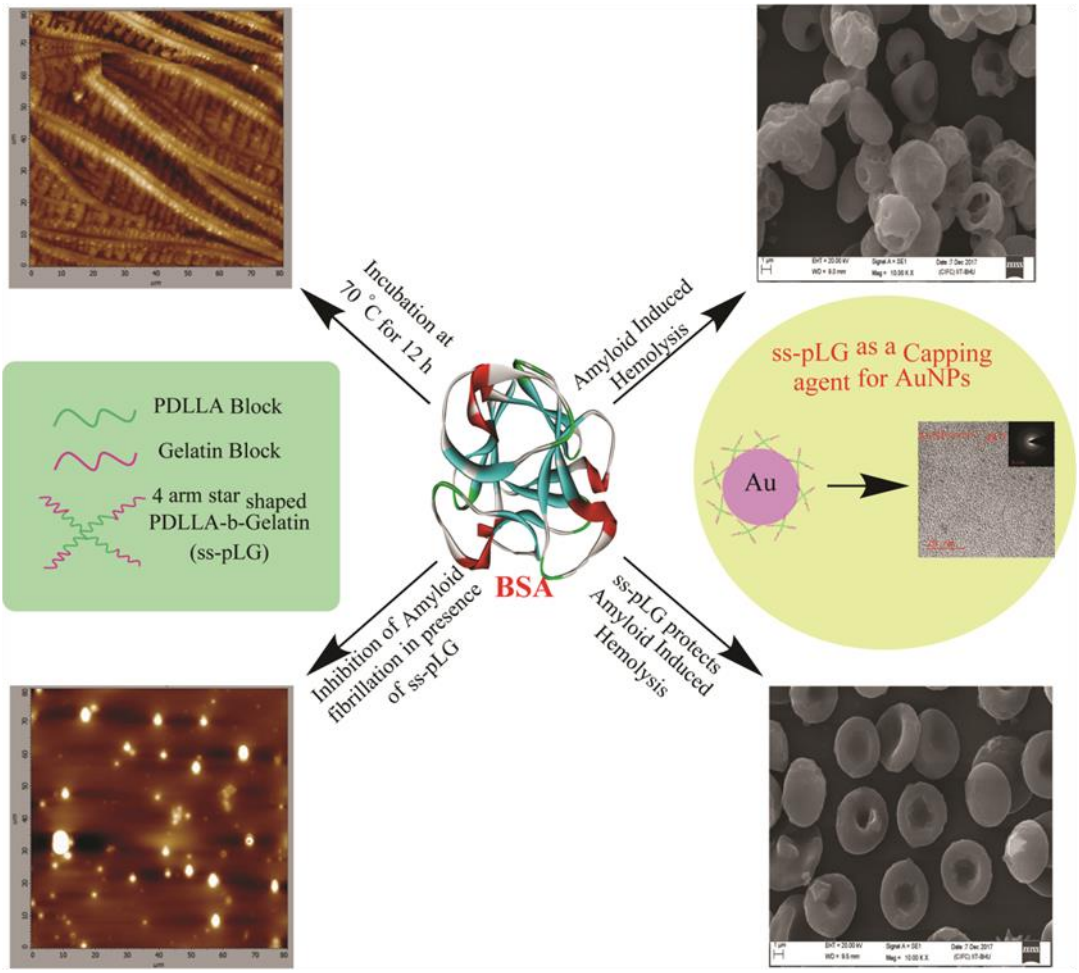
5. GELATIN GRAFTED POLY(D,L-LACTIDE) AS A ANTI-AMYLOIDOGENIC AGENT



This chapter describes the ss-pLG which can protect the amyloid fibrillation mechanism which was evidenced by physicochemical characterization techniques, amyloid induced neuroblastoma cells (MC-65 cells) and amyloid-induced hemolysis.

5.1. Overview

The conversion of physiological soluble proteins into higher-order insoluble aggregates is one of the fundamental causes for the existing health complications such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD).[144, 145] Until now, ~35 different proteins, including amyloid-β (Aβ), α -synuclein, and huntingtin from lesions of AD, PD, and HD, respectively, have been found to form β-amyloid aggregates, which lead to cause severe damage to neurons.[65, 146] Despite the differences in their primary structures, all amyloid proteins share common mechanisms to form morphologically and immunologically similar aggregates, such as ring-shaped amyloid oligomers or amyloid fibrils.[147] The formation of amyloid fibrils is considered as a consequence of protein misfolding. It is understood that fibrillation involves nucleation dependent polymerization mechanism, where amyloid monomers first polymerize into oligomers that further serves as nuclei for fibrils formation through addition of monomer.[148] It was observed that addition of molecules like chaperones could be one of the most effective strategies for targeting amyloid-linked diseases, which will rescue amyloid formation.[149] Understanding the fibrillation mechanism and developing aids to block the fibrillation process is considered to have high therapeutic values. In last few decades, several efforts have been made to inhibit amyloid formation through various molecules such as natural products[150-152] and selected peptides.[153, 154] In addition, nanometer-sized materials coated with specific molecules were also found to be capable of inhibiting the fibrillation process of proteins.[65, 155-158]

To the best of our knowledge very few articles have been reported concerning the synthetic polymers to inhibit the formation of amyloid fibrils.[159-161] It is widely studied that surface properties of polymers can influence the amounts and types of bound proteins, as well as the conformation, orientation or binding strength of the adsorbed protein.[162] In our previous study, we found gelatin grafting to PDLLA backbone improved its surface properties to hydrophilic nature. Studies have revealed that gelatin notably inhibits the formation of Insulin amyloid fibrils.[163] Therefore, it is essentially important to elucidate the biocompatibility of gelatin grafted poly(D,L-Lactide) (PDLLA) amyloid fibril formation in view of exploring the potential of synthetic polymers as anti-amyloidogenic agents.

Herein we assessed the hypothesis that ss-pLG might inhibit the amyloid fibrillation mechanism. We used bovine serum album (BSA) as a model protein, which is known to produce fibrils under high temperature (<70 °C). [65, 164, 165] Further, we assessed the potential of our synthesized ss-pLG in protecting amyloid fibril induced neurotoxicity in MC65 cells as well as in ameliorating amyloid fibril induced hemolysis. Finally, we also evaluated the efficiency of the ss-pLG as a capping agent on gold nanoparticles ($AuNPs^{ss\text{-}pLG}$)

5.2. RESULTS AND DISCUSSION

5.2.1. Effects of ss-pLG on inhibition of protein fibrillation

Amyloid aggregation of BSA was studied at 70 °C with the help of Thioflavin T by measuring the change in fluorescence intensity. **Figure 5.1** shows the effects of gelatin (Gel) and ss-pLG on inhibition of protein aggregation. The BSA followed strong

fibrillation process in the absence of Gel and ss-pLG, whereas substantial suppression of fibrillation was observed in the presence of Gel and ss-pLG, in a concentration

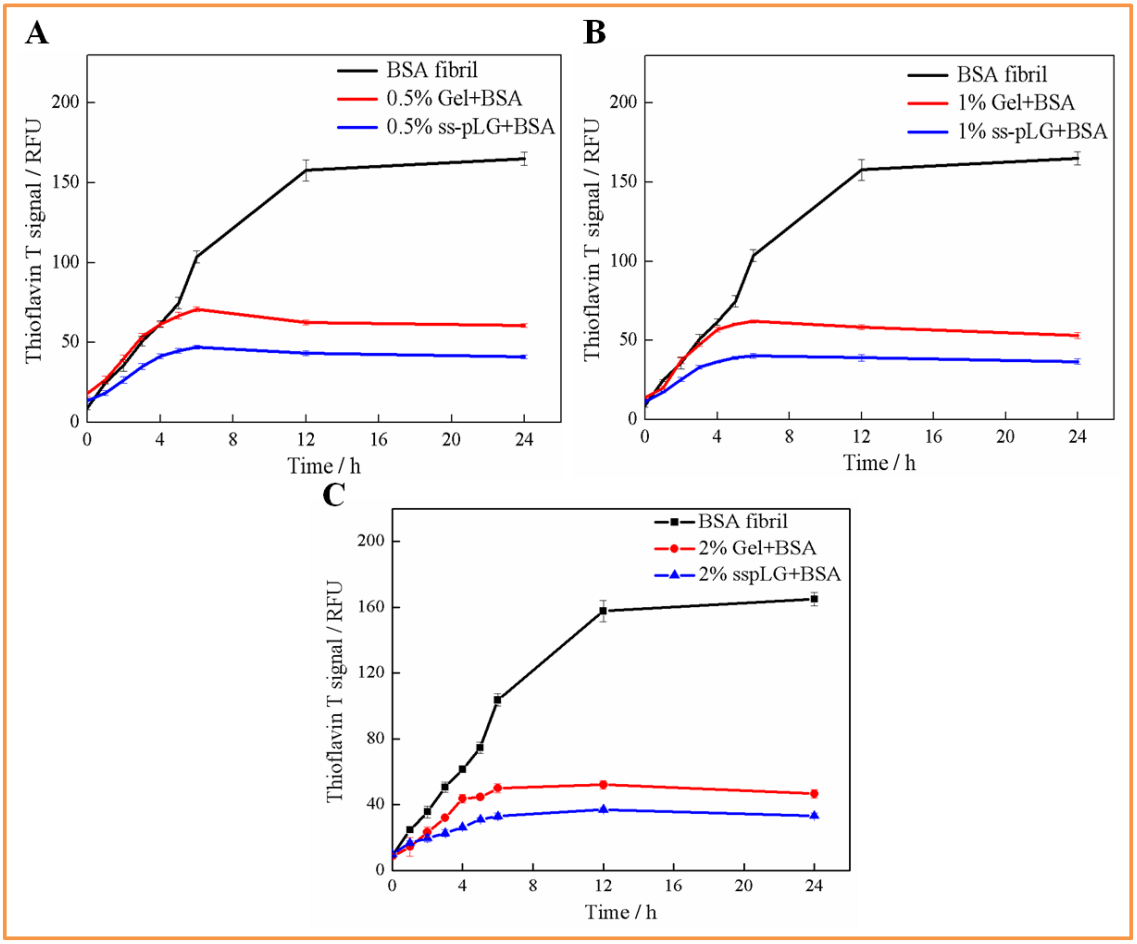


Figure 5.1 Effects of ss-pLG on inhibition of amyloid fibril formation shown through Thioflavin T as a marker, ex/em 440/480 nm. (A) 0.5% (B) 1% and (C) 2% of Gel and ss-pLG

dependent manner. Moreover, strong inhibition was observed with ss-pLG due to the structural organization and grafting of gelatin grafting onto four arms of PDLLA backbone. At lower concentration of Gel and ss-pLG such as 0.5% and 1%, the relative fluorescent unit (RFU) values were higher than 2% which indicates that higher concentration of polymer efficiently inhibit the fibrillation. BSA aggregation was also

studied using circular dichroism (CD) spectroscopy. BSA solution in PBS without polymer exhibited a considerable decrease in the intensity of CD bands around 208 (β-sheet) and 220 nm (α-helix) from its original that corresponds to the loss of BSA secondary structure.[65, 164] However, BSA incubated with polymers showed no decrease in the CD intensity, which further supports that gelatin grafted polymer, inhibits the fibrillation process (**Figure 5.2)**.

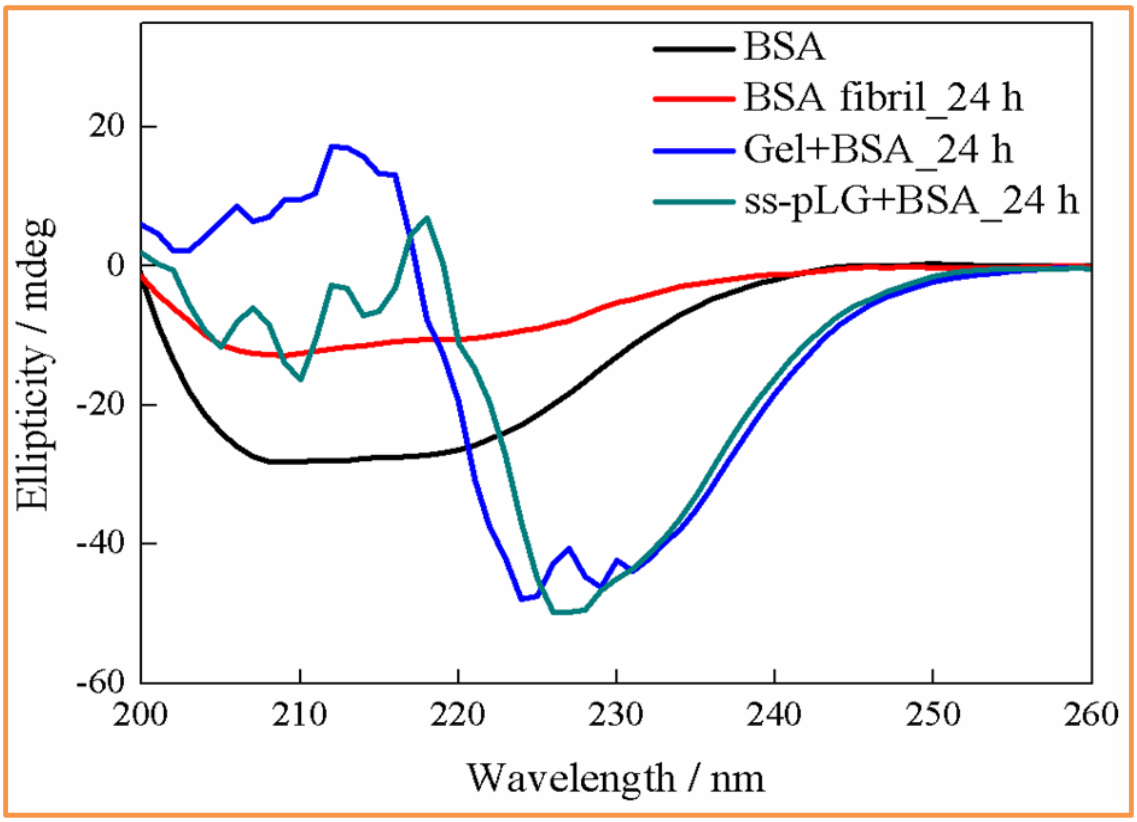


Figure 5.2 . Effects of ss-pLG on inhibition of amyloid fibril formation shown through CD spectroscopy. 2% of Gel and ss-pLG was used for the experiment.

Polymers without BSA were also studied as the control, where we observed CD intensity was increased after incubation with BSA.

To examine the morphology of BSA obtained from both with and without polymer after the aggregation reaction, we performed AFM. The images (**Figure 5.3**) evidenced that BSA with polymers efficiently inhibit the fibrillation mechanism, while BSA showed spherical shape and fibrils, before and after the aggregation reaction, respectively. These results reveal that Gel and ss-pLG polymers are highly effective in inhibiting the formation of amyloid fibrils that might be due to the strong interaction of BSA with Gel and ss-pLG.

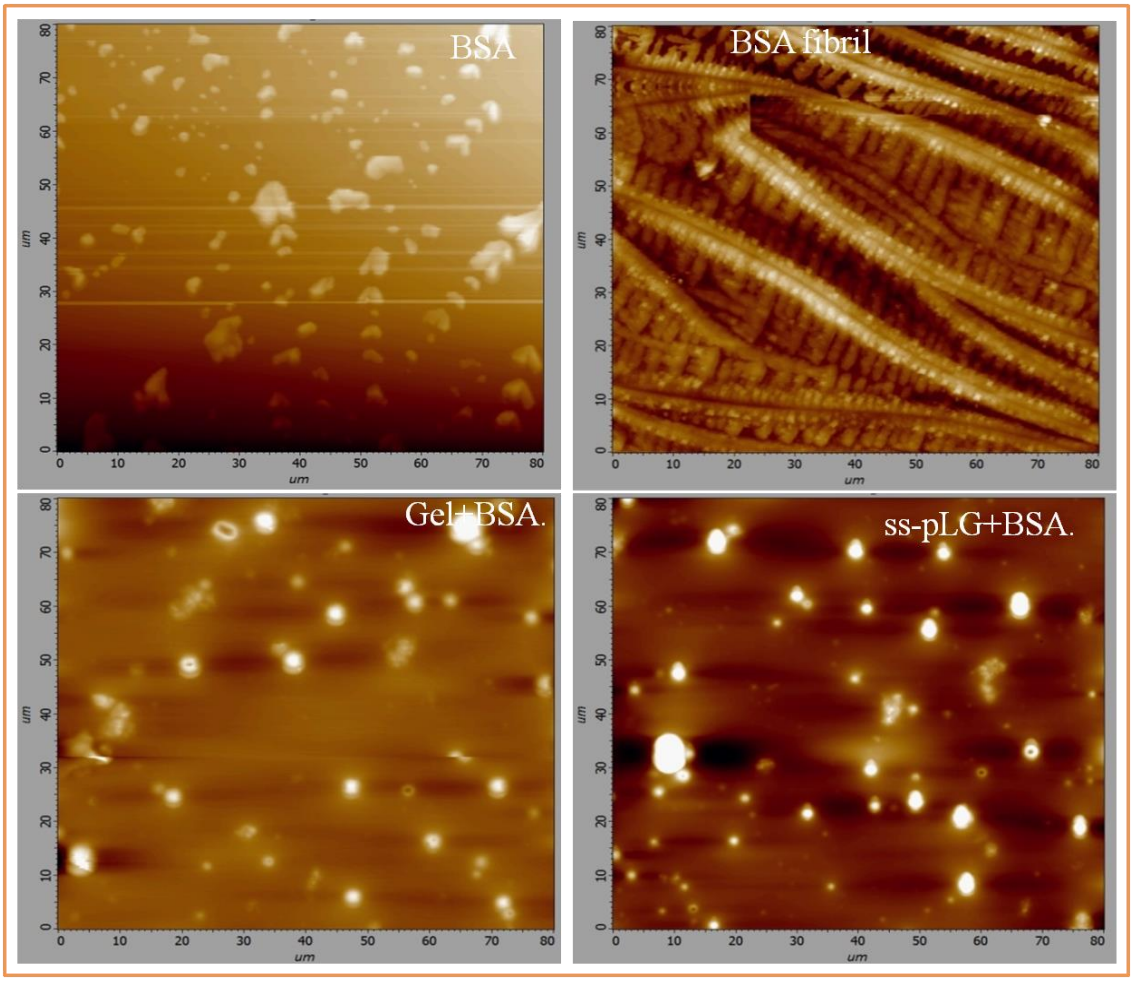


Figure 5.3 Effects of ss-pLG on inhibition of amyloid fibril formation shown through atomic force microscopy (AFM). 0.3 mg/mL of BSA and 2% of Gel and ss-pLG were used for all experiments. Occurrence of spherical oligomers were observed when BSA

was incubated with polymer, whereas without polymer BSA formed lengthy fibril. 2% of Gel and ss-pLG was used for the experiment.

5.2.2. Interaction of ss-pLG with Bovine serum albumin (BSA)

To corroborate our hypothesis, we studied BSA interaction with Gel and our synthesized polymer ss-pLG. It was already reported that dynamic quenching is largely caused by the collision and the energy transfer from BSA to ligands and consequently changes can be ruled out using UV spectroscopy.[166] Therefore, UV-Vis absorption spectra of BSA, Gel, ss-pLG and Gel+BSA, ss-pLG+BSA were taken. The absorbance peaks of BSA in PBS were found

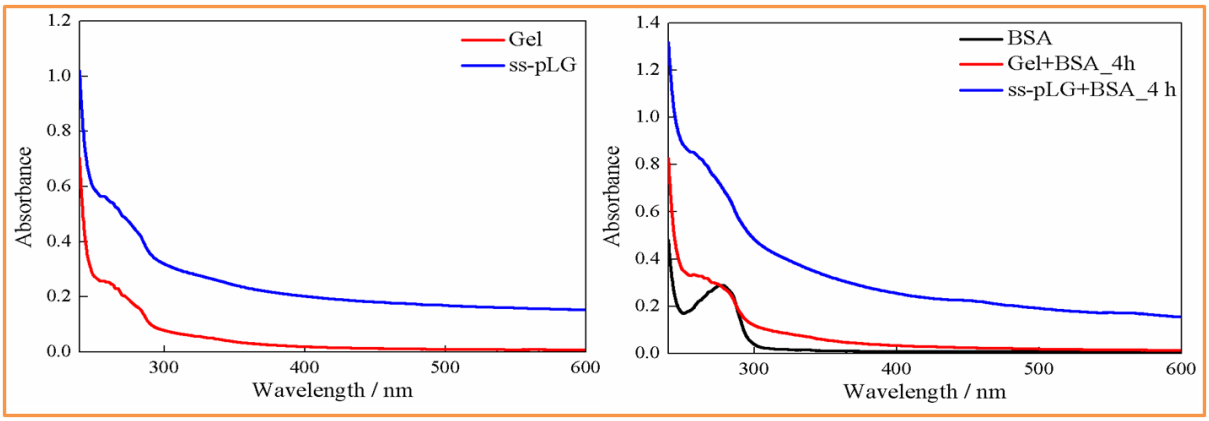


Figure 5.4. BSA-ss-pLG Interaction. Absorption spectra of BSA, Gel+BSA and ss-pLG+BSA after 4 h. 2% of Gel and ss-pLG was used for the experiment.

at ~280 nm. Polymers Gel and ss-pLG showed broad peak around 250-290 nm. The addition of BSA to the polymers showed a considerable change in absorption spectrum where the absorbance was increased up to 1.2 and 1.5 times higher than that of the respective Gel and ss-pLG (**Figure 5.4**). The observed change in UV-Vis absorption spectra indicates that the interaction is mainly due to the BSA–ligand complex formation. BSA shows a fluorescence band at 335 nm in PBS when excited at 290 nm

and observed to quench with the addition of Gel and ss-pLG with a blue shift about 45 nm in BSA interacted Gel and ss-pLG further supports the strong BSA-polymer interaction (**Figure 5.5**). Further, CD spectroscopy was used to study the changes in the secondary structure of the proteins. It was observed that the negative signal became

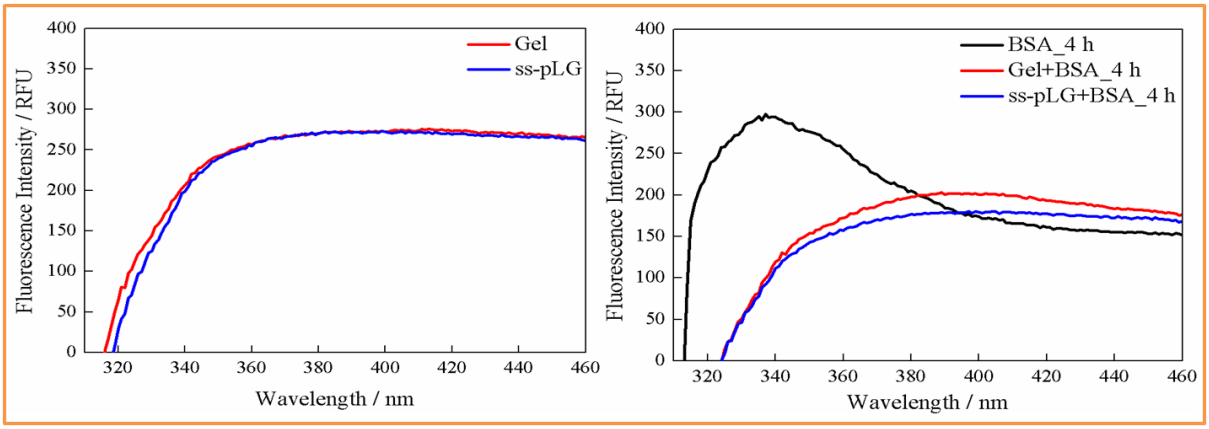


Figure 5.5 BSA-ss-pLG Interaction. Tryptophan fluorescence spectra of BSA, Gel+BSA and ss-pLG+BSA after 4 h (Ex 290 nm). 2% of Gel and ss-pLG was used for the experiment.

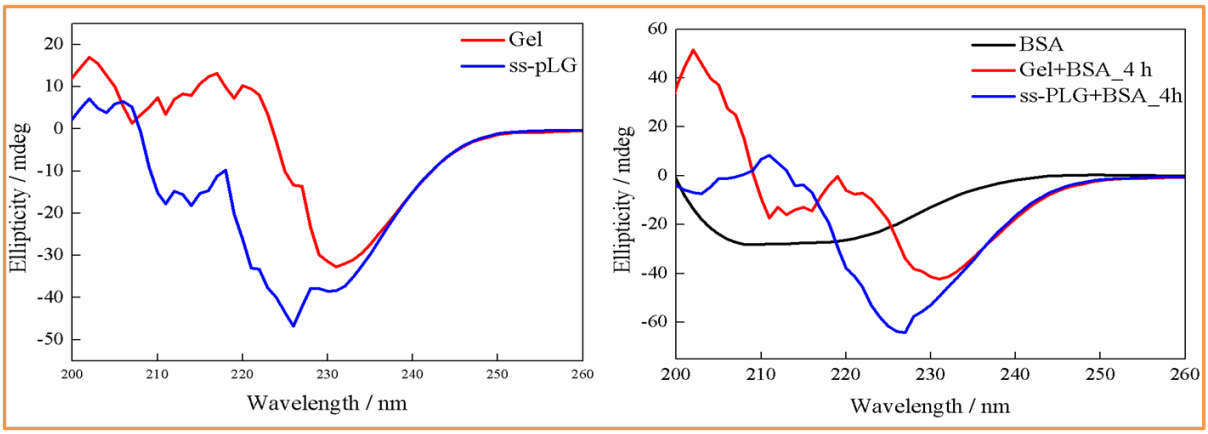


Figure 5.6 BSA-ss-pLG Interaction. CD spectra of BSA, Gel+BSA and ss-pLG+BSA after 4 h. 2% of Gel and ss-pLG was used for the experiment. 2% of Gel and ss-pLG was used for the experiment.

stronger after the addition of Gel and ss-pLG to BSA, indicating an increase or stabilization of the BSA-polymer interaction (**Figure 5.6**). Moreover strong interaction of BSA with ss-pLG was observed which could be due to the reduced lamella space/thickness in ss-pLG, which is due to the branched chemical structure.[36, 167-169]

5.2.3. Effects of polymer on rescuing toxicity of MC65 cells and amyloid-induced hemocompatibility

From the *in vitro* physico chemical characterization studies, we found that Gel and ss-pLG have the potency to inhibit the fibrillation mechanism. On account of the fact that Gel and our polymer ss-pLG likely possess anti-amyloidogenic property, we evaluated the protective efficiency of ss-pLG against Aβ indcued toxicity in MC65 cells, where cell death was induced by oligomeric Aβ peptides produced by the cells itself. In the absence of tetracycline (TC) in the culture medium, SβC gene is known to be induced in MC65 cells that leads to the production of C99 fragment of amyloid precursor protein and subsequently formation of Aβ peptides through its proteolysis by γ-secretase; that finally results in cell death. The addition of TC, an antioxidant, to the media of MC65 cells suppresses the production of C99 fragment and makes the cells survive. Considering this behavior of MC65 cells as the important basis, we evaluated toxicity or the rescuing ability of the ss-pLG against Aβ indcued cell death. [152, 170, 171] We used different concentration (0.05, 0.1 and 0.2%) of Gel and ss-pLG to screen the protective effects. As shown in **Figure 5.7**, protective effects of polymer followed a concentration dependent trend. 0.2% of Gel and ss-pLG exhibited remarkably higher protection efficiency than their respective polymers at lower concentration. However,

lower concentration (0.5%) of polymers also exhibited better rescuing of MC65 cells which was about > 60%. These results reveal that Gel and ss-pLG protects the cells most likely due to the possibility of appropriate interaction between the specific sites of cell membrane, resulting in suppression of the formation of Aβ peptides. Further, the better rescuing effect of ss-pLG compared to Gel may be attributed to the organization of Gelatin in the backbone of PDLLA and the polymer's lamellar thickness. It was reported that branching of polymers increase the lamella thickness because of the retarding effect of branching.[36, 167-169]

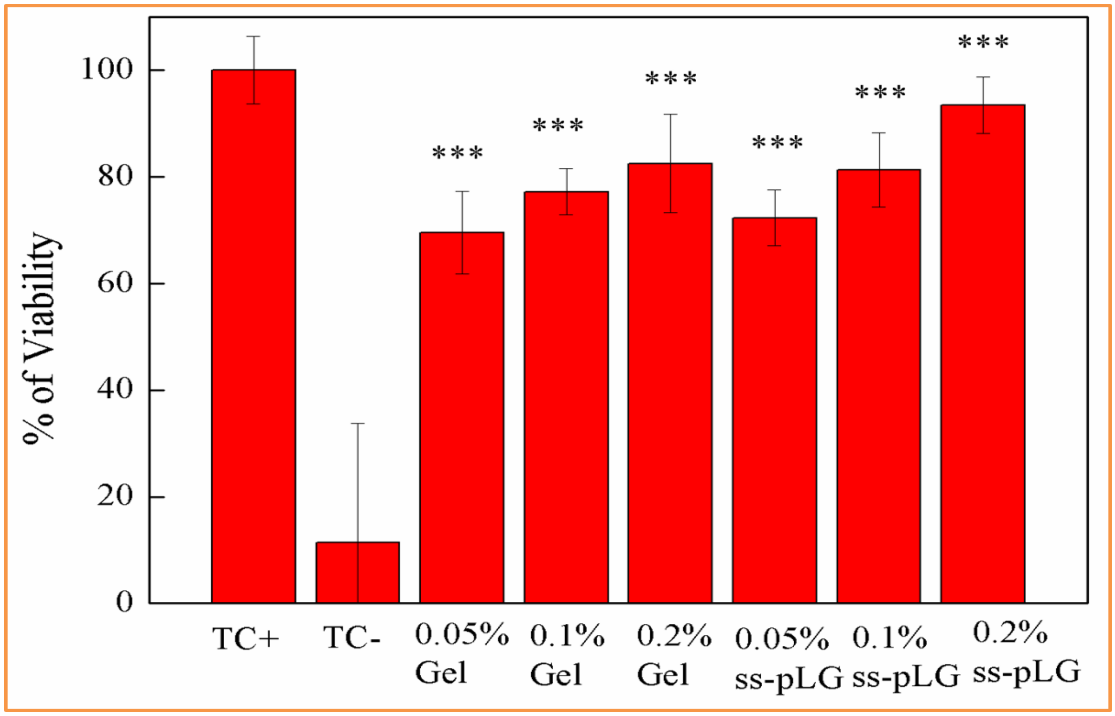


Figure 5.7 Effects of polymer on rescuing toxicity in MC65 cells . MTT assay of MC65 cells incubated with Gel and ss-pLG; MC65 cells without tetracycline (TC) was treated with Gel and ss-pLG over 48 h and viability was evaluated by MTT assay

5.2.4. Effects of polymer on amyloid-induced hemocompatibility

Further, we extended our study to evaluate the protective effect of amyloid linked biological complications in blood RBCs. Blood RBCs are more susceptible to the environment that will be lysed even when they come in contact with water. **Figure 5.8**

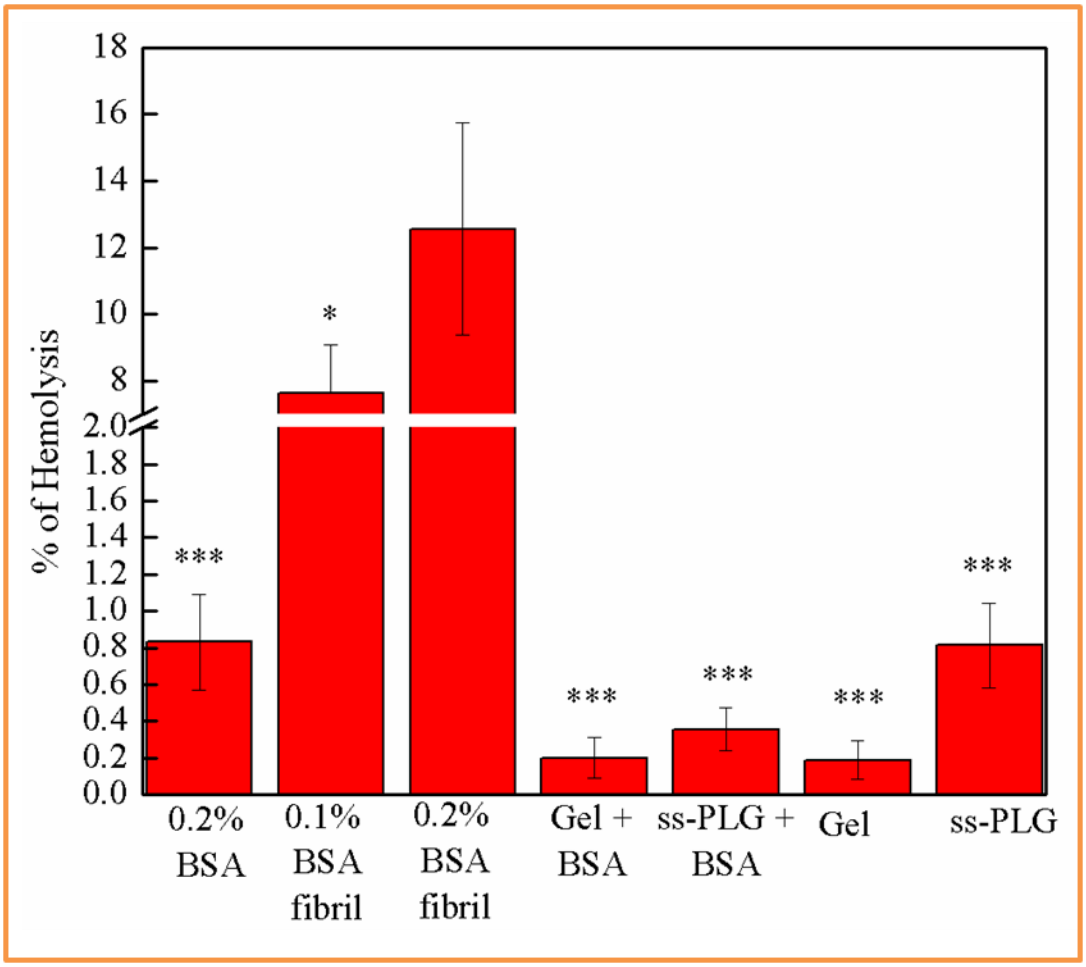


Figure 5.8 Hemolysis of RBCs with BSA, BSA amyloid fibril and BSA pre-incubated with Gel and ss-pLG.. All bars express mean values ± SD (n = 3); * $p<0.05$ and *** $p < 0.0001$.

shows the hemolysis of normal BSA, BSA fibrils and BSA incubated with polymers. The percentage of hemolysis after 4 h of incubation was found as 0.83±0.26, 7.63±1.46, 12.55±3.18, 0.2±0.11, 0.36±0.12, 0.18±0.1 and 0.81± 0.17 for 0.2% BSA, 0.1% BSA

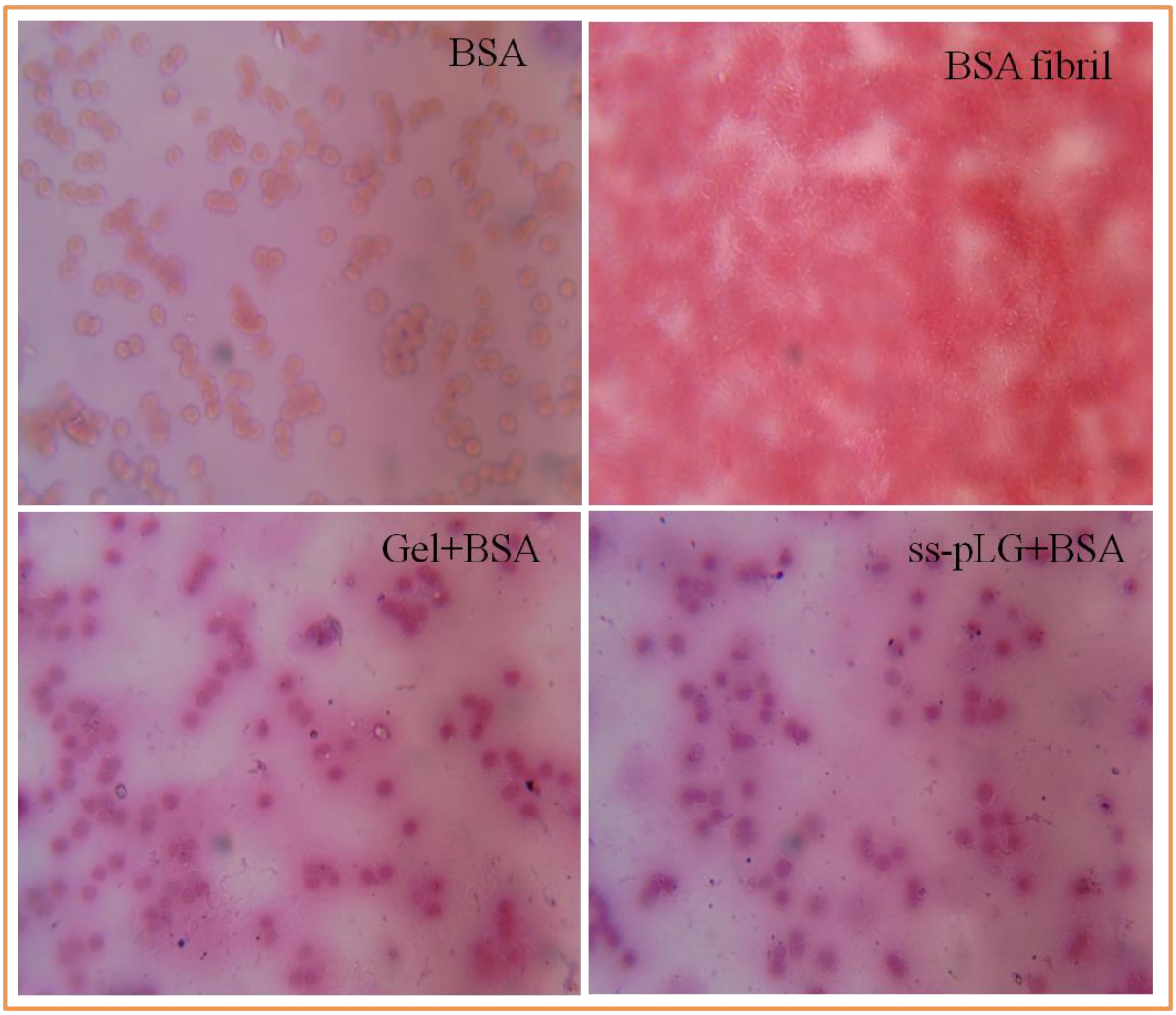


Figure 5.9. Evaluation of platelet aggregation by leishman stain.

fibril, 0.2% BSA fibril, Gel+BSA, ss-pLG+BSA, Gel and ss-pLG, respectively. It was reported that up to 10 % hemolysis is permissible for biomaterials,[119] thus 0.2% of BSA fibril is toxic to the blood RBCs and the percentage hemolysis of Gel+BSA and ss-pLG+BSA was significantly lower than BSA fibril that reveals that Gel and ss-pLG did not allow the BSA to follow fibrillation mechanism.

Further, the morphology was also observed using optical microscopy by leishman staining for the platelet aggregation. It was observed that there were no platelet

aggregation incase of BSA and BSA incubated with Gel and ss-pLG (**Figure 5.9**) that are equivalent to PBS incubated platelets (**Figure 5.11**B), whereas BSA fibril showed aggregated platelets and the morphology were also different as observed by other authors.[172] Thus, it signifies that Gel and ss-pLG do not cause any damage to platelets, most likely due to the selective inhibition of COX1 by Gel and ss-pLG that leads to decrease in TxA2 and in turn prevents platelet aggregation. [173]

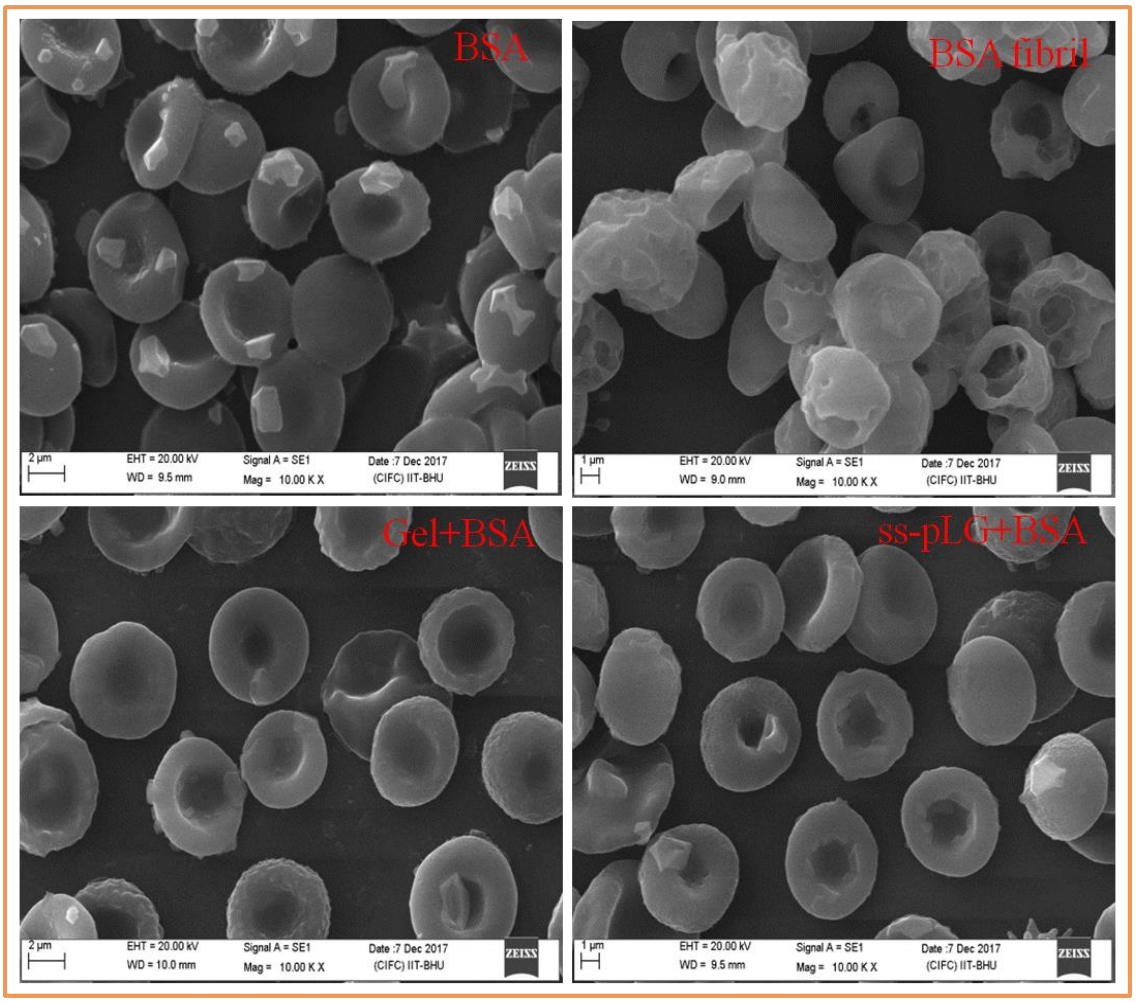


Figure 5.10. SEM morphology of RBCs after amyloid-induced hemolysis

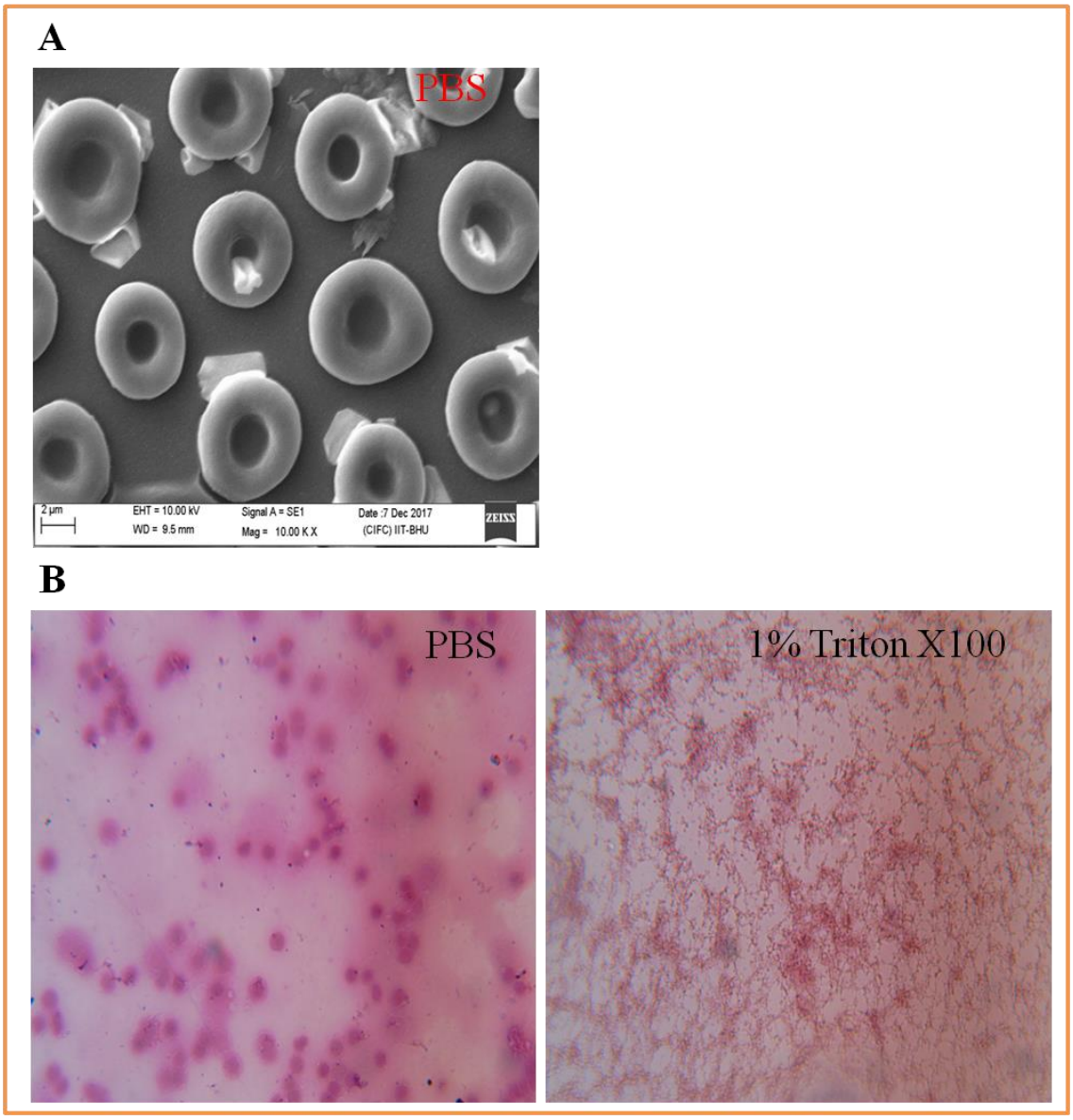


Figure 5.11. Hemolysis by positive and negative controls **(A)** SEM image of PBS incubated RBCs. (B). Optical microscopy image of Platelet aggregation by leishman staining method

The hemolysis behavior of RBCs were also qualitatively evaluated using SEM. The outcome revealed that, RBCs incubated with soluble BSA did not show any indication of cell lysis. In addition, we did not notice any indication of hemolysis when RBCs were treated with only Gel and ss-pLG (**Figure 5.10**) as observed with PBS (negative

control) (**Figure 5.11**A). However, in the presence of BSA amyloids, we observed lysed RBCs, i.e. change in discoid shape and rupture of cell membrane. Interestingly, BSA pre incubated with Gel and ss-pLG did not elicit any lysis of RBCs suggesting that polymer inhibits the amyloid aggregation process.

5.2.5. ss-pLG as an efficient biocompatible capping agent

Considering the biocompatibility characteristics of synthesized ss-pLG, we envisaged to use our ss-pLG in the synthesis of gold nanoparticles ($AuNPs^{ss\text{-}pLG}$) as a capping agent. We used simple, low-cost, and environmentally friendly strategies for the synthesis of AuNPs. We used $NaBH_4$ as a reducing agent and gelatin as a control capping agent which was already reported by various authors.[174-176]. The UV−visible spectra of these nanoparticles showed a SPR peak around ∼520 nm confirming the formation of nanoparticles (**Figure 5.12**A). DLS data of $AuNPs^{Gel}$_48 h and $AuNPs^{ss\text{-}pLG}$_48 h samples clearly showed a homogeneous population of nanoparticles with average hydrodynamic radius of ∼60 nm even after 48 h of incubation in room temperature (RT) (**Figure 5.12**B). Transmission electron microscopy (TEM) images further confirmed the formation of spherical uniform nanoparticles for $AuNPs^{Gel}$_48 h and $AuNPs^{ss\text{-}pLG}$_48 h, whereas the size of the NPs was found as 6±2 and 0.4±0.1 nm, respectively. (**Figure 5.12**C). The reduction in size of NPs in $AuNPs^{Gel}$ and $AuNPs^{ss\text{-}pLG}$ compared to AuNPs, could be due to the reducing ability of gelatine and PDLLA. Further, we performed SAED (selected area electron diffraction) pattern to assess the polycrystalline nature of the nanoparticles. (**Figure 5.12**D). The uncoated NPs showed clear crystalline structure and the ring patterns were not observed for $AuNPs^{Gel}$_48 h and $AuNPs^{ss\text{-}pLG}$_48 h that

further supports the functionalization of AuNPs. The synthesis mechanism involves two steps. The first step involves the reaction between $NaBH_4$ and $HAuCl_4$ for reduction and

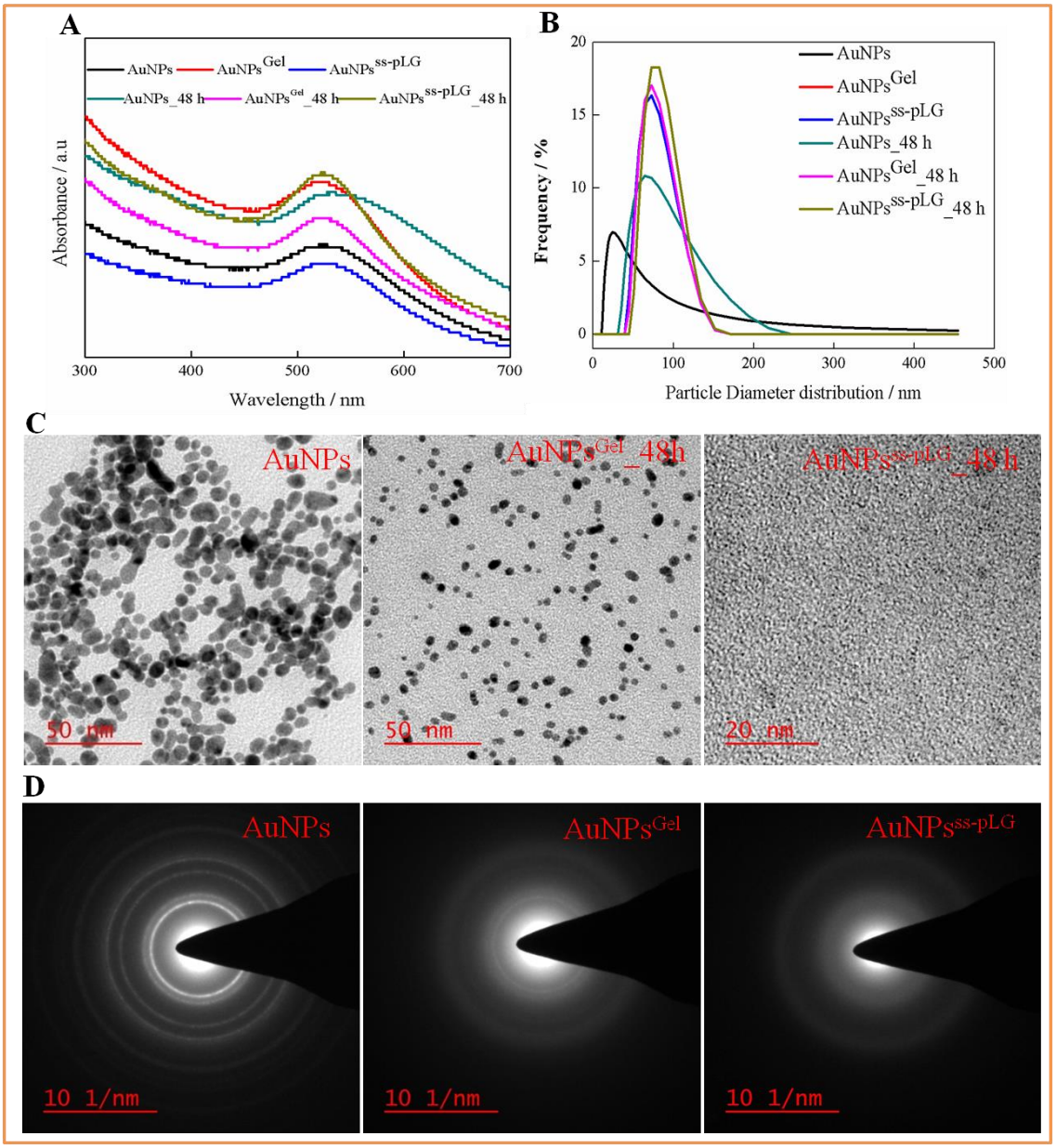


Figure 5.12. Effects of ss-pLG as a capping agent for AuNPs. (A) UV-visible spectrum of uncoated and polymer coated AuNPs. It confirms the synthesis of AuNPs by the observation of SPR peak ~520 nm. (B) Dynamic light scattering (DLS) behaviour of the synthesized AuNPs. Polymer coated AuNps show excellent colloidal stability. (C) TEM image of the synthesized AuNPs show spherical shaped NPs ranging from 0.4 to 30 nm.

(D) Selected area electron diffraction (SAED) pattern of uncoated and polymer coated AuNPs. Uncoated AuNPs show polycrystalline behaviour and AuNPs$^{ss\text{-}pLG}$ show highly amorphous behaviour.

the second step involves the stabilization of AuNPs using Gel and ss-pLG solution. The interaction between Gel/ss- pLG and the surface of AuNPs leads to form stable AuNPs, whereas uncoated AuNPs showed agglomeration that was observed through UV-Vis spec and DLS.

5.3. Conclusion

In this study, we evaluated the anti-amyloidogenic property of ss-pLG using BSA as a model protein. It was observed that ss-pLG possesses significantly higher potential than Gel to inhibit the formation of amyloids that may be attributed to the branched ss-pLG, which could reduce thickness of lamella. Further, ss-pLG did not induce hemolysis, which was evidenced by imaging RBCs using SEM and platelets using leishman stain. The rescuing of neurotoxicity was also observed in Aβ cell line model, MC65 cells. Though, we have established the anti-amyloidogenic potential of ss-pLG, the mechanism by which the aggregation is suppressed is yet to be explored. In addition, ss-pLG was shown to be an efficient capping agent for AuNPs. Thus, we anticipate that ss-pLG can be used as an efficicient biocompatible anti-amyloidogenic agent.