Amino acid-based polymeric hydrogel and nanoparticles for neuroregeneration and siRNA-drug loaded formulation for anticancer applications

A Dissertation

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by

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CHAPTER: 4

SUMMARY, CONCLUSIONS and FUTURE SCOPES

The study aimed to synthesize the amino acid-based polymeric hydrogel and nanoparticles to investigate its potential for neuroregeneration and anticancer activities.

The literature survey concludes that oxidative stress is a common denominator of neuronal cell loss and stimulator for neurological consequences like neurodegenerative diseases, TBI and spinal cord damage. Although, stem cells hold promise in regenerative medicine, but self-renewal, multipotency, and tumorigenicity during and post-therapy are also raising concerns. hydrogel offers key advantage in delivery of neurotrphic factor due to high porosity, providing a 3D micro-environment for neuronal adhesion, growth and proliferation. For neuronal development, the neurotrophic factor plays a crucial role in enhancing neurogenesis. However, the delivery of neurotrophic factor is challenging due to the requirement of trophic factors at a specific concentration, as the excessive dose may create other consequences. And hence, the development of stimulatory/biomimetic hydrogel with preconditioning effect is an exciting research area.

As per the reported literature, epidemiological studies revealed that low cancer rate observed in neurodegenerative disease however, exception are mentioned Rojas et al. And Pan et al. That melanoma, breast carcinoma have been observed with increased rate in patients with PD similarly brain metastases from TNBC have revealed the poorest treatment response. Any very few polymers are known for anticancer property. Hence, development of anti-cancer polymer

will provide the synergistic effect in cancer treatment. siRNA therapy offers key advantages but still application is limited in cancer therapy and not achieved the success rate in term of FDA approval.

With this literature survey we successfully deigned objectives and we conclude that the synthesized polymeric hydrogel and nanoparticles have been qualified for biomedical applications such as neuroregeneration, making formulation with siRNA, and drugs.

The overall conclusion for this dissertation was drown by summarizing the results mentioned in **Chapter-3 (Part I, Part II, Part III**, **Part IV)**

Chapter 3 (Part I and Part II)- neuroregenration

- Glycine based-acryloyl monomer has been polymerized to obtain neurogenic polymeric poly(N-acryloyl-glycine)-b-(acrylamide) [p(NAG-co-Ac)] hydrogel for regenerative applications (Part I).

- Furthermore, This hydrogel successfully modulated and neurogenic and angiogenic properties of glycine/glutamate have been imprinted in poly (N-Acryloylglycine)-co-(acrylamide)-co-(N-acryloyl-glutamate) co-polymeric hydrogel [p(NAG-Ac-NAE)] (Part II) and established its dual roles in angiogenesis and neurogenesis, compared to the only glycine containing p(NAG-co-Ac) hydrogel.

- Synthesized p(NAG-co-Ac) and p(NAG-Ac-NAE) nano-hydrogel characterized through various characterization tools such as SEM, HRTEM, FTIR, ^{1}H NMR, ^{13}C NMR spectroscopy, MALDI-TOF, DSC, TGA, and XRD.

- Particle size and morphology of the polymeric particles were confirmed through the FESEM and HRTEM. And both the p(NAG-co-Ac) and p(NAG-Ac-NAE) particle size

were calculated to be 20-50 nm, both are highly cross-linked and they are semicrystalline in nature. p(NAG-co-Ac) exhibits high swelling (~1500%) while p(NAG-Ac-NAE) exhibits very high swelling of 6188%..

- The obtained zeta potential result for p(NAG-b-A) is ξ -25 ± 4 mV and p(NAG-Ac-NAE) is of ξ ─36.9 (± 6.28) mV. Which showed that stability of particle increased with incorporation of acryloyl glutamate and revealed that the hydrogel particles are colloidal stable

- MALDI-ToF spectrum which evident that the maximum molecular weight (MW) of p(NAG-co-Ac) hydrogel ranging in between 1357 to 3106 Da . While for p(NAG-Ac-NAE) The *Mn* and *Mw* are calculated to be 1690 Da, and 1773 Da, respectively (*PDI*) of 1.04.

- Rheological characterization of the hydrogels was performed. The viscosity and the shear rate with the various concentrations of hydrogel samples at 34 ^{o}C, 37 ^{o}C and 43 ^{o}C. The frequency sweep tests were conducted to evaluate the change in the viscoelastic modulus as a function of angular frequency (0.1-100 rad s^{-1}) with an oscillating strain of 5%. At the physiological temperature the storage modulus for p(NAG-b-A) hydrogel was found to be varied from 2.9 kPa to 3.7 kPa at 0.1 to 100 rad sec^{-1} and loss modulus was found to be varied from 2.4 kPa to 2.3 kPa. While for for p(NAG-Ac-NAE) Storage modulus (G') values were slightly decreased calculated to be varied from 2.3 kPa to 2.7 kPa at 0.1 to 100 rad sec^{-1} and loss modulus (G") varied from 0.12 kPa─0.16 kPa, at 0.1 to 100 rad sec^{-1}. It can be noted that 1 kPa - 3 kPa favours astrocyte differentiation, [58] and modulus of 2 kPa can increase the differentiation of immature to mature neurons.

Which could be the reason for more astrocyte like structure observed in p(NAG-co-Ac) while mature neuron observed in p(NAG-Ac-NAE) hydrogel.

- Both of the hydrogel share the Shear thinning behavior which allows injectability and possesses an advantageous role in drug delivery, cell encapsulation, granular hydrogel formation and in tissue engineering

- *In vitro* cell viability assay reveled that both the p(NAG-co-Ac) and p(NAG-Ac-NAE) copolymers were cytocompatible for Hek293, and HepG2 cell lines and accelerated the proliferation of PC12 up to 150% at concentration of 250 μg mL^{-1} p(NAG-co-Ac) and at the concentration of 500 μg mL^{-1} p(NAG-co-Ac).

- Macroscopic images revealed both Poly(NAG-co-Ac) and p(NAG-Ac-NAE) nanohydrogel provides a stable 3D extracellular mimetic environment and promotes healthy neurite growth for primary cortical neurons by facilitating cellular adhesion, proliferation, actin filament stabilization and neuronal differentiation.

- poly(NAG-co-A) hydrogel promoting nerve regeneration via the GSK3β inhibition.

- **Similarly both the** hydrogel promotes cellular proliferation and the healthy network formation of actin filament and confirms very high stability of the cytoskeleton network compared to the control sample.

- Both the hydrogel possess the anti-oxidant property, however the mechanism for both are slightly differ.

- Like in p(NAG-co-Ac); Presence of free radicals in stress conditions may cause the oxidation of p(NAG-co-Ac) hydrogel. These free radicals can attack to ─C=O bond of

polymer and can oxidize the p(NAG-co-Ac) and can release CO_2 and water as byproducts. Further the conversion of secondary amine is primary amine is occurred due to the attack of $OH^{\cdot}$ radical which has been confirmed from the FTIR band shifting from 1658 cm^{-1} (pNAG-co-Ac) to 1637 cm^{-1} (H_2O_2 treated p(NAG-co-Ac)) This interaction of ROS and stimulatory effect on astrocyte assists the neuroprotective role. Further, controlled synthesis and purification of (pNAG-co-Ac) hydrogel with different molecular weights can provide the clear insight about the neurogenesis and neuroprotection efficacy of the hydrogel.

- p(NAG-Ac-NAE) mainly trigger the molecular event. The p(NAG-Ac-NAE) and p(NAG-co-Ac) hydrogels show a protective role via scavenging ROS generation and mitochondrial membrane depolarization in oxidative stress conditions. We also observed a higher cellular proliferation in p(NAG-Ac-NAE) hydrogel compared to the p(NAG-co-Ac) hydrogel, due to the excitatory role of glutamate and cooperative phenomena of glycine and glutamate. A significant increase in cell proliferation is observed in p(NAG-Ac-NAE) hydrogel, whereas a decrease in proliferation is observed in the case of p(NAG-co-Ac) hydrogel treated PC12 cells. Our results further demonstrated that the p(NAG-Ac-NAE) hydrogel promotes stable cytoskeleton network formation.
- The p(NAG-Ac-NAE) hydrogel induces higher pro-angiogenic activity compared to the p(NAG-co-Ac) hydrogel and shows neurogenic potential in primary neuronal cells. Neo-angiogenic activity of glutamate is reduced by the development of p(NAG-Ac-NAE) hydrogel and showed cytotoxic behaviour on aggressive cancer cell lines. The results are clear shreds of evidence that the p(NAG-Ac-NAE) hydrogel is angiogenic, while p(NAG-co-Ac) hydrogel is anti-angiogenic in nature. The results further indicate that the

angiogenic potential could arise due to the presence of excitatory neurotransmitter glutamate imprinting.

- In conclusion, the obtained results revealed that the synthesized p(NAG-Ac-NAE) and p(NAG-co-Ac) hydrogel shows excellent cytocompatibility, hemocompatibility, favourable mechanical stability, and neuroprotective in nature. Its difference in angiogenic properties useful in use of different neurological conditions for repair mechanism,

In the Part 3 Chapter 4, the main objective addressed is: Development of glycine based Anti-cancer bio-polymeric poly(N-acryloyl glycine-acrylamide) hydrogel for poor prognosis heparanase driven malignancies.

The main findings of this part have been summarized below pointwise.

- Motivation of this part of the work is: at a molecular level proteases and heparanase are implicated in an invasive tumour progression by remodelling the extracellular matrix and worsening the prognosis by promoting metastasis and angiogenesis. Glycine plays a pivotal role in cell survival and proliferation. Most of the anti-cancer agents alter the glycine metabolomics and suppress cell proliferation.
- Therefore, in the present part, the geometrical and electronic properties of Glycine, N-acryloylglycine monomer (NAG), and polymeric units of poly [(N-acryloylglycine)-co-(acrylamide)] p(NAG-co-Ac) are rationalized by density functional theory (DFT) with B3LYP/ 6-311g* and experimental evidences have been provided for their uses in cancer therapy.
- The Frontier Molecular Orbital theory (FMO) reveals that the introduction of acrylamide/ divinyl benzene in the polymer has increased the biological activities by lowering the band gap energy.
- Linear and cross-linked co-polymeric rearrangements show the protease inhibitory activities of biodegradable p(NAG-co-Ac) hydrogel. It is revealed that the cross-linked

homo-polymeric tetrameric arrangements interacted with the heparanase binding domain II (HBDII) with more affinity dock score of -11.08 kcal mol^{-1} in nanomolar concentration.

- Experimentally, it is found out that the hematocompatable p(NAG-co-Ac) hydrogel shows anti-proliferative and migratory inhibitory activity towards the invasive aggressive cancer such as triple negative breast cancer (TNBC) by intracellular ROS production and leads the programmed cell death.
- *In vitro* cytotoxicity of p(NAG-co-Ac) hydrogel has been studied and the results were compared with cytotoxicity of glycine and N-acryloyl glycine. The p(NAG-co-Ac) hydrogel shown higher cytotoxicity towards aggressive cancer cell lines then the normal healthy cell lines. N-acryloylglycine shown higher cell killing efficiency in HEK293, however it reduces by the formation of p(NAG-co-Ac) hydrogel. We observed the lack of cell killing effect of p(NAG-co-Ac) hydrogel in MCF-7 cell line, which may be observed due to the low expression of heparanase in MCF-7, then the MDAMB-231 and LN229. In LN299 cells, the glycine induces the necrotic death which may be due to the excessive ROS generation, while p(NAG-co-Ac) hydrogel induces the programmed cell death by modulating the ROS production at therapeutic level.
- We further observed the reduced vessel area, total number of junctions, junction density and total vessel length which demonstrate the anti-angiogenic behaviour of p(NAG-co-Ac) hydrogel in; in-ovo system . This behaviour may be observed due to the heparanase inhibitory potency of p(NAG-co-Ac) and the heparanase is tightly involved in the angiogenesis. As database shows, the highest expression of heparanase was observed in esophageal cancers, bladder cervical and colorectal cancers etc.,
- In conclusion, p(NAG-co-Ac) with anti-angiogenic and anti-tumorigenic capabilities has made it as a future potential anticancer polymer for heparanase-driven

invasive malignancies and synergistic delivery system which further could improve the prognosis.

- **<u>Part 4</u>: Synthesis and characterization of polymeric nanoparticles for siRNA and drug delivery for the treatment of cancer.**

- P(NAPA), P(LME) And p(NAPA-co-LME)Nanoparticle were synthesized with narrower size i.e below 100nm compared to already published literature. And The zeta potential for P(NAPA) was -35mV, for P(LME) is -38mV, while with for formation of p(NAPA-co-LME) the zeta potential was decreased and was of -28mV.
- Side chain containing amino acid derived polymer exhibits the significant properties such as stimuli responsiveness, self-assembling characteristic behaviour, presence of amino group and -COO favour the interactions with drug and small molecule siRNA.
- p(NAPA-co-LME) is cytocompatible in normal healthy cell line like PC12 and HepG2 and L929 while cytotoxic toward the MDAMB-231 and LN229.
- Individually P(NAPA), P(LME) are show the heparanase activity, while increased activity observed in formation of p(NAPA-co-LME) copolymer.
- p(NAPA-co-LME) cellular internalization through the LAT1 transporter and clathrin mediated endocytosis and follow the endosomal lysozomal escape mechanism.
- Presence of aromatic rings in the polymer enhance the loading efficiency of siPOL2RA up to 70% and high loading efficiency observed for drug along with the silencing of POL2RA. Chemical modification and entrapment in nanoparticles induce the serum stability of siRNA. POL2RA expression is also correlated with other cancer overexpressed marker cyclin dependent kinase and silencing of POL2RA alter the expression of CDK finally induce programed cell death.

- In conclusion, this siRNA/drug loaded novel polymeric nanoparticle separately or combination could be a multi-targeting tool in advance stage cancer therapy in a targeted manner.

4.2. FUTURE SCOPES

A series of Polymeric nanomaterials such as polymer hydrogel, polymer capsules etc have been reported in this dissertation. All the materials are biocompatible and tested their uses for various applications in cell based study only.

For the clinical applications these materials should be tested in the in vivo models.