Chapter 7
Summary

7 Summary

The outcomes of the study of specifically designed nanoscaffolds could be a promising approach for treating Alzheimer's disease, bearing tremendous potential for localized actions for controlling symptoms of AD, and recovery of neurons simultaneously. The outcomes of the study are enlisted below:

- Simple and effective N-TIPS method has been used to develop nanoscaffolds loaded with Memantine HCL, in which PLGA (50:50/75:25) was used as a release control polymer and Pluronic F127 as a surfactant, stabilizer, and pore-forming agent.
- Based on the experimental design and the constraints applied, the Box-Behnken design predicted the optimal concentration of PLGA 19.18 mg/mL, Pluronic F-127 4.98 mg /mL, and rotation speed 500 rpm for the preparation of nanoscaffolds with predicted responses: drug loading of 11.6 % and Porosity of 82.7 %.
- Experimentally, drug loading and porosity of optimized memantine-loaded nanoscaffolds were calculated to be 10.31% and 86.61% respectively with a relative error of -1.28 and + 3.9, respectively (from the predicted values).
- The statistical analysis: ANOVA and regression analysis showed that there was significant effect of PLGA and interactive effect of Pluronic F-127 and rotation speed on drug loading and porosity.
- Pluronic F-127 is a non-ionic surfactant that behaves as a surface stabilizer and pore-forming agent, and an optimum concentration of Pluronic F-127 (4.97 % w/v) and rotation speed (500rpm) result in the formation of uniform minute-sized pores with high porosity.
- PAMPA studies showed improvement in permeability of Memantine upon loading in nanoscaffolds with permeability coefficient (Pe= 12.4 x 10-6 cm/s) as compared to pure drug memantine (Pe= 5.2 x 10-6 cm/s) and a controlled release pattern was obtained. Drug polymer and surfactant compatibility was studied by FTIR and the spectra of drug, polymer and excipients showed no interaction between drug, polymer and excipients.
- Also, thermal analysis (TGA/DTG curve) indicated enhanced thermal stability of (PEG-MEM-PLGA) SANs as compared to PLGA 50:50/75:25, Pluronics F127 due to presence of integrated polymeric structure of nanoscaffolds.
- Surface morphology and particle size distribution showed polyhedral self-assembled 3D structures in nano-size range of 242.1±130.9 nm with 0.377 polydispersity index and 23±4.51 mV zeta potential representing polydisperse and stable self-assembled

nanoscaffolds.

- In addition, the porosity and entrapment efficiency of (PEG-MEM-PLGA) SANs respectively was 82.33%±0.25 and 90.61%±0.48 showing good entrapment of Memantine within the porous polymeric matrix of (PEG-MEM-PLGA) SANs.
- The release studies from self-assembled nanoscaffolds showed a release of 94.52±0.23% of Memantine up to 72 h with Korsmeyer-Peppas drug release kinetics with correlation coefficient (R^2) of 0.9901 indicating sustained delivery of the drug.
- high swelliblity index (94.52±0.23 %) and drug release (84.8±0.051 %) of (PEG-MEM-PLGA) SANs were obtained at pH 6.8 due to wettability of PEG and PLGA, along with time and pH-dependent increase in biodegradability of (PEG-MEM-PLGA) SANs by hydrolysis into lactic acid and glycolic acid, maximum being at basic pH.
- Accordingly, phosphate buffer saline (pH 6.8) can be proposed as a suitable vehicle for administration of (PEG-MEM-PLGA) SANs which will facilitate self-assembly in 3D form with high drug release.
- The observed ideal storage condition for (PEG-MEM-PLGA) SANs was 25±2°C, 60±5%RH at which no significant change in entrapment efficiency (90.21±0.40 %) and % porosity (81.86±0.82 %) after 3 months of storage of SANs While decrease in entrapment efficiency and % porosity was observed at 40°C/75% RH suggesting that high temperature induces degradation and is not suitable for long term storage.
- Moreover, the behavioral study on scopolamine-induced amnesia model of mice treated with (PEG-MEM-PLGA) SANs and (PEG-MEM-PLGA) SANs-BMSc administered intrathecally showed significantly better learning capacities in mice as compared to free drug MEMp along with good escape latency patterns (the time for the animal to find the platform and escape the maze) indicating overall improvement in cognitive behavior.
- The neurobehavioral study such as Ymaze and Morris water maze study on mice treated with PEG coated memantine loaded PLGA nanoscaffolds showed a significant neurobehavioral effect (***$p<0.0001$) as compared to pure memantine drug and diseased control group by increasing % spontaneous alteration behavior in Y maze with decrease in time and lower distance movement for finding platform in water maze study indicating the improvement of cognitive function of mice.
- Furthermore, the microscopic study of section of mice brain treated with PEG coated memantine-loaded PLGA nanoscaffolds showed a decrease in the accumulation of amyloid β plaque and degenerated neurons and improvement in memory and learning skill of treated mice. It showed appearance of eosinophilic (degenerating) Purkinje

neurons with condensed nuclei and bright eosinophilic cytoplasm along with vacuolation within the overlying molecular layer suggesting concurrent swelling/degeneration of Purkinje neuron dendrites. The neuronal recovery were observed at 3rd, 6th and 9th week of treatment with (PEG-MEM-PLGA) SANs.

- Moreover, the enzyme kinetic inhibition assay revealed the mechanism which might be probably involved in AD. PEG coated Memantine-loaded PLGA nanoscaffolds showed higher inhibition for butyrylcholinesterase and β secretase compared to acetylcholinesterase indicating the probable retention of butyrylcholine, APP, and acetylcholine in the brain that might help to preserve cognitive functions and improve the mental condition in AD.
- The Lineweaver–Burk plot of AChE enzyme inhibition assay for memantine-loaded PLGA nanoscaffolds showed a decrease in the Vmax values from 0.403 to 1.112 μM/min, and the Km value changed from 25.46 to 76.36 μM, indicating non-competitive mode of inhibition with a Ki of 7.74 μM-1.
- The Lineweaver–Burk plot for BUChE enzyme inhibition assay for memantine-loaded PLGA nanoscaffolds showed an increase in Vmax value from 1.073 to 3.355 μM/min alongwith an increase in Km value from 15.5 to 23.93 μM-1 indicating non-competitive mixed inhibition with a Ki of 6.53 μM-1.
- The Lineweaver-Burk plot of β secretase enzyme inhibition assay for memantine-loaded PLGA nanoscaffolds shows an increase in value of Vmax (1.080 to 1.166 μM/min) and Km from 66.93 to 203.710 μM-1 indicating non-competitive mixed inhibition with a Ki of 6.11 μM.
- The maximum inhibitory effect of memantine-loaded nanoscaffolds on AChE, BUChE, and β secretase in the brain was found to be 61.38%, 83.83%, and 75.43% respectively. The half maximally inhibitory concentration (IC_{50}) of memantine-loaded nanoscaffolds in brain acetylcholinesterase (AChE), butyrylcholinesterase (BUChE) and β secretase were 3.653±0.81 μM, 6.035±0.085μM, and 5.68±0.144 μM respectively.
- The Lineweaver–Burk plot of AChE enzyme inhibition assay for PEG coated memantine loaded PLGA nanoscaffolds showed a decrease in the Vmax value from 0.868 to 0.0425 μM/min, though the Km value remain unchanged at 47.261 μM, indicating non-competitive mode of inhibition with Ki value of 6.32 μM-1.
- The Lineweaver–Burk plot of for BUChE for PEG coated memantine loaded PLGA nanoscaffolds showed a decrease in Vmax value from 0.192 to 0.0773 μM/min with an unchanged Km value of 51.40 μM-1 value indicating non-competitive inhibition with

an increase in Ki value from 6.35 to 21.8 μM-1.

- The Lineweaver-Burk plot of (PEG-MEM-PLGA) SANs for PEG coated memantine loaded PLGA nanoscaffolds showed increase in value of Vmax (1.046 to 1.206 μM/min) and Km from 2.0652 to 119.40 μM-1 indicating non-competitive mixed inhibition with increase in Ki value from 2.46 to 10.35μM.
- The inhibitory activity of AChE, BUChE and β secretase increased with a graded increase in inhibitor concentration (0, 2.5, 5, 10, 20 μM). The maximum inhibitory effect of (PEG-MEM-PLGA) SANs on AChE, BUChE and β secretase for cortex homogenate was found to be 65.38 %, 85.83 % and 73.43 % respectively, whereas for Hippocampus it was 89.92%, 90.32% and 84.63% respectively.
- The half maximally inhibitory concentration (IC_{50}) of (PEG-MEM-PLGA) SANs for cortex and Hippocampus homogenate of acetylcholinesterase (AChE) was 8.653±0.81 μM and 6.64±0.73 μM respectively.
- The half maximally inhibitory concentration (IC_{50}) of (PEG-MEM-PLGA) SANs for cortex butyrylcholinesterase (BUChE) and Hippocampus BUChE were 4.15±0.15 μM and 3.035±0.085μM respectively.
- The half maximally inhibitory concentration (IC_{50}) of (PEG-MEM-PLGA) SANs for cortex β secretase (βS) and Hippocampus βS were 3.68±0.144 μM and 2.90±0.10 μM respectively.
- This study showed higher inhibition of hippocampus AChE, BUChE and β secretase activity by (PEG-MEM-PLGA) SANs as compared to that of cortex region.
- (PEG-MEM-PLGA) SANs showed good hemocompatibility with 1.534 % haemolysis (low intensity red colour) as compared to pure Memantine drug (MEMp) and (MEM-PLGA) SANs indicating no rupture of RBC cells up to 12 mg/mL of (PEG-MEM-PLGA) SANs Memantine-loaded PLGA self-assembled nanoscaffolds show higher hemocompatibility (7.187 % at 12 mg/ml) as compared to pure memantine drug (9.26 % at 12 mg/ml).
- PEG coated PLGA nanoscaffolds (PEG-MEM-PLGA) SANs were the most hemocompatible due the stealth characteristic provided by addition PEG to the PLGA nanoscaffolds. PEG hydrates the phospholipid membranes and stabilizes the RBC membrane and also minimizes surface interaction of the nanoscaffolds with opsonin, preventing macrophage uptake and clearance from the body
- In addition, decrease in IL-1β, IL-6, IL-10, and TNF-α in was observed upon

administration of PEG coated memantine-loaded nanoscaffold.

- TNF-α concentration was found to be significantly higher in cerebral and hepatic tissue of AD induced mice as compared to normal healthy mice which was reduced after administration of MEMp, marketed (Admenta), (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs and (PEG-MEM-PLGA)-BMSc SANs. (PEG-MEM-PLGA)-BMSc SANs showed almost 14-fold and 15-fold higher reduction in cerebral and hepatic TNF-α level as compared to MEMp, marketed (Admenta), (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs.
- TNF-α level in renal tissue varied non-significantly amongst the groups because TNF-α were predominately expressed in cerebral and hepatic cells after induction of AD whereas TNF-α was lesser expressed in serum and renal tissue as compared to IL-1β, IL-6, IL-10.
- Potential activity of memantine and memantine incorporated nanoscaffolds showed significant effect on reduction of TNF-α, IL-1β, IL-6, IL-10 and thus contribute to reduction of neuroinflammation.
- Additionally, significant ($p^{***} < 0.001$) effect of MEMp, (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs and (PEG-MEM-PLGA)-BMSc on reduction in the levels of insoluble amyloid-β, hyperphosphorylated tau was observed via decrease in IL-1β concentration which leads to inhibition of NF-κB activity to restore cognition.
- (PEG-MEM-PLGA) SANs and (PEG-MEM-PLGA)-BMSc SANs treated animal group has shown prominent reduction in IL-1β level from 25.15 ± 1.57 pg/ml to 17.48±6.9 pg/ml in serum, 28.04 ± 4.2 pg/ml to 26.34±6.1 pg/ml in cerebral tissue, 93.33 ± 2.6 pg/ml to 28.29±2.4 pg/ml in hepatic tissue as compared to (MEM-PLGA) SANs.
- (PEG-MEM-PLGA)-BMSc exhibits the potential in reduction of neuroinflammatory response via regulating proinflammatory cytokines levels mostly in serum and cerebral tissue. (PEG-MEM-PLGA)-BMSc SANs reduces IL-1β in serum from 237.29±0.805 pg/ml to 10.98±2.71 pg/ml and cerebral tissue from 296.83±0.898 pg/ml to17.24±5.7 pg/ml.
- (PEG-MEM-PLGA)-BMSc SANs showed 20-fold, 17-fold and 1.5-fold reduction in IL-1β level in serum, cerebral and hepatic tissue respectively which indicates the maximized therapeutic effect in brain and blood with less distribution in other organs because BMSc facilitates neuronal recovery by regeneration confined to brain that helps in treatment of AD most effectively upon incorporation in nanoscaffolds loaded with memantine.

- Non-significant effect of IL-6 was observed in cerebral, hepatic and renal tissue in AD mice treated with MEMp, (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs, (PEG-MEM-PLGA)-BMSc SANs.
- Significant reduction in IL-6 serum level from 180.74±2.1pg/ml to 85.14±1.67 pg/ml, 28.37±6.03 pg/ml, 20.03±6.68 pg/ml and 15.02±1.67 pg/ml was obtained after administration of MEMp, (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs, (PEG-MEM-PLGA)-BMSc SANs respectively.
- The reduction of serum IL-6 concentration is mostly due to regulation of pro-inflammatory cytokines after administration of MEMp, (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs, (PEG-MEM-PLGA)-BMSc SANs. (PEG-MEM-PLGA)-BMSc SANs showed 10-fold, 5-fold and 3-fold reduction in serum IL-6 level as compared to MEMp, (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs.
- Furthermore, it was observed that level of IL-6 in cerebral tissue of mice treated with (PEG-MEM-PLGA) SANs (277.12±3.34 pg/ml) and (PEG-MEM-PLGA)-BMSc SANs (280.96±7.73 pg/ml) was elevated even after following the treatment protocol which indicates the contributory role in AD and higher than that of standard group treated with MEMp ((148.07±4.86 pg/ml) indicating the neuroregenerative capacity of nanoscaffolds containing bone marrow stem cells.
- The elevated IL-6 level indicates the neurodegenerative condition in AD mice initially without treatment and later neuroprotective behaviour of IL-6 was noticed after following treatment protocol with (PEG-MEM-PLGA) SANs and (PEG-MEM-PLGA)-BMSc SANs.
- IL-10 concentration in cerebral tissue and serum were reduced in animals subjected to administration of MEMp, (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs, (PEG-MEM-PLGA)-BMSc SANs.
- IL-10 concentration was significantly reduced in serum from 280.66±0.98 pg/ml to 36.32±0.33 pg/ml, and cerebral tissue from 139.24±1.91 pg/ml to 23.39±3.55 pg/ml in AD induced animal group treated with (PEG-MEM-PLGA)-BMSc SANs which showed 4-fold and 5-fold efficacy in comparison to standard, marketed, (MEM-PLGA) SANs and (PEG-MEM-PLGA) SANs respectively.
- (MEM-PLGA) SANs, (PEG-MEM-PLGA) SANs and (PEG-MEM-PLGA)-BMSc SANs treated group also showed slight increase in hepatic tissue IL-10 level as compared to AD mice.
- Similarly, IL-10 level in renal tissue was found to be slightly reduced in AD induced

animal groups treated with marketed formulation (Admenta) (135.24±2.59 pg/ml), (MEM-PLGA) SANs (107.21±5.62pg/ml, (PEG-MEM-PLGA) SANs (135±2.1 pg/ml) and (PEG-MEM-PLGA)-BMSc SANs (150.32±5.73 pg/ml compared to untreated AD mice (158.06±0.84pg/ml)

- The study demonstrated that the (PEG-MEM-PLGA)SANs, (MEM-PLGA)SANs and Admenta showed 12-fold, 9-fold and 3-fold increase in plasma half-life of memantine which prolonged its availability up to 39 h, 28 h and 17 hours respectively, as compared to memantine drug suspension.
- (PEG-MEM-PLGA)SANs (56.16±1.42h) showed greater elimination half-life as compared to (MEM-PLGA)SANs (41.34±1.32h), MEMp (20.61±2.01h) and Admenta (30.56±1.91h) which represents higher retention of drug in body for prolonged period [258]. This surface modification resulted in 2.8 fold increase in plasma half-life of nanoscaffolds over the parent particle (MEMp).
- Cmax and AUC (total) of drug for (PEG-MEM-PLGA)SANs were found to be significantly higher (9.53 μg/ml and 0.659 μg/ml*h, ***$p < 0.001$) than the (MEM-PLGA)SANs (6.78 μg/ml and 0.23 μg/ml*h), MEMp (6.38 μg/ml and 0.057 μg/ml*h) and Admenta (6.35 μg/ml and 0.136 μg/ml*h), which indicates prolonged and sustained-release effect.
- The relative bioavailability of (PEG-MEM-PLGA) SANs was found to be 2.26 and that of (MEM-PLGA) SANs was 1.69 as compared to Admenta. The improved bioavailability could be attributed to site specific delivery by intrathecal route of administration, and surface functionalization with PEG
- Eventually, diminished clearance, elimination rate constant and Vd of the (PEG-MEM-PLGA)SANs, and higher Cmax and AUC (total) than (MEM-PLGA)SANs, MEMp and Admenta indicated prolonged and sustained-release. Further, memantine pure drug suspension (MEMp) and marketed memantine tablet (Admenta) were minimally distributed in brain because of their inability to cross blood-brain barrier and maximally distributed in liver.
- Moreover, memantine pure drug suspension (MEMp) and marketed memantine tablet (Admenta) were observed in kidney and liver 30 minutes post dosing indicating its excretion and clearance from these organs.
- In pharmacodynamic studies, a significant reduction in amyloid plaque accumulation was observed. Biodistribution study showed higher retention of memantine in the brain, followed by liver and kidneys following treatment protocol with (PEG-MEM-

PLGA)SANs in AD induced mice.

- The intrathecal route of administration facilitates movement of drug across the blood-brain barrier and can be found in the brain within a short span of time. After 30 minutes of dosing the concentration of (PEG-MEM-PLGA) SANs increased being maximum at 72 h, after which it declined and remained in brain up to 120 h.
- The concentration of drug from (PEG-MEM-PLGA) SANs (2.995±1.99 μg/g) was approximately 1.7 times higher than that obtained from (MEM-PLGA) SANs (1.781±0.44 μg/g) in the brain after 72 h of similar dose exposure (10 mg intrathecally). However, the concentration of drug released from (PEG-MEM-PLGA) SANs was negligible in the liver and kidney but 72 h post-dosing the concentration increased up to 120 h.
- Memantine from pure drug suspension (MEMp) and marketed memantine tablet (Admenta) accumulated mainly in kidney and liver after 30 minutes of dosing. Meanwhile the memantine from pure drug suspension (MEMp) and marketed memantine tablet (Admenta) were minimally distributed in brain because of inability to cross blood-brain barrier.
- Liver function indicator enzymes ALT, AST, and bilirubin total in serum of mice administered with (PEG-MEM-PLGA)SANs (37.0±6.40 IU/Lt, 34.7±5.56 IU/Lt, and 1.7±0.05 mg/dl) were lower than those given (MEM-PLGA)SANs (39.2±1.37 IU/Lt, 39.8±1.79 IU/Lt and 2.46±0.09 mg/dl) and both were approximate similar to the non-treated control group (32.11±0.75 IU/Lt, 30.5±1.36 IU/Lt and 1.2±0.08 mg/dl) which indicates the safety of (PEG-MEM-PLGA)SANs and (MEM-PLGA)SANs.
- Kidney function indicators blood Urea and uric acid evaluated in (MEM-PLGA) SANs (34.80±0.05 mg/dl and 5.2±1.67 mg/dl) and (PEG-MEM-PLGA) SANs (30.5±0.08 mg/dl and 2.6±0.80 mg/dl) were found to be similar to the control group (30.2±0.02 mg/dl and 2.2±1.41 mg/dl); respectively which indicates no renal toxicity with the treatment.