate buffered saline (pH 7.4). A more detailed description of the aggregation of amyloid beta peptide, evaluation of the aggregates, formulation and evaluation of hydrogels of amyloid beta peptide can be found in the experimental methods described in 2.2.2 (Chapter 2).

4.2 Results and discussion

4.2.1 Aggregation/ fibrillisation of amyloid beta peptide

The aggregation of amyloid beta peptide was induced at neutral pH at 37 ºC.

4.2.2 Formulation and evaluation of hydrogels of amyloid beta peptide

Hydrogel systems of amyloid beta peptide was formulated with low concentrations of gelator at the pH where the amyloid beta tends to aggregate.

The formulation process involved the dissolution of the nucleoside gelator in phosphate buffered saline (pH 7.4) with the aid of gentle heat and allowed to cool at room temperature. During this process, the energy supplied in the form of heat overcomes the energy barriers for the aqueous dissolution of the gelator molecules and upon cooling, the gelator molecules self-assembles into a 3D network of entangled fibres.[150] Thus a stable hydrogel was formed. The main driving force for the self- assembly of gelator molecules included π-π stacking, Van der Waals force and other non-covalent interactions. These supramolecular fibrous structures formed entraped water molecules by capillary forces and the peptide molecules are considered to be entrapped in these fibrous structures.[95,109,151]

The ability of gelator molecule to form a stable hydrogel in the presence or absence of peptides were evaluated by vial inversion test. From the vial inversion test it was found that both the concentrations of gelator formed a stable gel in the absence of the peptide while with the peptide it formed a weak gel (Figure 4.2). The formation of a weak gel with peptide may be due to the alteration in molecular assembly and mechanical properties of the self-assembled gels by the peptide molecules. This phenomenon was previously reported by Nadeem *et al* for aromatic peptide hydrogels using two model proteins beta-lactoglobulin and bovine serum albumin.[152]

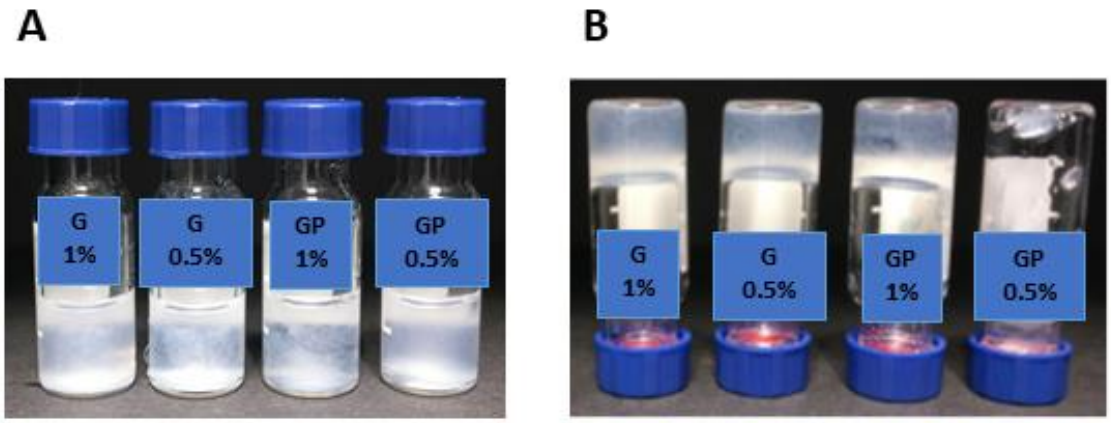

Figure 4.2: Hydrogels in the presence and absence of amyloid beta peptide **A)** Vials with and without peptide G 1% denotes hydrogel (1% w/v) without peptide, GP 1% denotes hydrogel (1% w/v) with peptide. Similarly for 0.5% w/v. **B)** Inverted vials of A

4.2.3 Evaluation of the aggregates/fibrils of amyloid beta peptide in the solution and in the nucleoside based hydrogel

Several spectroscopic techniques have been used to study the fibrillization of amyloid beta peptide. The main analysis technique used in our study was Thioflavin-T binding assay.

Thioflavin T fluorescence assay identifies and quantifies amyloid fibrils.[153] Thioflavin T is basically a benzothiazole salt. The addition of this fluorescent dye to samples containing amyloid fibrils leads to the emission of a strong fluorescent signal at excitation wavelength of 440 nm and emission wavelength of 482 nm.[154] However in the absence of fibrils it shows only a weak fluorescence. The intercalation of thioflavin T molecule within the channels of the amyloid fibrils is the main reason underlying the strong fluorescence signal.[155,156]

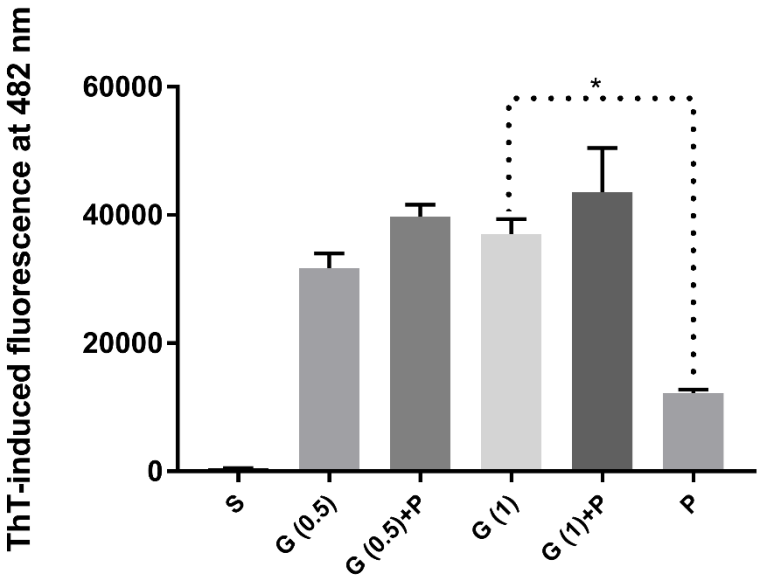

Figure 4.3. ThT fluorescence spectra of samples and controls of amyloid beta peptide (solution/ pH 7.4 PBS (S), gel (0.5% w/v) (G (0.5)), gel with peptide (0.5% w/v)(G (0.5) +P), gel (1% w/v) (G (1)), gel with peptide (1% w/v) (G(1) +P), peptide-beta amyloid peptide (P) excited at 440 nm and emission measured at 482 nm after 1 hour. * indicates significant difference with P-value of 0.0001 by one way ANOVA followed by post hoc Tukey's multiple comparison test. Data shown are mean ±SD, in all cases n is 3

From our experimental results, it was observed that the incubated samples of amyloid beta peptide (with amyloid fibrils) gave an average fluorescence intensity of 12227, whereas the gel samples (control) alone produced a strong fluorescent signal (about 31703 and 37014) for different concentrations (Figure 4.3). This fluorescence signal was 2-3 times higher than that of amyloid beta fibrils/aggregates and completely masked any detectable signal from the aggregates. The high fluorescence signal of the gel may be due to ThT associated with the hydrophobic core of the gel fibres.[107] Thus the use of this fluorescent dye was limited for our studies and unfortunately we were not able to conclude the role of gelator in the inhibition of aggregation of peptides by this method.

Further analysis by microscopic methods were also not feasible as the gel by itself has a fibre structure. Hence, we were not certain as to whether we could

differentiate the gel fibres from the amyloid fibrils. Thus the study was not progressed further.

4.3 Conclusion

Unfortunately, we were not able to get a clear conclusion whether the gelator possess the ability to inhibit peptide aggregation or not due to the limitations in the methods of characterization and evaluation of peptide fibrils/aggregates.

Thus, for further studies we decided to carry out our analysis on model proteins which were reported to aggregate rapidly.

CHAPTER 5

PROTEIN AGGREGATION INHIBITION BY A LMWG

In this chapter, the ability of *N*4-octanoyl-2'-deoxycytidine (LMWG) in inhibiting protein aggregation is evaluated.

5.1 Introduction

Protein pharmaceuticals are one of the most rapidly growing class of clinical therapeutics, where they have a significant therapeutic role in cancer, inflammatory diseases, neurological disorders and vaccines.[157,158] Despite their importance, the application of protein and peptide therapeutics is limited by the propensity of these molecules to form aggregates. Protein aggregation as discussed earlier in Chapter 2 is the process by which proteins form a complex consisting of two or more protein molecules, held together by non-covalent forces.[159] These aggregates can severely impact the biological activity of the protein, impairing the quality and safety of the formulation and hence reducing the efficacy of the therapeutic.[160] Hence, a key challenge in the biopharmaceutical industry is the development of a stable protein formulation that minimizes or prevents protein aggregation.[161]

As discussed earlier the main current strategies used to inhibit protein aggregation are the addition of solute excipients and/or co-solvents (stabilizers) such as glycerol [65], arginine hydrochloride [60], polysorbate 80 [57] and others to the protein solution.[162] While some stabilizers, such as glycine are well-known to stabilise antibodies[163], they have the limitation of destabilising other proteins such as myoglobin. Thus, the action of stabilisers often depends on the nature of the target protein and hence the choice of an effective stabiliser and selection of the stabiliser concentration for a specific protein can be an exhaustive process. [164,165] In addition, large amounts of

stabilisers can be required to obtain the desired effect and this may increase the cost of preparation or potentially lead to toxicity.
Thus, there is an urgent need for the development of novel methods to prevent protein aggregation which may be applicable to many proteins at low concentrations and which are also relatively economic, biocompatible and biodegradable.

5.1.2 Lysozyme aggregation

Lysozyme is an antimicrobial enzyme which is found in certain secretions of human body like tears, saliva, milk, mucus etc.[166] It is a thermostable enzyme with a melting point of 72 ºC and has an isoelectric point of 11.3.[167] It has a molecular weight of 14.3 kDa. It is a protein that shows high propensity for aggregation at several conditions such as alkaline pH, acidic pH, higher temperatures, different solvents e.g. ethanol, guanidine hydrochloride, different additives, and physical treatment (for example UV).[168]

In our studies, we used alkaline pH (pH 12.2) as the trigger for the aggregation of lysozyme. At alkaline pH, lysozyme was reported to form higher amorphous aggregates as a result of increased exposure of hydrophobic surfaces and negligible net charge.[169] The negligible net charge is due to the balance of positive and negative charges which reduces the repulsive forces and hence the attractive forces predominate.[170] The process of aggregation in lysozyme follows a polymerization type mechanism. The alkaline pH triggers the rapid unfolding of native lysozyme monomers. This results in the formation of exposed hydrophobic surfaces on the monomers i.e. the amide or carbonyl groups (which are the backbone of protein) are exposed on the surface. This process initiates polymerization wherein monomers with exposed surfaces form non covalent bonds with each other resulting in the formation of small oligomers. These oligomers grow larger by the further association of monomers and finally form stable amorphous aggregates. The non-covalent bond formed is further strengthened by intermolecular disulphide bonds. Thus unlike amyloid beta aggregation, lysozyme aggregation process has no lag phase or a rate limiting step.[171]

5.1.3 Beta-lactoglobulin aggregation

Beta-lactoglobulin is a highly structured protein present in bovine milk/bovine whey.[172] It is globular in nature [173] and has a molecular weight of 18.3 kDa. It is composed of 162 amino acid residue and has an isoelectric point of 5.1. Beta- lactoglobulin forms aggregates rapidly at several conditions such as acidic pH, high temperature, and the presence of additives, for example NaCl and guanidine hydrochloride.[174].
In this study we used acidic pH (pH 4.6) and NaCl to induce aggregation in beta-lactoglobulin.[123] The mechanism of aggregation of beta-lactoglobulin is similar to that of lysozyme aggregation. Here the aggregation is mediated by multifunctional polymerization. The aggregation process can be explained by two steps. In the first step, the monomers form dimers and these dimers further form aggregates of intermediate size by electrostatic interactions. This step is followed by association of the formed intermediates which leads to the formation of large amorphous aggregates. The salt additive assists the aggregation process by aiding the formation of clusters.[123]

Herein, we investigated whether protein aggregation could be prevented through weak non-covalent interactions between the protein and the gelator. To investigate this hypothesis we chose two model proteins, lysozyme and beta-lactoglobulin, that are well known to aggregate.

The aggregation of proteins, evaluation of the aggregates, formulation and evaluation of hydrogels of proteins were performed according to the experimental methods described in 2.2.3.

5.2 Results and discussion

5.2.1 Aggregation of lysozyme and beta-lactoglobulin

The aggregation of lysozyme was induced at alkaline pH, while aggregation in beta-lactoglobulin was induced at acidic pH at room temperature.

As discussed earlier in Chapter 1 protein aggregation cannot be categorized as a single step process, it involves several stages which results in the formation of several intermediates. [121] Proteins exhibit a strong tendency to aggregate at a pH close to its isoelectric point. The isoelectric points of lysozyme and beta-lactoglobulin are approximately 11 and 5.1. At or around this pH, the charges exhibited on the surface of protein become balanced. This balance in charges causes attraction forces to predominate causing aggregation and precipitation of proteins.[175] Thus on exposure to alkaline pH condition (pH 12.2), lysozyme forms amorphous aggregates. Similarly at acidic pH (pH 4.6), beta-lactoglobulin forms aggregates. These aggregates grow rapidly with time and this growth was reported to be directly dependant on the concentration of protein.[169]

5.2.2 Formulation and evaluation of hydrogels

Hydrogel systems of both the proteins were formulated with low concentrations of gelator at physiological pH and pH where the proteins tend to aggregate. The detailed procedure was described in 2.2.3.3. The mechanism of formation of hydrogels were discussed earlier in section 4.2.2 (Chapter 4).

Initially, the model proteins at the concentration of 3% w/v were incorporated into hydrogels prepared in phosphate buffered saline (pH 7.4) at 37 °C and then allowed to cool at room temperature. These hydrogels with lysozyme and beta-lactoglobulin at different concentrations of gelator in phosphate buffered saline are shown in Figure 5.1. The gelator formed viscoelastic gels stable to vial inversion at physiological pH in the presence of both proteins.

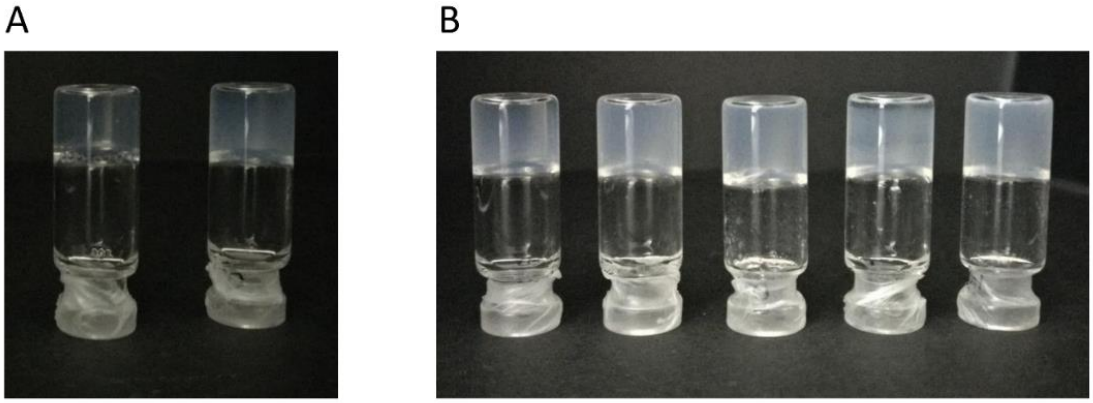

Figure 5.1: Vial inversion of hydrogel with protein at a concentration of 30 mg/ml in physiological pH 7.4 sodium phosphate buffered saline for **A)** beta-lactoglobulin with gelator concentration 0.15, 0.3% w/v (left to right) **B)** lysozyme with gelator concentration 0.3, 0.5, 0.8, 1, 1.25% w/v (left to right).

This was followed by formulation of hydrogel with/without proteins at the previously reported condition where both the proteins tend to aggregate i.e. 100 mM sodium phosphate solution pH 12.2 for lysozyme [121] and 1M sodium chloride pH 4.6 for beta-lactoglobulin.[123] Different concentration of gelators with and without lysozyme were prepared from concentrations ranging from 0.3%, 0.5%, 0.8%, to 1% w/v with 100 mM phosphate solution, pH 12.2. It was observed that at pH 12.2, the gelator did not gel as confirmed by the vial inversion (Figure 5.2). This was likely due to deprotonation of the acidic ribose hydroxyl groups which has been previously reported for nucleosides [176], converting the gelator into an ionized state and hence causing it to remain in solution.

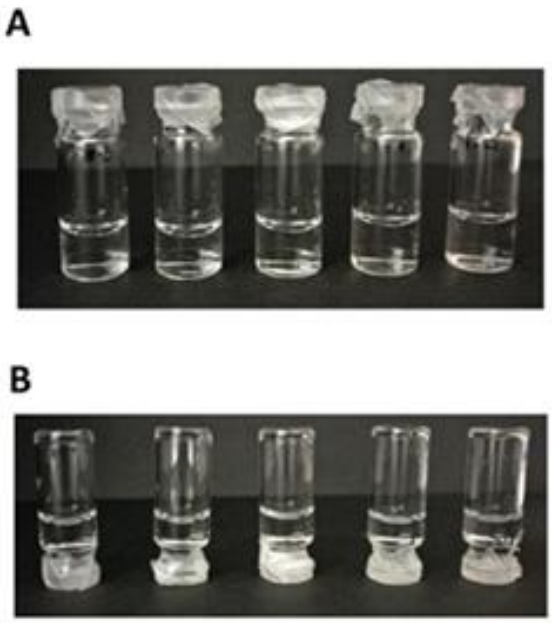

Figure 5.2: Visual observation of hydrogel in 100 mM pH 12.2 sodium phosphate solution (I = 0.7 M) at different gelator concentrations after mixing at 37 °C and allowing to cool at room temperature for 10 min. **A)** Nucleoside gelator without protein. Gelator concentration from left to right: 0.3%, 0.5%, 0.8%, 1.0% and 1.25% w/v **B)** Inverted vials of gelator (A)

Similarly, different concentration of gelators with and without beta-lactoglobulin were prepared from concentrations ranging from 0.15%, 0.3%, 0.4%, 0.8%, to 1% w/v with 1M sodium chloride, pH 4.6. All the concentrations of gelator formed a hydrogel stable to vial inversion (Figure 5.3 A) and with beta-lactoglobulin they formed a weak gel (Figure 5.3 B, D). It was evident that all these gels produced cloudiness and this was found to increase with increasing gelator concentration (Figure 5.3 A). This is considered to be due to the precipitation of gelator with pH variation.[177] The decreased solubility of gelator molecule in acidic pH condition could be the reason for precipitation.[178]

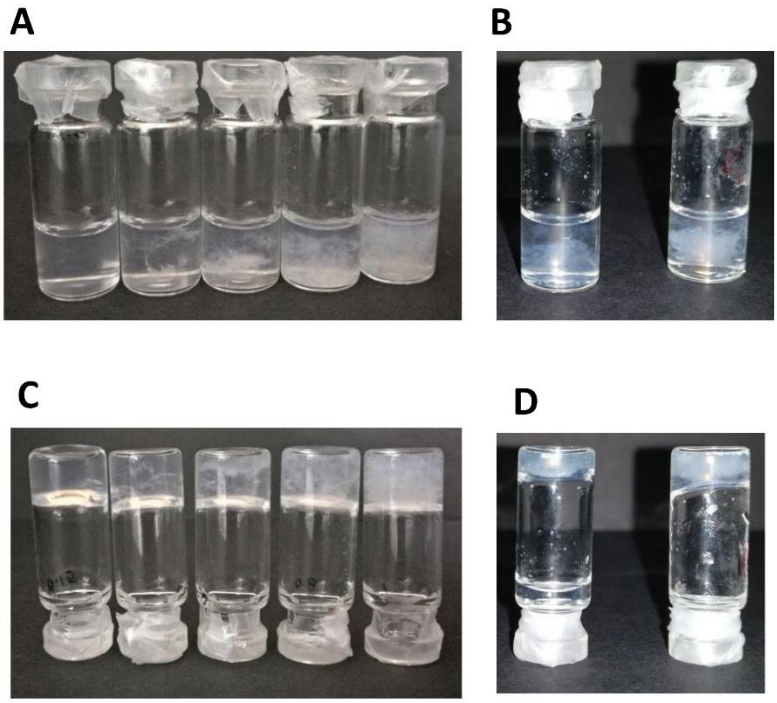

Figure 5.3: Visual observation of gelator only and gelator with 30mg/ml beta-lactoglobulin in 1M NaCl (pH-4.6, I = 2 M) at different gelator concentrations after mixing at 37 °C and allowing to cool at room temperature for 10 min. **A)** Nucleoside gel without beta-lactoglobulin. Gelator concentrations from left to right 0.15%, 0.3%, 0.5%, 0.8% and 1% w/v **B)** nucleoside gel with beta-lactoglobulin. Gelator concentrations from left to right 0.15, 0.3% w/v showing the intensity of cloudiness. **C)** Inverted vials of A **D)** Inverted vials of B

5.2.3 Evaluation of protein aggregates

5.2.3.1 Evaluation of protein aggregates of lysozyme

5.2.3.1.1 Visual observation

The lysozyme aggregates in the vials were large enough to be visualised by the naked eye and could be visually compared with lysozyme in the presence of different concentrations of gelator in the same buffer. It was observed that in the presence of 0.3% w/v gelator, the aggregation

induced cloudiness was reduced when compared to lysozyme in pH 12.2 solution alone. With further increases in the concentration of gelator the cloudiness further reduced and at concentration of 0.8% w/v, 1% w/v and 1.25% w/v there was no visible cloudiness (Figure 5.4).

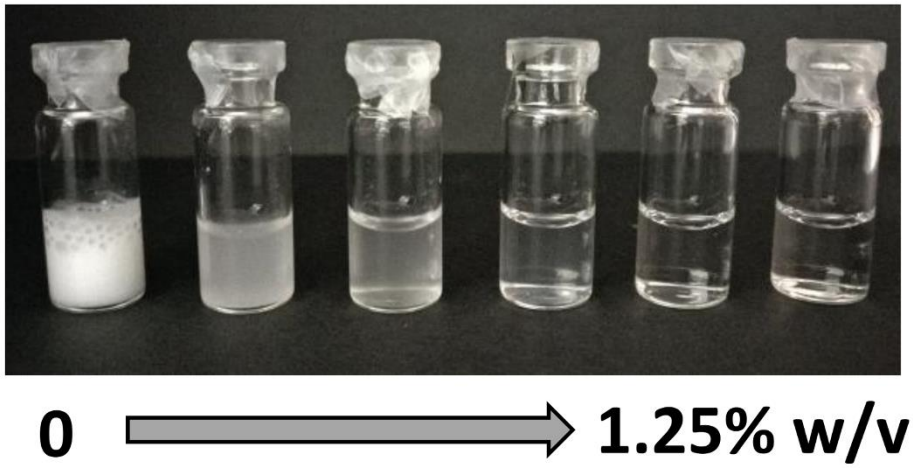

Figure 5.4: Photographs of the nucleoside gelator with lysozyme at different gelator concentrations (left to right 0, 0.3, 0.5, 0.8, 1.0 and 1.25% w/v) with 3% w/v lysozyme in 100 mM pH 12.2 sodium phosphate solution (I=0.7 M) after mixing at 37 ºC and allowing to cool at room temperature for 15 min.

5.2.3.1.2 Turbidimetry

The visual observations were confirmed analytically with turbidity measurements. The turbidity of the gelator solutions (control) and solutions of gelator with lysozyme were measured at 500 nm as previously reported .[179,180] The turbidity of lysozyme is an indicator of protein aggregation as the protein in physiological pH did not exhibit any turbidity (Figure 5.5 A). The gelator by itself showed very low levels of turbidity and hence it did not interfere with the turbidity measurement of aggregates. Indeed, it was found that for gelator samples with protein, increasing gelator concentration led to a decrease in the turbidity of the sample. At the highest gelator concentration (1.25% w/v), the turbidity of the sample was found to be nearly zero (Figure 5.5 B). This clearly

demonstrated the inhibition of protein aggregation in the presence of this nucleoside gelator.

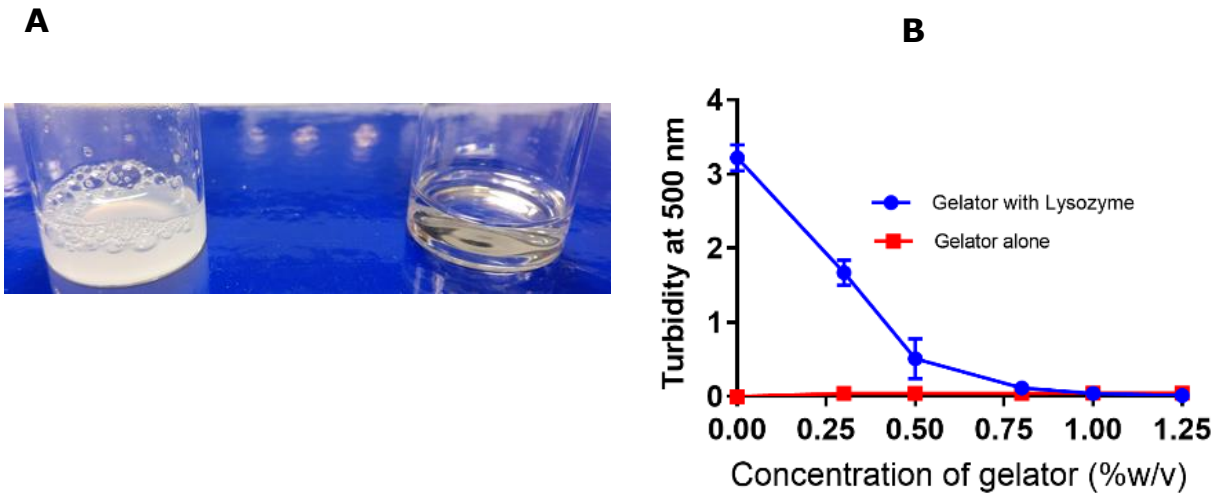

Figure 5.5. A) Visual observation of lysozyme in pH 12.2 sodium phosphate solution and in physiological pH (7.4) sodium phosphate buffered saline left to right. **B)** Turbidity measured at different gelator concentrations for samples containing gelator alone and gelator with 3% w/v lysozyme in 100 mM pH 12.2 sodium phosphate solution (I = 0.7 M) after mixing at 37 °C and allowing to cool at room temperature for 15 min. Data shown are mean ±SD, In all cases n is 3.

5.2.3.1.3. Optical/Light microscopy

The inhibition of lysozyme aggregation in the presence of the gelator was further confirmed by optical microscopy. Optical microscopy was favoured as the lysozyme aggregates were visible to the naked eye. This technique was previously used as an effective strategy for assessing protein aggregates in pharmaceutical formulations.[67,181] From the microscopy images, it was observed that the lysozyme aggregates without any gelator present showed densely packed spherical structures (Figure 5.6 A) measuring approximately 5-8.8 µm. These amorphous aggregates were found to be larger than the aggregates reported by Satish *et al.* using AFM and this is likely due to the

higher concentration of proteins (5.5 times more) used here.[121] These aggregates were also observed in the presence of 0.3%, 0.5% and 0.8% w/v gelator although their numbers were lower (Figure 5.6 B, C, and D). In contrast, in samples containing 1% and 1.25% w/v gelator concentrations, these structures were absent suggesting that lysozyme does not aggregate under these conditions (Figure 5.6 E and F).

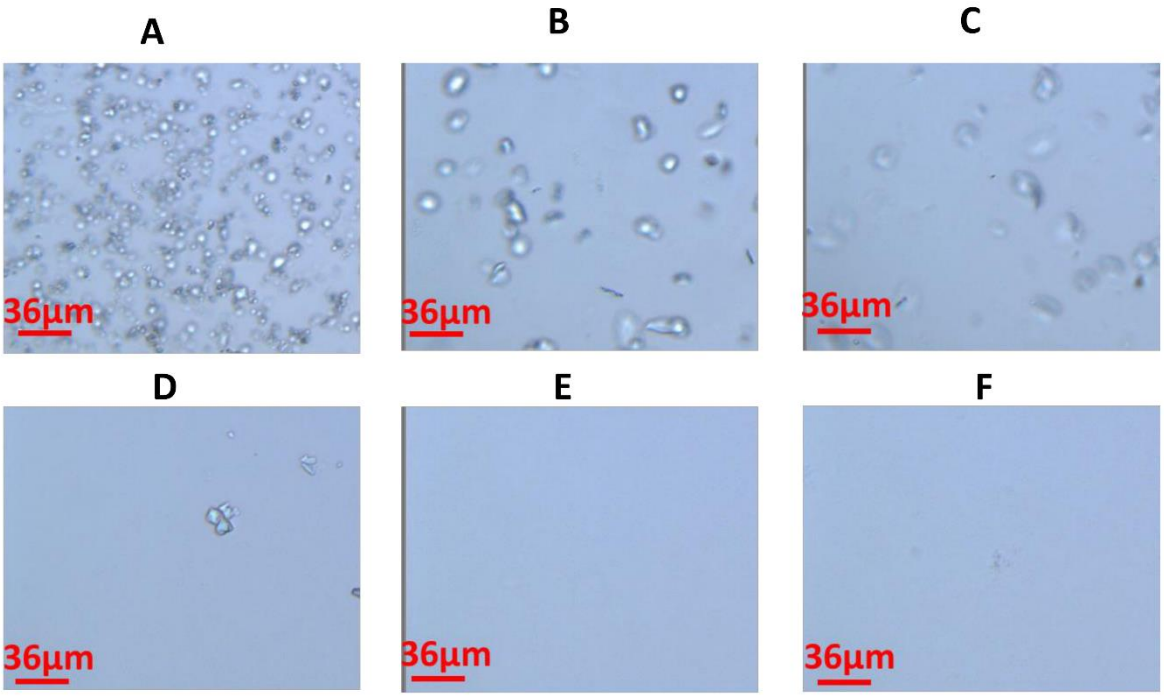

Figure 5.6: Optical microscopy of 30 mg/ml lysozyme aggregates and 30 mg/ml lysozyme in 100 mM pH 12.2 sodium phosphate solution (I = 0.7 M) at different gelator concentrations. Lysozyme **A)** without any gelator and with gelator **B)** 0.3% w/v **C)** 0.5% w/v **D)** 0.8% w/v **E)** 1% w/v **F)** 1.25% w/v.

5.2.3.1.4 ANS fluorescence spectroscopy

Extrinsic fluorescent dyes like ANS, thioflavin T were reported to be used for the characterization of protein aggregates due to their high sensitivity, ease of use and flexibility. [72,76] Characterisation of lysozyme aggregates by ANS spectroscopy was reported to be successful in previous studies.[74,121,182] Thus the ability of the gelator to decrease or inhibit protein aggregation was further explored using an ANS (8-anilino-1-napthalene sulfonic acid) binding assay using fluorescence spectroscopy and microscopy.

Upon protein aggregation, ANS is known to display an increase in its fluorescence intensity when bound to hydrophobic regions in the protein that become accessible upon aggregation.[121] The fluorescence intensity of the samples and controls were recorded at the emission wavelength of 476 nm, 5 hours after addition of ANS to allow the diffusion of ANS into the solution and its interaction with the aggregated protein. Protein samples (P) prepared at pH 12.2 showed increased fluorescence intensity, demonstrating that the assay was able to report the aggregation of the protein. A significantly lower fluorescence signal was observed for lysozyme in these conditions in the gelator solution (G) (Figure 5.7), consistent with a lower ANS binding sites for the fully folded proteins in the gelator solution. The significant difference in fluorescence intensity was demonstrated using an one way ANOVA followed by post hoc Tukey's multiple comparison test (P value less than 0.0001). The control samples, of solution (S) at pH 12.2 and 7.4 clearly indicate that the pH of the solution does not affect the ANS fluorescence. Further control samples of gels containing no proteins (G) and samples of gelator with preformed protein aggregates (G+A) confirmed the ability of the ANS to detect aggregated protein in the gel. This ANS fluorescence data is further confirmation of the ability of the gelator to inhibit protein aggregation.

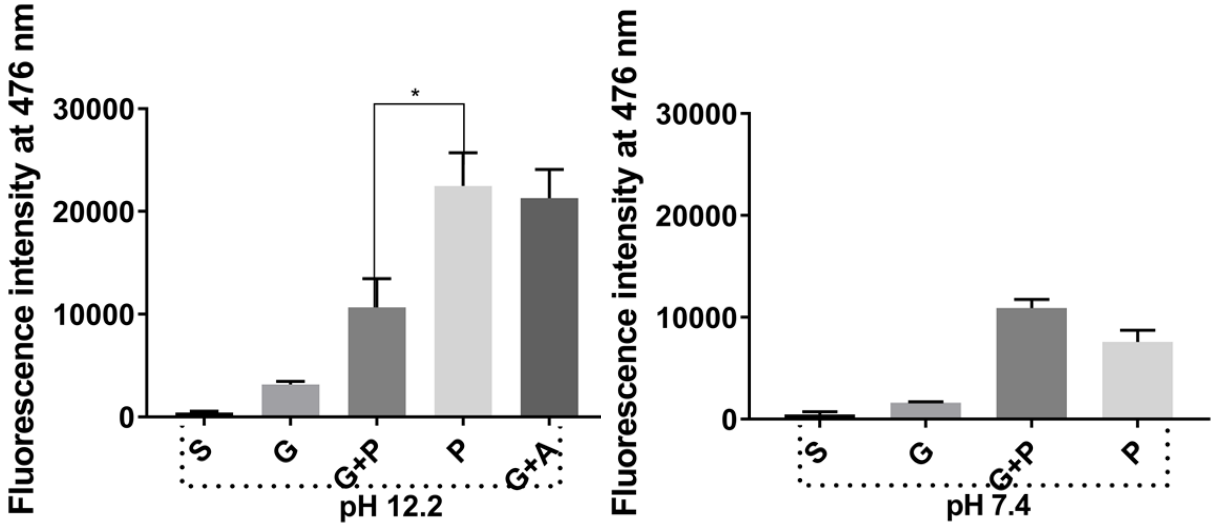

Figure 5.7: ANS Fluorescence spectra of samples and controls of lysozyme (solution(S), gelator (G), gelator with protein (G+P), protein (P), and gelator with aggregates (G+ A)) at pH 12.2 (aggregation inducing) and pH 7.4 (no aggregation), excited at 370 nm and emission measured at 476 nm after 5

hours. * indicates significant difference with P-value of 0.0001 by one way ANOVA followed by post hoc Tukey's multiple comparison test. Data shown are mean ±SD, in all cases n is 3.

5.2.3.1.5 Fluorescence microscopy

Fluorescence microscopy was the final analytical method used to confirm the protein aggregation inhibition by LMWG and this method complemented the spectroscopic analysis. In this technique, the samples of aggregates were visualized by staining with ANS. The exposed hydrophobic region of protein aggregates were bound to the dye and thus exhibited strong fluorescence and this fluorescence was detected by a fluorescence microscope. Thus this method gave a better visualization of the presence or absence of aggregates.[183]

The fluorescence microscopy images of the aggregates (P) showed the presence of small fibre-like structures, which were interconnected with each other and spread throughout the sample whereas the gelator (1.25% w/v) with lysozyme (G+P) showed the absence of these structures. In the control gelator sample (G) these structures were also absent (Figure 5.8). Further controls of protein at pH 7.4 (no fibre–like structures) and preformed aggregated protein mixed into the gel (presence of fibre-like structures) are shown in Figure 5.8. These images clearly demonstrate that the gelator formulated with the protein inhibited protein aggregation.

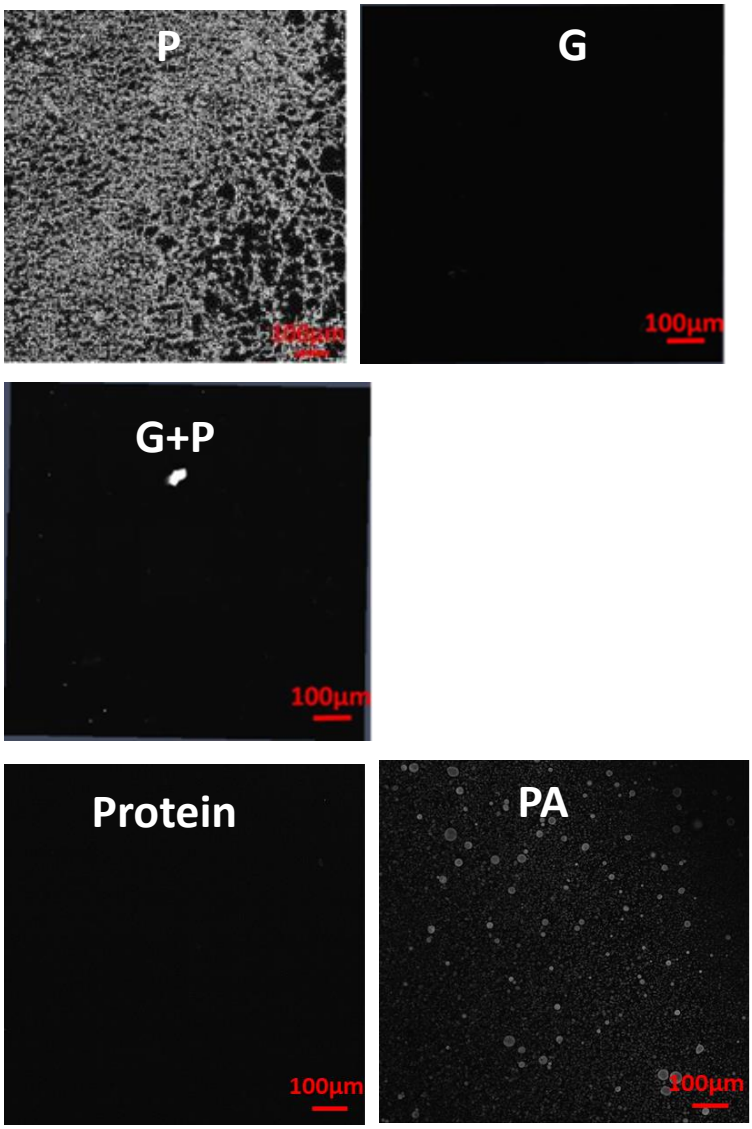

Figure 5.8: Fluorescence microscopy images of lysozyme (P), gelator solution (G) and gelator with lysozyme (G+P), 1.25% w/v gelator with preformed aggregates (PA) at pH 12.2 and lysozyme at physiological pH 7.4 in sodium phosphate buffered saline (Protein).

5.2.3.2 Evaluation of protein aggregates of beta-lactoglobulin

To further demonstrate the broad application of the gelator in inhibiting protein aggregation, we investigated the gelator's ability to inhibit aggregation at an acidic pH using beta-lactoglobulin.

5.2.3.2.1 Visual observation

To investigate protein aggregation in this system, we therefore compared cloudiness of the hydrogels and the hydrogels with protein at the same concentration of gelator. The 0.15% w/v gel with beta-lactoglobulin visually exhibited more cloudiness than the gel without protein and thus was thought to contain protein aggregates. For the 0.3% w/v sample, the cloudiness was found to be almost similar suggesting protein aggregation inhibition (Figure 5.9).

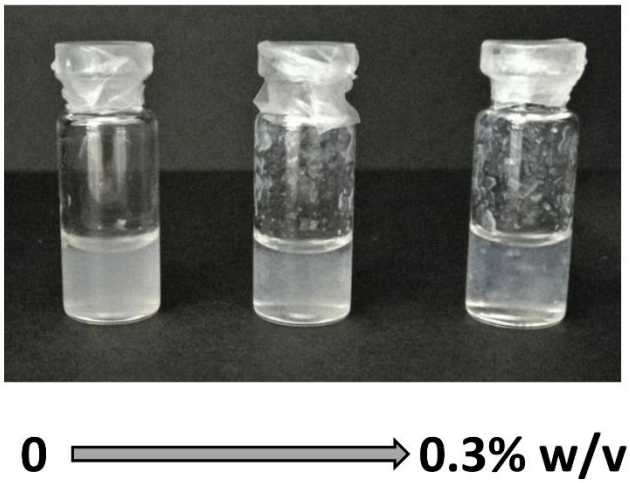

Figure 5.9: Photograph of nucleoside gelator with beta-lactoglobulin showing the intensity of cloudiness. Gel with 3% w/v beta-lactoglobulin in 1M NaCl at pH 4.6 (I = 2 M) at different gelator concentrations from left to right 0, 0.15 and 0.3% w/v after mixing at 37 °C and allowing to cool at room temperature for 15 min.

5.2.3.2.2 Turbidimetry

To confirm the visual observation, the turbidity of the gels with and without beta-lactoglobulin and aggregates without gelator were compared using a similar method to that reported Yan *et al*. [124] From the results it was observed that the turbidity of gels without beta-lactoglobulin increased with increasing gelator concentration (Figure 5.10). The turbidity of 0.15% w/v gelator sample with beta-lactoglobulin was significantly higher than that of the hydrogel

without proteins whereas the turbidity of 0.3% w/v hydrogel with and without the protein were similar. Protein containing samples with higher gelator concentrations were not evaluated as the hydrogel itself exhibited increasing turbidity values with higher gelator concentrations. These turbidity measurements were in agreement with the visual observations.

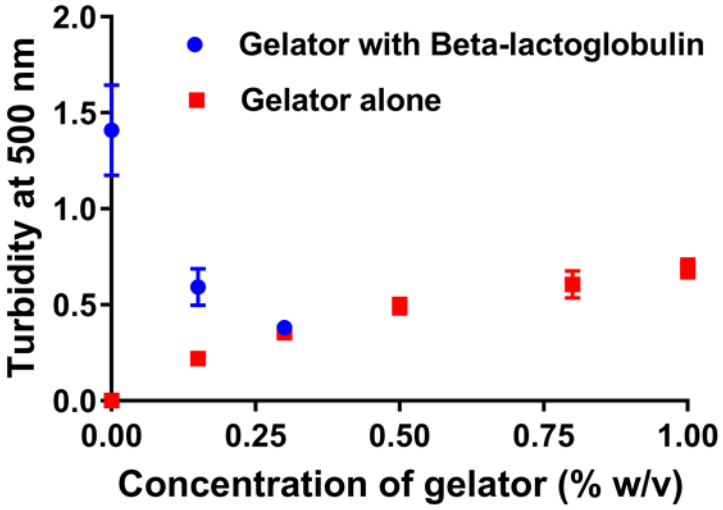

Figure 5.10: Plot of gelator concentration versus turbidity for gelator alone and gelator with 3% w/v beta-lactoglobulin in 1M NaCl at pH 4.6 (I = 2 M) at different gelator concentrations after mixing at 37 °C and allowing to cool at room temperature for 15 min. Data shown are mean± SD, in all cases n=3.

5.2.3.2.3 Optical/Light microscopy

To further validate our observations, optical microscopy was carried out. However, from these images, it was not possible to clearly distinguish between the protein aggregates and the gel structure as the gel itself exhibited structures similar in appearance to that of protein aggregates (Figure 5.11).

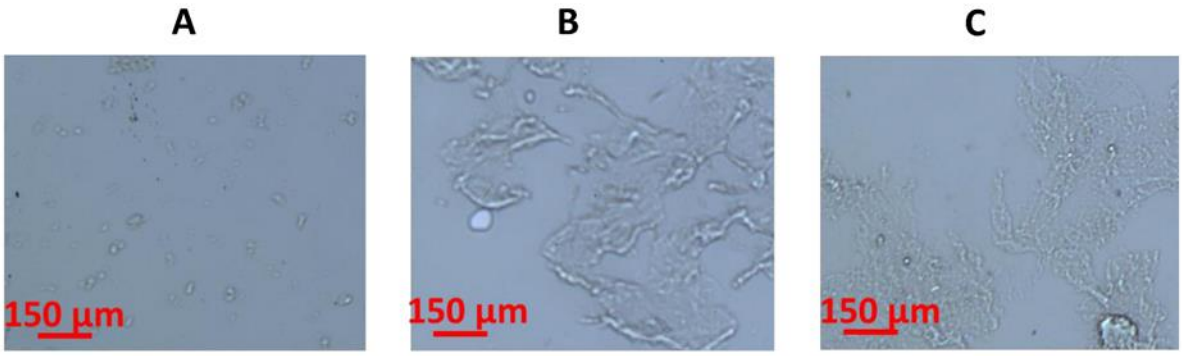

Figure 5.11: Optical microscopy images of beta-lactoglobulin aggregates at a concentration of 30 mg/ml in 1M NaCl (pH 4.6, I = 2 M) and in 0.3% w/v gelator concentrations. A) beta-lactoglobulin alone B) 0.3% w/v nucleoside gel C) beta-lactoglobulin in 0.3% w/v nucleoside gel.

5.2.3.2.4 ANS fluorescence spectroscopy

The hydrogel's ability to inhibit protein aggregation was further verified by an ANS binding assay and fluorescence microscopy in a procedure similar to that described for lysozyme. The results of ANS fluorescence binding assay revealed a remarkable decrease in fluorescence intensity of the hydrogel containing beta-lactoglobulin (G+P) when compared to the solutions of protein (P) only in the same conditions (Figure 5.12). It was also evident that neither the pH nor the presence of the gel induced ANS fluorescence as no significant fluorescence intensity was detected in the gel alone or the solution itself.

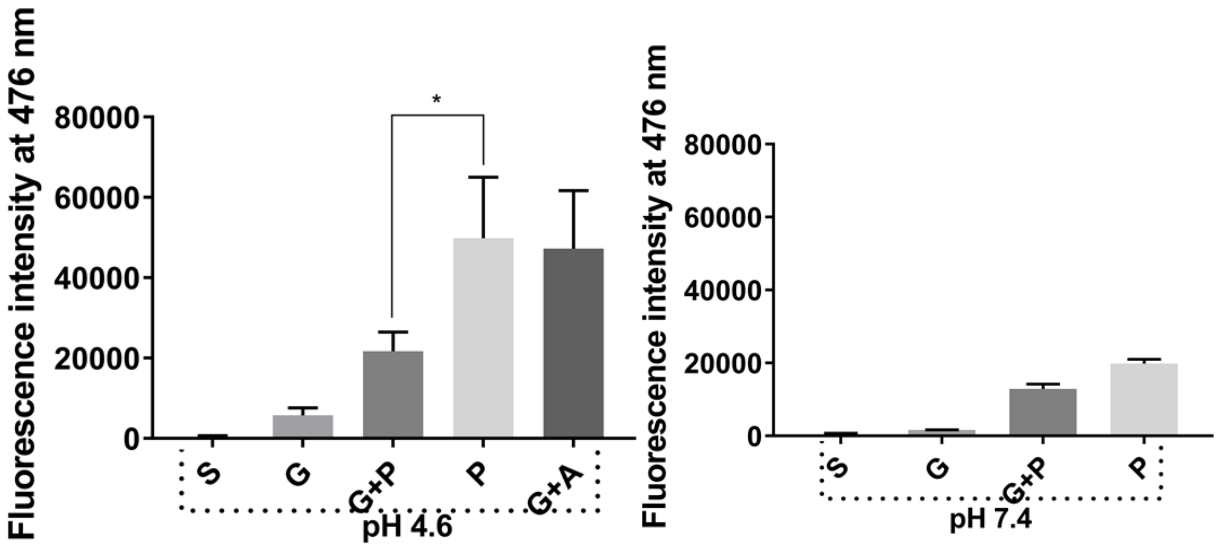

Figure 5.12: ANS Fluorescence spectra of samples and controls of beta-lactoglobulin (solution (S), gel (G), gel with protein (G+P), protein (P), and gel with aggregates (G+ A)} at pH 4.6 (aggregation inducing) and pH 7.4 (non-aggregation inducing), excited at 370 nm and emission measured at 476 nm after 5 hours. * indicates significant difference with P-value of 0.0001 by one way ANOVA followed by post hoc Tukey's multiple comparison test. Data shown are mean± SD, in all cases n=3.

5.2.3.2.5 Fluoroscence microscopy

The fluorescence microscopy images of the protein in the presence of hydrogel (G+P) did not exhibit any fibre-like interconnected structures such as those observed for the aggregated protein (P) (Figure 5.13). Further controls of protein at pH 7.4 (no fibre –like structures) and preformed aggregated protein mixed into the gel (presence of fibre-like structures) are shown in Figure 5.13. These data further proved the hypothesis that the gelator has the strong ability to inhibit protein aggregation.

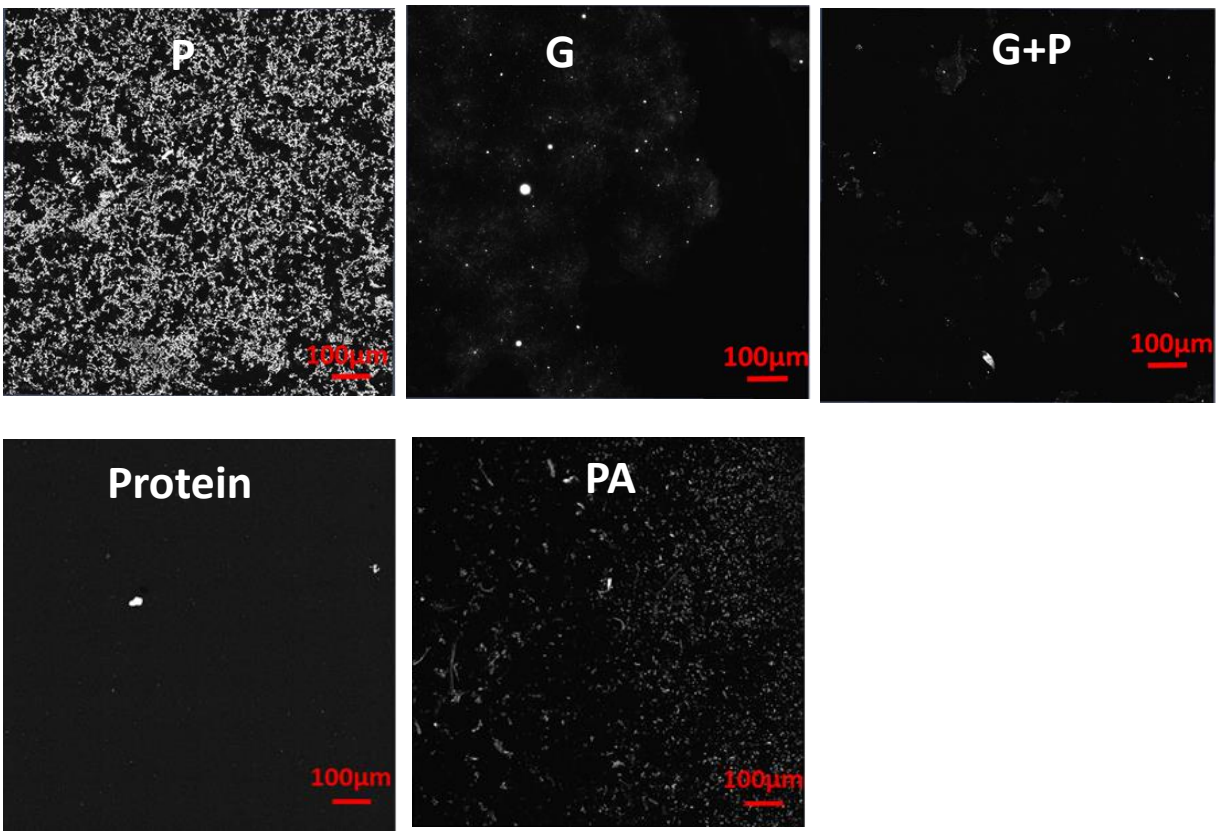

Figure 5.13: Fluorescence microscopy images of beta-lactoglobulin (P), gel (G), gel with beta-lactoglobulin (G+ P), protein and gel with preformed aggregates (PA) at pH 4.6.

5.2.4 Activity assay of proteins

Since we do not have a mechanistic understanding of the gelators role in protein aggregation inhibition, there was a need to analyse whether the proteins are functional in the hydrogel. For assessing this, we checked the activity of lysozyme in the hydrogel by a lysozyme enzymatic assay as previously reported.[11,180]

5.2.4.1 Lysozyme enzymatic activity assay

The enzymatic activity of the lysozyme incorporated in the hydrogel system was assessed by using *Micrococcus lysodeikticus* cells. The lysozyme exerted

the unique ability to cleave of the glycosidic bonds between the components of this bacterial cell wall. Any degradation in the bacterial cell wall was measured by a decrease in the turbidity of cell suspension (decrease in absorbance) at 460 nm.[180]

From the results, it was observed that the absorbance of hydrogel samples without protein showed a stable absorbance throughout the duration of study whereas the hydrogel sample with lysozyme showed a reduction in absorbance with time. A similar trend was observed for control samples i.e. free lysozyme. Thus from the data it was concluded that the proteins in the hydrogel system were in their functional state.

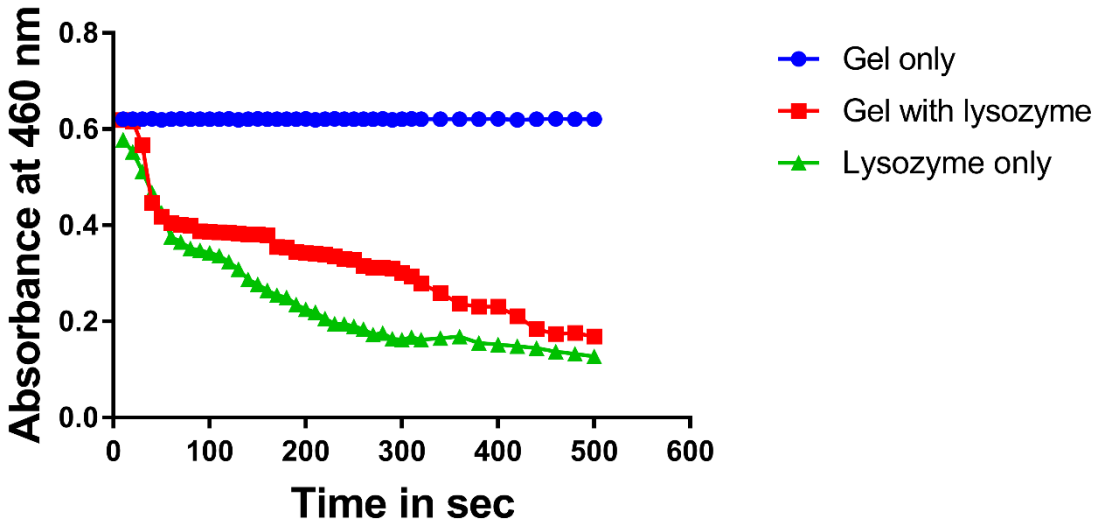

Figure 5.14: Lysozyme activity assay in the presence of gel alone, gel with protein and protein alone with *Micrococcus lysodeikticus* cells with 3% w/v lysozyme and 1% w/v of gelator at the absorbance wavelength of 460 nm at pH 7.4.

5.3 Conclusion

Based on our experimental results we were able to conclude that the supramolecular 2'-deoxycytidine based gelator exhibited the ability to suppress the aggregation of proteins with a broad range of isoelectric points

(alkaline and acidic). The results of activity assay of proteins also showed that the proteins are in their functional state inside the hydrogel system.

To our knowledge, this is the first report of using a low molecular weight gelator for the inhibition of protein aggregation. Since, this 2'-deoxycytidine based hydrogel is only is stable for 3 days, the potential using in this gelator for a commercial product is limited However, this work proves the potential of LMWG to be used for the formulation of proteins in preventing protein aggregation.

CHAPTER 6

GENERAL DISCUSSION, CONCLUSION AND FUTURE PROSPECTIVE

6.1 General discussion

6.1.1 Background

Protein and peptide therapeutics possess several advantages over other drugs which includes their high specificity and low toxicity.[184,185] Despite these advantages their application is limited due to their high propensity to form aggregates or fibrils. This reduces the therapeutic efficacy of biologicals and impairs the quality and safety of the formulations.[160] Many excipients have been used extensively to suppress aggregation, however the limitations associated with them (discussed in chapter 1) prevent their use.[162,163] Thus, there is a necessity for the development of novel methods that can inhibit protein aggregation in a wide range of proteins at low concentration and in a relative economic way. Herein, we have evaluated the ability of a novel supramolecular nucleoside gelator as a hydrogel formulation to prevent protein aggregation to address the limitations of excipients used currently.

LMWGs, particularly the peptide based molecules, have been evaluated as platforms for protein delivery but not as an approach for the prevention of protein aggregation.[100,186] Li *et al* in their review have discussed the principles of designing peptide based low molecular weight hydrogels for delivery of proteins. Moreover, they described most recent efforts in utilizing these gelators for protein delivery using, for example, a peptide based nanofibrous hydrogel[102] and an injectable two layered peptide hydrogel system[103]

These LMWGs form hydrogels by self-assembly into a cross-linked network of nanofibres capable of retaining large amounts of water and thus form a 3 dimensional structure. The self-assembly process involves the formation of

micelle-like aggregates through solvophobic forces. These structures further rearrange, forming cylindrical fibres which are then stabilized by hydrogen bonding.[107] Some unique properties of these hydrogels such as the high water content, soft and porous nature, and biocompatibility made them ideal reservoirs for large quantities of water-soluble molecules such as peptides and proteins. [109,187]

In the study, we investigated whether the novel nucleoside based LMWG, *N*4-octanoyl-2'-deoxycytidine could prevent protein aggregation through weak non-covalent interactions between the protein and the gelator. To investigate this hypothesis we chose a model peptide, amyloid beta and two model proteins, lysozyme and beta-lactoglobulin that are well known to aggregate. The gelator molecule used was previously developed by our group as an injectable, self-healing hydrogel.[109] This hydrogel system possesses the ability to shear thin in response to a stress, and then reforms its structure (mechanical properties) when the stress is removed by the formation of new non-covalent interactions.[108] Thus the hydrogel can be effectively injected through a syringe and later it can form a gel. This property of the hydrogel makes it an ideal candidate for the delivery of biologics.

6.1.2 Findings of the study

The initial stability study of the 2' deoxycytidine gelator in the hydrogel system clearly demonstrated the physical instability (in the form of precipitates) of the gelator with time. However, the chemical degradation studies by LC-MS revealed that the gelator is physically and chemically stable only for a period of 1-3 days and thus the ability to inhibit protein aggregation can only be evaluated within a 3 day time frame.

Based on this, model peptide and proteins were incorporated into hydrogels prepared in respective buffers at 37 °C, allowed to cool at room temperature and analysed within 3 days. In our study, aggregation was facilitated by shifting the solution pH to the proteins isoelectric point, where the charge balance causes attractive forces between proteins to be predominant.[175]

Firstly, we assessed the ability of the nucleoside hydrogels to inhibit protein aggregation of amyloid beta peptide at pH 7.4. We used various evaluation techniques such as a fluorescence spectroscopic assay but unfortunately we were not able to get a clear picture of aggregation inhibition due to the limitations in the methods of characterisation of peptide aggregates. Moreover we found that the reported aggregation condition were not that efficient in inducing aggregation.

We further extended our studies to model proteins. Initial experiments were carried out with lysozyme that aggregated at a pH of 12.2. The preliminary evaluation criteria was by visual observation. The aggregation of proteins induced cloudiness in the solution and this was found to decrease with increasing concentration of gelator (from 0.3% w/v to 0.8% w/v). A further increase in concentration showed negligible cloudiness. The visual observations were confirmed analytically with turbidity measurements. From the turbidity measurements, it was found that for gelator samples with proteins, increasing gelator concentration led to a decrease in the turbidity of the sample. At the highest gelator concentration (1.25% w/v), the turbidity of the sample was found to be nearly zero. This clearly demonstrated the inhibition of protein aggregation in the presence of this nucleoside gelator. From the microscopic images, it was observed that the lysozyme aggregates without any gelator present showed densely packed spherical structures. These structures were also observed in the presence of 0.3%, 0.5% and 0.8% w/v gelator although their numbers were lower. In contrast, in samples containing 1% and 1.25% w/v gelator concentrations, these structures were absent suggesting that lysozyme does not aggregate under these conditions. The ability of the gelator to inhibit protein aggregation was further explored using an ANS (8-anilino-1-napthalene sulfonic acid) binding assay using fluorescence spectroscopy and microscopy. Protein samples prepared in the aggregating condition showed increased fluorescence intensity. A significantly lower fluorescence signal was observed for lysozyme in these conditions in the gelator solution. This suggests that there is less ANS binding to the fully folded proteins in the gelator solution. The fluorescence microscopy images of the aggregates showed the presence of small fibre-like structures which were

interconnected with each other and spread throughout the sample whereas the gelator (1.25% w/v) with lysozyme showed the absence of these structures. These images clearly demonstrated that the gelator formulated with the protein inhibited protein aggregation.

Hence, to further demonstrate the broad application of the gelator in inhibiting protein aggregation, we investigated the gelator's ability to inhibit aggregation at an acidic pH using another protein beta-lactoglobulin. The aggregation of beta-lactoglobulin was studied at pH 4.6. From the visual observation, it was evident that all the gels produced cloudiness at this pH and this was found to increase with increasing gelator concentration. Thus the cloudiness of hydrogel was compared with hydrogel with protein at the same concentration. From this observation, it was found that the 0.15% w/v gel with beta-lactoglobulin visually exhibited more cloudiness than the gel without protein and thus was thought to contain protein aggregates. However, for the 0.3% w/v sample, the cloudiness was found to be almost similar suggesting protein aggregation inhibition. To confirm the visual observation, the turbidity of the samples were measured. The gel by itself showed increased turbidity with increasing concentration. Thus the gels with and without beta-lactoglobulin and aggregates without gelator were compared. The turbidity of 0.15% w/v gelator sample with beta-lactoglobulin was found to be significantly higher than that of the hydrogel without proteins whereas the turbidity of 0.3% w/v hydrogel with and without the protein were similar. To further validate our observations, optical microscopy was carried out. However, from these images, it was not possible to clearly distinguish between the protein aggregates and the gel structure; since the gel itself exhibited structures similar in appearance to that of protein aggregates .The hydrogel's capability to inhibit protein aggregation was further verified using the ANS binding assay and fluorescence microscopy. The results of ANS fluorescence binding assay revealed a remarkable decrease in fluorescence intensity of the hydrogel containing beta-lactoglobulin when compared to the solutions of protein only in the same conditions. The fluorescence microscopy images of the protein in the presence of hydrogel did not exhibit any fibre-like interconnected structures such as those observed for the aggregated protein. These data further proved the

hypothesis that the gelator has the strong ability to inhibit protein aggregation. The mechanism of inhibition of aggregation may be through the physical entrapment of the protein molecules within the fibrous network or by the interaction of gelator molecule with the protein by forming non covalent bonds.

6.1.3 Limitation of the study

In this study, we were not able to use certain evaluation techniques to characterize aggregates like Thioflavin T assay or TEM. Firstly, since the gelator has a hydrophobic core, it limits the use of fluorescence spectroscopic evaluation techniques which involve measuring the fibrilisation of the peptide by hydrophobic dyes such as thioflavin T. The hydrogel itself showed high fluorescence as the dye was probably bound to the hydrophobic core of gelator nanofibres.. Importantly, the nucleoside hydrogel has its own CD spectra and hence was not appropriate for these studies. Also, electron microscopy techniques were not applied as a method of evaluation because our hydrogel system itself has fibres which makes it difficult to distinguish it from the amyloid fibres formed as a result of peptide/protein aggregation. Moreover high concentration of buffer salts used in our experiments could have crystallised out and thus obscured the protein aggregate visualisation.[107]

The other main limitation was the instability of the gelator when stored for a long time. The gelator tends to precipitate with storage. These precipitates were found to increase with time. Thus, we could not clearly demonstrate how long the gelator can prevent protein aggregation.

6.2 Conclusions

Based on our experimental results we have shown that a supramolecular low molecular weight 2'-deoxycytidine based gelator exhibits the ability to suppress the aggregation of proteins with a broad range of isoelectric points (alkaline and acidic). To our knowledge, this is the first report of using a low molecular weight gelator for the inhibition of protein aggregation. Even though

the novel 2'-deoxycytidine based hydrogel has the potential to solve one of the most important formulation challenges of protein aggregation and thereby improve the therapeutic efficacy of biotherapeutics, its limited physical stability would preclude it being used in a commercial product.

However, these studies have shown the potential of supramolecular gelators in general as an effective formulation approach for inhibiting aggregation in a wide range of proteins at lower concentration. Thus, further research is needed to evaluate whether the phenomenon is observed for other physically stable supramolecular gels.

6.3 Future prospective

It is clear from the study that there is need for further investigations on the mechanism of stabilisation of the proteins and to further evaluate why the gelator precipitates after 3 days.

The further studies that could be carried out are:-

a) Evaluation of the stability of proteins in the hydrogel system.

The stability of proteins in the hydrogel system can be studied by using the different techniques used to study protein aggregation. The hydrogel system with proteins can be stored at different storage conditions for long periods of time and at each respective time intervals the samples could be evaluated for protein aggregation using the turbidimetry, fluorescence spectroscopy and microscopy

b) Mechanistic study of protein aggregation inhibition by the gelator molecule.

A mechanistic study could be carried out by examining the interaction of proteins with nanofibers in the hydrogel system by structural analysis techniques like SAX (small angle X-ray scattering) and SANS (small angle neutron scattering).

c) Investigation of the effect of gelator with other model peptides/proteins

The effect of gelator on the inhibition of aggregation in other model proteins such as bovine serum albumin (BSA), insulin, ovalbumin, or haemocyanin.

d) Investigation of the effect of gelator with proteins that aggregate at different aggregation conditions such as the addition of surfactants and heat.

e) Evaluation of other established supramolecular hydrogels like diacylated uridinophosphocholine derivative, 2' deoxyadenosine and ribonucleoside derivative, 2' deoxyadenosine derived gelator and polymer hydrogels with better physical stability to inhibit protein aggregation.

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