

# Impact of aggregation triggering ultrashort self-assembling peptide motifs on the solubility of proteins

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## Abstract

The intracellular solubility and folding properties of proteins are influenced by numerous extrinsic and intrinsic factors. One of the intrinsic parameters is the presence of an aggregating motifs within the amino acid sequence of the protein which makes the protein intrinsically disordered. Here we have studied the effect of an ultrashort self-assembling peptide (SSP) motif consisting of four amino acids, Isoleucine-Valine-Phenylalanine-Lysine, on the aggregation propensity of intracellular expression and folding of small ubiquitin-like modifier (SUMO) protein by C-terminal conjugation with the SSP motif. Conjugation of the SSP motif directed SUMO accumulation into the inclusion bodies of *E. coli*. SSP conjugated inclusion bodies of SUMO have revealed a highly ordered, rich fibrous structure, when observed under scanning electron microscopy. FTIR analysis of SSP conjugated SUMO has also confirmed that the composition of self-assembling fibrous material is mainly beta sheet rich. This observation has confirmed our hypothesis that SSP motifs can lead to the aggregation of soluble proteins, when exposed to favorable conditions.

## Introduction

Proteins are vital for virtually all biological processes and, since native expression does not yield sufficient amounts for structural and functional characterization, recombinant protein production is indispensable. Moreover, protein stability and yield are important prerequisites for a wide range of applications including an increasing number of therapeutic applications such as monoclonal antibodies, enzymes, cytokines and hormones [1,2].

Expression of recombinant proteins is hampered due to their poor solubility in heterologous hosts. Intracellular misfolding and poor solubility leads to the formation of inclusion bodies in the submicron sized species of bacteria [3]. Sometimes inclusion bodies based protein expression is deliberate mainly to boost the recombinant protein production [4] or reduce the toxicity of target protein to the host [5]. Some protein-based drugs are also packaged in inclusion bodies [6]. However most of the time recombinantly produced proteins are required to be soluble and fully folded. For this purpose, several N-terminal fusion tags like Maltose Binding protein (MBP), trigger factor (TF), Thioredoxin (TRX), transcription termination/antitermination (NusA), Ubiquitin protein (UB), glutathione S-transferase (GST) and small ubiquitin-like modifier (SUMO) have been used to improve the solubility of protein in heterologous prokaryotic and eukaryotic hosts [7]. Use of SUMO fusion have been reported to increase protein yield, solubility, and purification in prokaryotes [7,8].

Protein-based pharmaceuticals are one of the innovative pharmaceuticals that hold promise as a multibillion dollar industry in the health sector [9]. Protein aggregation, however, causes a major economic and technical burden on pharmaceutical and biotechnology industries. For instance, protein based pharmaceutical products face major challenges due to their aggregation over time, as proteins have an inherent tendency to aggregate [10]. Protein aggregation is a process during which misfolded proteins accumulate into insoluble aggregates. In order to be functional, proteins should be fully folded.

Therefore, if the protein of interest is to be used as a therapeutic product, it should have long term stability. There are many in vitro and in vivo factors affecting the stability of proteins in certain solutions. These interactions are mainly non-covalent which include enhanced hydrogen bonding, hydrophobic attractions and van der Waals forces among backbone and side-chain atoms, low steric clashes and minimize high energy interactions between amino acids and other components in solution [11].

Several neurodegenerative are related to aggregation of intrinsically disordered proteins such as Parkinson's, Alzheimer's, Dementia with Lewy bodies and the transmissible spongiform encephalopathies and frontotemporal dementias etc. [12]. Typical common characteristics of prion and amyloid diseases include ordered protein aggregate formation mostly in the form of long fibrils [13]. Most of these proteins are intrinsically disordered and lack stable native structure which in turn allows them the flexibility to adopt many conformations and perform important physiological functions. One example of such proteins is microtubule associated protein TAU which is involved in the regulation and stabilization of microtubules. However, dysfunction of TAU can lead to oligomerization and fibril formation in the brain causing many of the diseases mentioned above [14]. Many extrinsic and intrinsic factors modulate the aggregation behavior of intrinsically disordered proteins. Osmolytes are examples of extrinsic factors

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regulating the aggregation properties of intrinsically disordered proteins [15,16].

The intrinsic aggregation propensity of proteins is directly related to presence of aggregation prone regions which reduces the solubility of these proteins [17]. In silico aggregation prediction methods are widely used and often rely on the formation of intermolecular beta sheets [18]. Recent studies have revealed the presence of at least one fibril forming segment in about 1% of human proteome [19]. Fourteen aggregation prone motifs have been identified in human immunoglobulin G [20]. However, complete understanding of the mechanism underlying protein aggregation is still under debate [21]. Herein we describe, the impact of the aggregation triggering ultrashort self-assembling peptide (SSP) motif, consisting of four amino acids Isoleucine-Valine-Phenylalanine-Lysine (IVFK), derived and modified from microtubule associated protein, TAU, on the solubility of SUMO. Multiple copies of aggregation triggering peptides were attached to SUMO in different constructs and their effect on the aggregation of SUMO was studied.

## Materials and methods

### Construct designing and preparation

Single stranded DNA oligonucleotides coding for corresponding USPs were first optimized using genart Invitrogen online codon optimization software. These single stranded DNA fragments and their reverse complementary strands with forward primer having 5' AGGT and 3' TAAT overhang. Similarly the reverse primer has 5' CTAGATTA overhang as shown in table 1. These DNA fragments were synthesized from Sigma-Aldrich. Approximately 600pM of each oligonucleotide was phosphorylated using 5 units of T4 polynucleotide kinase and 1x T4 DNA ligase buffer at 37 °C for 45 minutes in a small Eppendorf tube. The reaction was stopped by heating the tube at 65 °C for 20 minutes. After phosphorylation equimolar concentration of complementary oligonucleotides were annealed in buffer containing 40mM Tris HCl pH 8.0, 10mM MgCl<sub>2</sub>, 50mM NaCl. The mixture was first heated at 99 °C and gradually cooled to 50 °C. Annealed double stranded DNA fragments were then purified by 1.5% agarose gel electrophoresis and Qiagen gel extraction kit.

**Vector Preparation:** Current studies were carried out using pE-SUMO vector with kanamycin resistance from life sensors. pE-SUMO vector was restricted with type IIS restriction enzyme Bsa1 overnight at 37 °C. The restricted vector was then the phosphorylated using alkaline phosphatase to avoid self-ligation of the vector.

For ligation 75-100ng of pE-SUMO was mixed with 100x molar excess of double stranded DNA fragments. As both vector and insert have complementary sticky ends, therefore T4 DNA ligase was used for ligation and the ligation reaction was incubated overnight at 16 °C. About 4ul of ligation reaction was transformed to *E. coli* DH5a cells and transformation was performed as per stranded protocol and six colonies were screened by miniprep for each construct to find successful clones. Positive clones were confirmed by DNA sequencing with T7 forward primer.

### Expression of SUMO-SSP constructs

Expression of SSP1, SSP2, SSP3 and SSP5 as well as control constructs was carried out in *E. coli* BL21(DE3) cells. About 200ng of each plasmid were transformed into BL21(DE3) cells and next day 3-4 colonies from plate were inoculated in 50ml of LB (Kanamycin) cells were grown to visible turbidity. These primary cultures were then diluted 100 times in 2L flasks containing 1L of LB + Kanamycin and the

cells were grown to OD<sub>600</sub> of 0.8 at 37 °C before being induced with 0.5M isopropyl thio-β-D-galactoside (IPTG) at 25 °C for 16 hours. Cells were then harvested by pelleting them at 6000g for 20minutes.

### Inclusion body isolation and Characterization

For isolation of inclusion bodies, cell pellets containing expressed constructs were resuspended in 50mM Tris+ 100mM NaCl+EDTA-free 1x protease inhibitor, pH 8.0. Cells were then sonicated for 20 cycles of 10 seconds-on and 20 seconds-off on ice to break the cells. Sonicated solution of lysed cells was spun at 13000g for 40 minutes. Pelleted inclusion bodies were then washed twice with 50mM Tris+100mM NaCl+0.5% triton x-100, pH 8.0 and once with 50mM Tris+100mM NaCl, pH 8.0 to remove cell debris. For denaturation of inclusion bodies, they were resuspended in denaturation buffer containing 50mM Tris+8M urea, pH 8.0 at 2mg/ml concentration using tissue homogenizer and bath sonication. 50mM Tris+6M GdnHCl, pH 8.0 was also used to denature inclusion bodies.

### Refolding and Purification of SUMO-SSP peptides

Denatured inclusion bodies solution for each SUMO-SSP construct were filtered through 0.45um filter before being loaded on to His-trap (5ml) affinity column under denaturing condition in 50mM Tris+8M urea, pH 8.0 buffer using AktaStart from GE healthcare. Protein coding for each construct was eluted from the column in 50mM Tris+100mM NaCl+2M urea+ 500mM Imidazole pH 8.0 by gradient elution. Elution fractions were screened by SDS-PAGE gel electrophoresis.

### Characterization of SUMO-SSP peptides by scanning electron microscopy

The morphology of SUMO-SSP was determined using an FEI Magellan XHR or Quanta 600 scanning electron microscope (SEM) with an accelerating voltage of 2-5kV. The SEM samples were prepared either by lyophilization of SUMO-SSP solution followed by coating of powdered sample on sticky carbon tape or by putting directly a drop of SUMO-SSP on a silicon wafer and drying in a vacuum desiccator for overnight. Lastly, the dried samples from both sample preparation methods were sputter coated with 5 nm Iridium prior to imaging.

### Fourier-transform infrared spectroscopic analysis of SUMO-SSP fusion proteins

FTIR measurements of SUMO-SSP fusion proteins were taken by Thermo Scientific FTIR-ATR iS10. A background scan was measured before the sample. The spectrum was collected in the range 500-4000 cm<sup>-1</sup>, with a 1 cm<sup>-1</sup> interval. Both background and sample measurements were taken as average over 10 scans

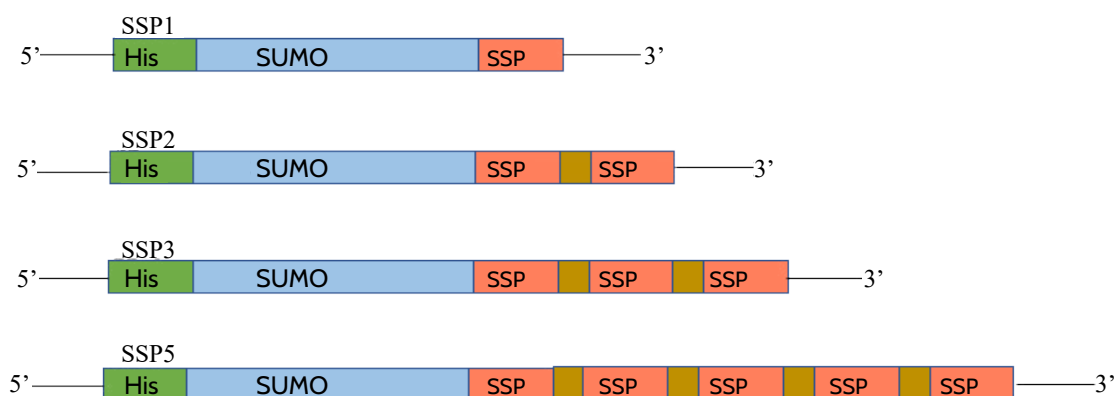
## Results and discussion

### Gene design and vector construction

Codon optimized single stranded DNA fragments coding for SSP motifs were designed in such a way that the SSP motifs were directly conjugated to the SUMO protein sequence (Figure 1). For this purpose pE-SUMO was restricted with type IIS restriction enzyme Bsa1 in such a way that it created a four nucleotide overhang on lower strand (3'). Complementary four nucleotides were added to the 5' end of all forward DNA fragments. The single stranded DNA sequence, as described in the table 1, were synthesized from Sigma-Aldrich. All the lyophilized DNA fragments were solubilized in autoclaved MilliQ H<sub>2</sub>O at a concentration of 100 μM. To facilitate the annealing, phosphorylation of all single stranded DNA fragments was carried out

**Table 1.** DNA sequences coding for SSPs. These sequences were synthesized from Sigma-Aldrich

Name	Oligonucleotide sequence
SSP1_F	AGGTATTGTTTTAAATAAT
SSP1_R	CTAGATTATTTAAAAACAAT
SSP2_F	AGGTATTGTTTTAAAGGTCTGGCATCGTGTAAATAAT
SSP2_R	CTAGATTATTTAAACACGATGCCAGGACCTTTAAAAACAAT
SSP3_F	AGGTATTGTTTTAAAGGTCTGGCATCGTGTAAAGGCCAGGTATTGTGTCAAATAAT
SSP3_R	CTAGATTATTGAACACAATACCTGGGCCTTTAAACACGATGCCAGGACCTTTAAAAACAAT
SSP5_F	AGGTGGTCCGGGTATTGTTTTAAAGGTCTGGCATCGTGTAAAGGCCAGGTATTGTGTCAAAGGACCGGGAATAGTATTCAAAGGCCCTGGGATTGTA TTTAAAGGTCCCGGATAAT
SSP5_R	CTAGATTATCCGGGACCTTTAAATACAATCCCAGGCCTTTGAATACTATTCCCGGTCTTTGAACACAATACCTGGGCCTTTAAACACGATGCCAGGACCTTTA AAAACAATACCCGGACC

**Figure 1.** Template of sumo-conjugated SSPs. Separate constructs were made with different number of copies of SSPs. SSP1 contains one copy of motif, SSP2 with two copies of motif separated by GPG linker, similarly SSP3 and SSP5 are with three and five copies of motif. Each motif copy is separated by GPG linker

using T4 polynucleotide kinase. These single stranded DNA fragments were then annealed together. Because the DNA fragments coding for SSP motifs are small compared to the size of the vector pE-SUMO, 100x molar excess of these fragments was used in the ligation reaction. *E. coli* DH5 $\alpha$  cells were transformed with the ligation reaction and produced colonies on LB agar plates containing Kanamycin antibiotic. Since the pE-SUMO vector was dephosphorylated using alkaline phosphatase to avoid the self-ligation of the vector, the colonies appeared on the kanamycin containing LB agar plates were predominately SSP motifs ligated vector. Further confirmation of these clones was confirmed by DNA sequencing of 4-6 plasmids harvested by miniprep. DNA sequencing of these plasmids confirmed the successful insertion of the DNA sequence coding for SSP motifs.

### Expression of SUMO-SSP constructs

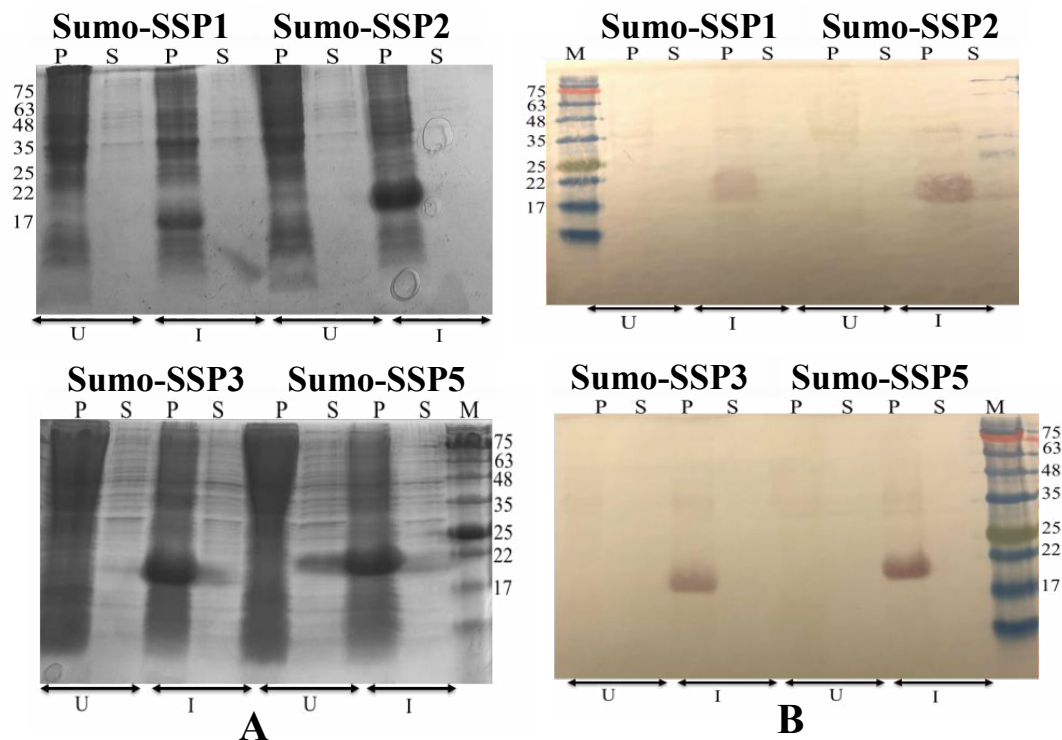
Once confirmed by DNA sequencing, plasmids containing the SSP motifs were expressed in *E. coli* BL21(DE3) cells. As expected SUMO-SSP conjugated proteins expressed at higher levels when induced with IPTG. It was observed by western blotting that all of the SSP conjugated SUMO protein appeared in the inclusion bodies of *E. coli*, when blotted against anti-histidine antibodies. There was no band observed for soluble fraction which indicates that the presence of SSPs in the recombinant protein leads to its misfolding and the formation of inclusion bodies upon translation. Since the Histidine tag is on the N-terminus of SUMO protein, it can be said that soluble SUMO protein once attached to the SSP motifs aggregates and forms inclusion bodies. This observation has confirmed our hypothesis that SSP motifs, when exposed to favorable conditions can lead to the aggregation of soluble proteins (Figure 2).

### Refolding and characterization of SUMO-SSP conjugated proteins

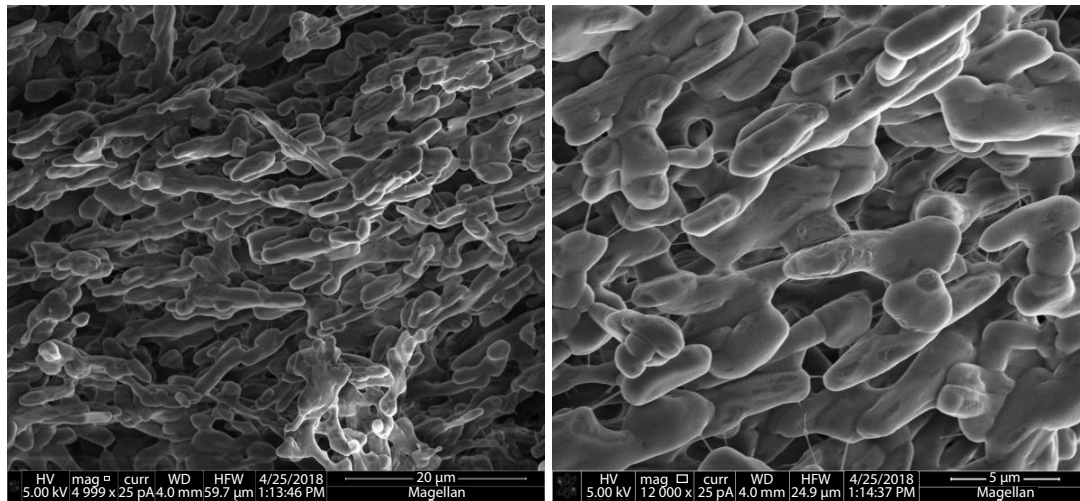
For further characterization of SUMO-conjugated SSP motifs, expression of these conjugated proteins was scaled up to 1L and inclusion bodies were prepared by cell disruption. Since most of the inclusion bodies contains small fraction of impurities, the mild detergent solution was used to remove impurities. Intact inclusion bodies were similar to those reported in the literature when observed by SEM (Figure 3)[22-24].

These inclusion bodies were then denatured using denaturants like GnHCl and Urea. It was observed that SUMO-conjugated SSP motifs were more soluble in urea compared to GnHCl. It was also noted that inclusion bodies tend to solubilize more in basic denaturant solution when compared to acidic solution. It was also found that inclusion bodies adhesiveness increased with an increasing number of SSP copies i.e. SUMO-SSP1<SUMO-SSP2<SUMO-SSP3<SUMO-SSP5. It means SUMO-SSP1 inclusion bodies were easily soluble and more stable in denaturant solution compared to SUMO-SSP5. Solution containing ammonium acetate and acetic acid was less effective compared to Tris and Urea. The denatured inclusion bodies for SUMO-conjugated SSP motifs, when observed under SEM showed similar structure (Figure 4).

Refolding was initially attempted by dialyzing the denatured SUMO-SSP solution against buffers without denaturant at 4 °C, however it was observed that SUMO-SSP proteins tend to precipitate during overnight dialysis. Refolding was therefore carried out by nickel affinity chromatography. SUMO-SSP solutions were applied to the His-trap 5ml column under denaturing conditions and proteins were refolded and eluted from column in a buffer containing reduced urea concentration (1M) and imidazole. Eluted proteins when observed by



**Figure 2.** Expression of SUMO-SSPs constructs. **Panel A** SDS-PAGE followed by Coomassie staining **Panel B** SDS-PAGE followed by western blotting against anti-his antibodies. **P** stands for sample from pellet after cell lysis, **S** stand for supernatant after cell lysis. **U** stands for Un-induced samples, **I** stands for induction of samples with IPTG after cell density reach to OD600 0.8. **M** is pre-stained protein marker and prominent bands are indicated by their size in kDa



**Figure 3.** Scanning electron microscopy of inclusion bodies of SUMO-SSP5

SDS-PAGE gel electrophoresis, produced bands for purified SUMO-SSP conjugated proteins (Figure 5). His-trap affinity chromatography based purification results have shown that high quality purification and refolding of SUMO-SSPs can be performed in a single step. These refolded and purified proteins did not precipitate when dialyzed. This is indicative of the increased stability of the SUMO-SSP conjugated proteins after refolding.

Secondary structure of SUMO-SSP5 was analyzed by FT-IR spectroscopy. The results showed that the conjugated protein is primarily comprised of beta sheets, as the peak was found at 1625

$\text{cm}^{-1}$  (Figure 5). One of the main reasons behind the beta sheet-rich secondary structure is the presence of SSP motifs on the c-terminus. The SSP motif is comprised of three hydrophobic amino acid residues and one hydrophilic amino acid at the end. This combination of amino acids allows for non-covalent interactions, particularly, hydrogen bonding and aromatic stacking, between other copies of the motif's residues on other molecules of SUMO-SSP in a solvent exposed state. The hydrogen bonding takes place between the N group of hydrophobic residues in the unfolded state of one monomer to the COO<sup>-</sup> group of residue from another monomer. The aromatic stacking mainly takes place between the side chains of phenylalanine on opposing monomers or opposing beta sheets in the same monomer in the unfolded state.

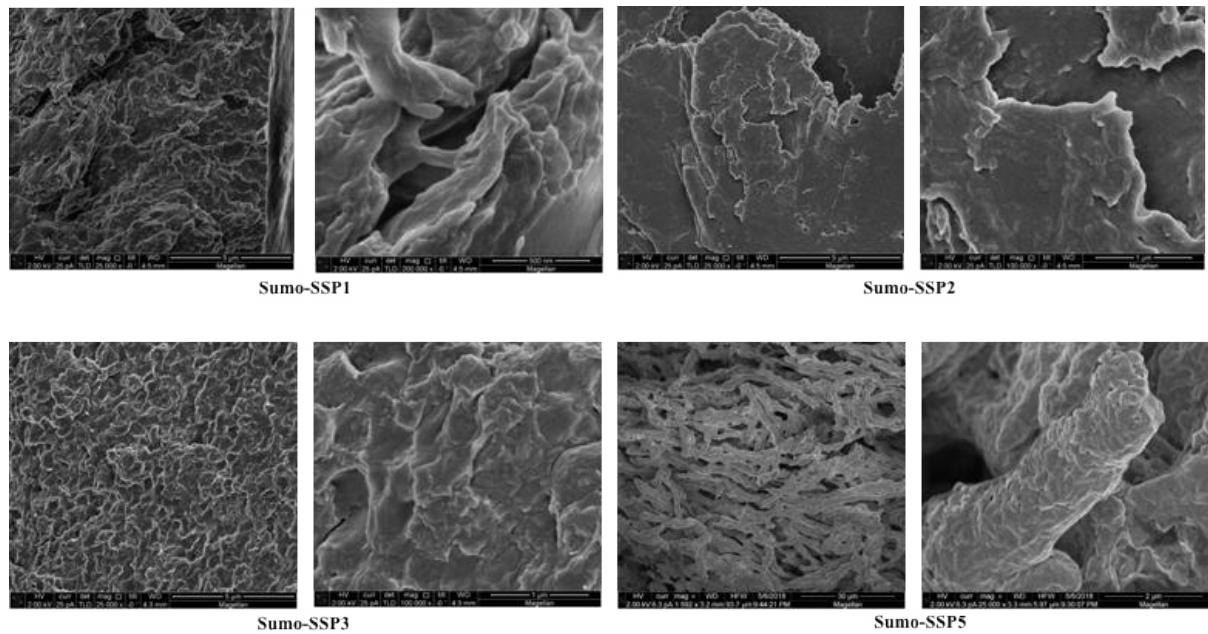


Figure 4. Morphology characterization of SUMO-SSPs inclusion bodies solubilization by scanning electron microscopy in the presence of denaturant 8M urea

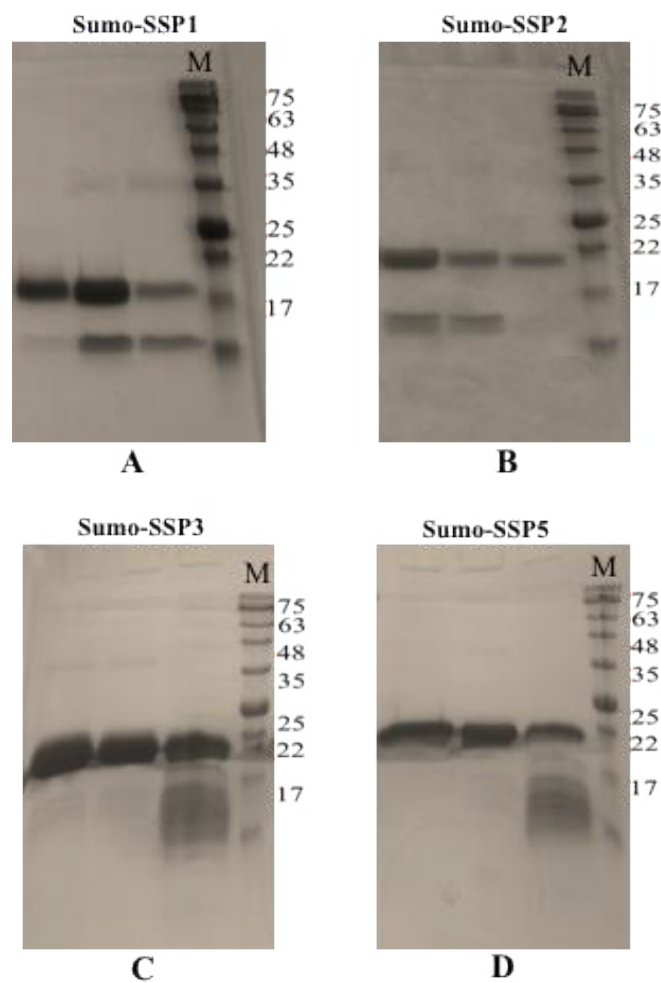


Figure 5. Purification of SUMO-SSPs. A SUMO-SSP1, B SUMO-SSP2, C SUMO-SSP3, D SUMO-SSP5. M is pre-stained protein marker and prominent bands are indicated by their size in kDa

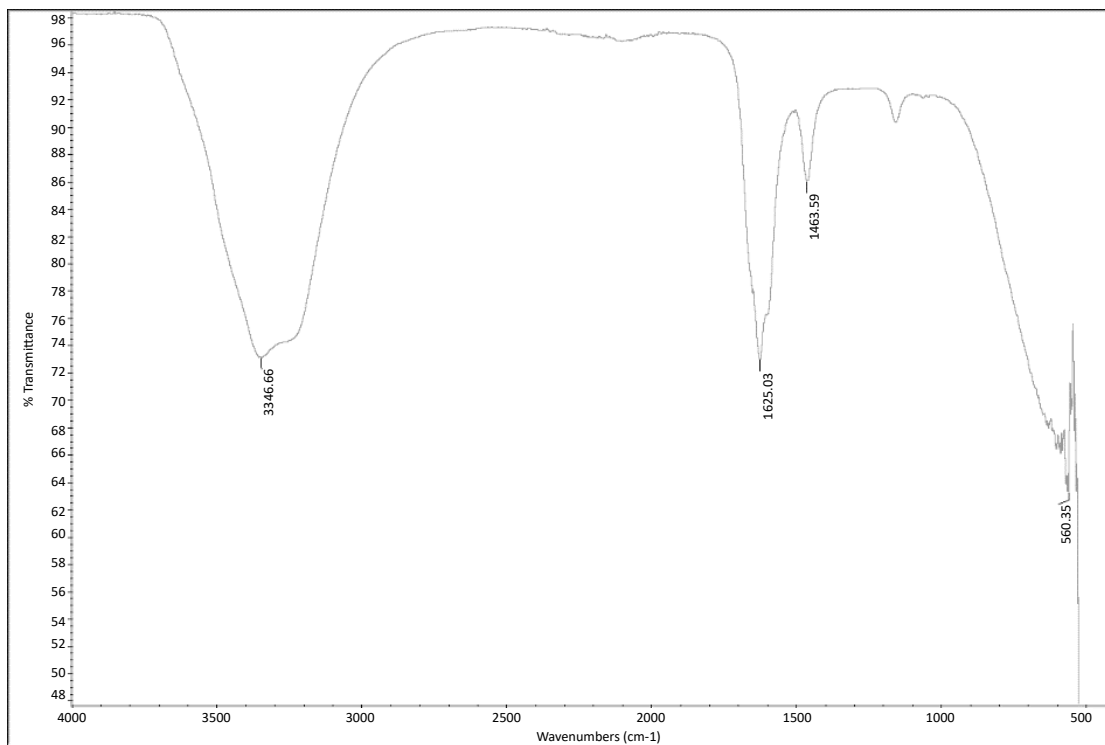


Figure 6. Secondary structure characterization of SUMO-SSP5 using FTIR

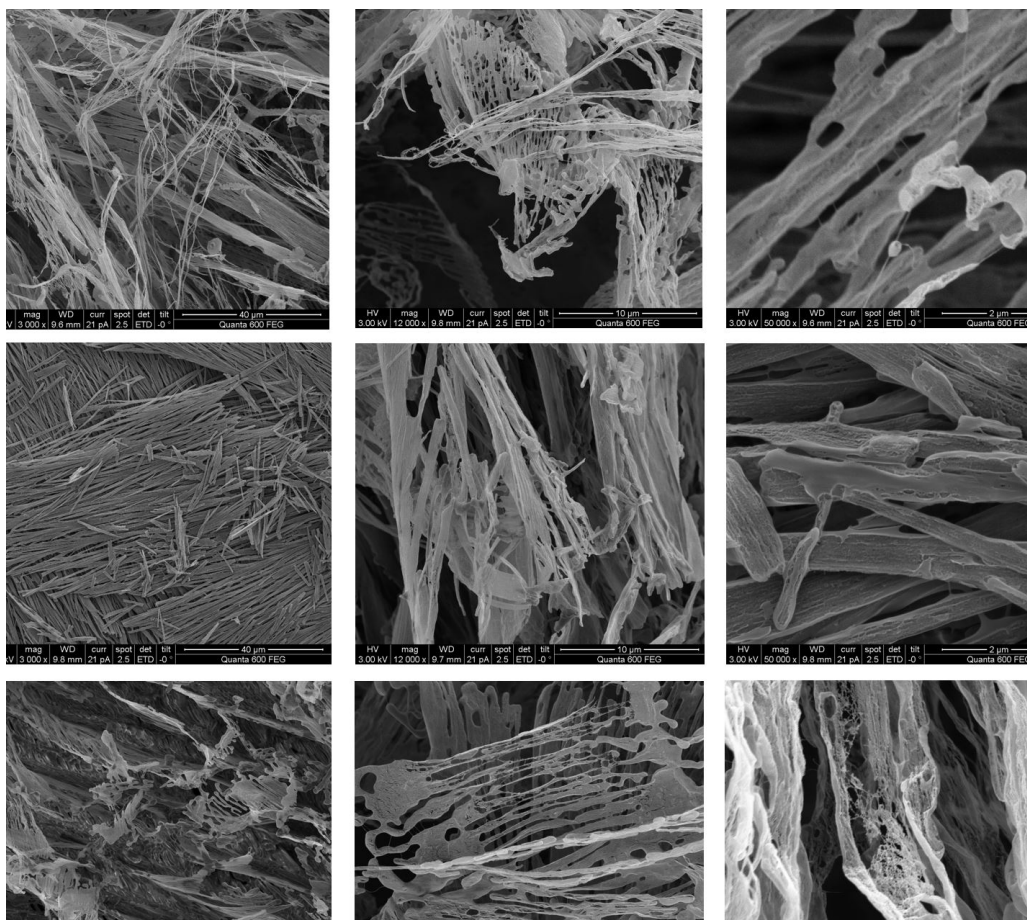


Figure 7. Morphology characterization of refolded SUMO-SSPs by scanning electron microscopy. A. SUMO-SSP1. B. SUMO-SSP2. C. SUMO-SSP3. All refolded SUMO-SSPs have shown highly fibrous structure

## Morphological characterization of SUMO-SSPs conjugated proteins by SEM

It was found that the material was highly fibrous when refolded SUMO-SSPs was observed under SEM (Figure 5). The length of fibers was more than 100µm long while the diameter varied for different SUMO-SSPs. The SUMO-SSPe fibers were flat and crystalline, while SUMO-SSP1 and SUMO-SSP3 were thin and long. The protein morphology was significantly different compared to denaturing condition, which shows that upon refolding protein properties change and SUMO-SSPs exhibit the fibrous structure due to monomer-monomer interaction and stacking (Figures 6 and 7).

## Conclusion

Many proteins aggregates due to self-association of molecules in the form of particles, precipitates and fibers. The aggregation prone motifs within the protein sequence plays critical role in the association of molecules [25]. During this study, we have tried to conjugate the ultrashort self-assembling peptide motif to a highly soluble protein, SUMO, and studied its impact on the solubility of SUMO [26,27]. We observed that almost all of the SUMO expression was directed to the inclusion bodies in the bacterial cells. This research highlights the importance of the identification of aggregation prone motifs within the protein sequences. Truncation and mutation of such motifs can improve the solubility and stability of proteins especially if the protein of interest is biotherapeutic. However, if the target recombinant protein is toxic to the cells or to be used in the fibrous form, then such a ultrashort self-assembling peptides can be conjugated to the target protein.

## Author contributions

CAEH conceived the idea. MS, SSQ and HHS performed the experiment. MS and CAEH wrote the manuscript.

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## Conflict of Interest

The authors declare no conflicts of interest

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