Development of Amino acid based Copolymeric Nanoparticles for Organ Targeting Delivery of Drugs and Tissue Regeneration

A Dissertation

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by

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Chapter 4

Summary, Conclusions and Future Scopes

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SUMMARY, CONCLUSIONS, AND FUTURE SCOPES

4.1. Summary and Conclusions

A brief of this dissertation work has been discussed concisely in this chapter. This dissertation work aims to develop a library of amino acid-based copolymer nanoparticles (NPs) with various compositions. The designed NPs are qualified for multiple biomedical applications such as organ targeting drug delivery, wound healing and tissue regeneration, and also act as a suitable model polymer to develop a gold standard method for angiogenesis analysis. Particularly, multiple drug-loaded carriers in a combination mode are studied for TNBC treatment. Based on the experimental demands, some computational works, such as *in silico* docking, Network Pharmacology, and Molecular Dynamics Simulation, have been performed.

However, chapter wise results and key findings as a summary and conclusions for this dissertation are described below.

Chapter -1 discuss the introduction and literature review based on the dissertation work such as polymer and polymer NPs; amino acids with their bio functions; biomedical applications of poly(amino acid)s; integration of computational methods in healthcare therapeutics; global statistics on TNBC; role of amino acid based nanocarriers in TNBC; origin, categories and phases of wounds; polymers used for wound healing, tissue regeneration, angiogenesis, and methods used for analyse angiogenesis followed by motivation, research gaps, and objectives with proper justifications.

Chapter 2 is based on the “Materials and Methods” used in this work. A detailed synthesis and characterization followed for the copolymer NPs and nanoformulations (NFs) are described. The detailed steps followed for molecular docking, network pharmacology, and MD simulation

are explained here. Further, this chapter discusses the process of drug loading, release, and theories behind the mathematical model are described. All steps followed for *in vitro* cell-based studies are thoroughly described. Furthermore, the *in vivo* protocols used in this work are given. Additionally, the analysis methods and codes used for the *in ovo* CAM assay study are also appended.

Chapter 3 This chapter is divided into four subparts which describe methodologies, results, and discussion on four different objectives of this dissertation.

Part 1: In this part, "A library of organ targeting drug delivery NPs (OTDDNs) based on poly[(*N*-acryloyl glycine)-co-(*N*-acryloyl-*L*-phenylalanine methyl ester)]" has been developed with different targeting capacities to major organs, and further treatment efficacy of 1:4 composition of breast targeting nanocarriers has been evaluated by encapsulating with phyto drugs.

The key findings are summarized below.

- A library of organ targeted amino acid-based amphiphilic copolymer NPs, i.e., p(NAG-co-NAPA)$_{(x:y),}$ are synthesized via mini-emulsion free radical polymerization.
- The physicochemical characterizations of the synthesized p(NAG-co-NAPA)$_{(x:y)}$ NPs are conducted to ensure the synthesis and subsequently screened for their potential to act as a drug delivery vehicle to the different organs.
- *In vivo* organ based biodistribution is studied to reveal the best OTDDNs targeting breast, i.e., p(NAG-co-NAPA)$_{(1:4)}$ NPs (P4).
- In addition to this, an *in silico* study is also performed to justify the selection of piperine and DHA, since they target 14 genes that are responsible for TNBC.
- The prepared piperine (PP4) and DHA (DP4) loaded NFs possess excellent drug loading and pH-dependent drug release that enhances the targeted delivery in the TNBC site by minimizing the systemic side effects.

- Further, the low IC_{50} values in combination treatment, i.e., with Co-NPs(7:3), revealed the synergetic treatment efficiency of TNBC rather than antagonistic behaviour.
- The flow cytometry results revealed that the inhibition of TNBC by Co-NP(7:3) formulation is accomplished due to the mitochondrial membrane potential mediated apoptosis by arresting the G2-M phase in the cell cycle.
- Further, the p(NAG-co-NAPA)$_{(x:y)}$ NPs of the OTDDS library represent various potential healthcare applications, which are useful for a number of organ based targeted therapies such as liver, heart, lungs, and kidney.

Part 2: In this part**,** "Folate conjugated poly[(*N*-acryloyl glycine)$_1$-co-(*N*-acryloyl-*L*-phenylalanine methyl ester)$_4$]" Copolymer NPs have been used to co-deliver of Dihydroartemisinin and Piperine for the enhanced treatment of TNBC. A detailed *in vitro* study was performed on the MDA-MB-231 cell line to identify the genes responsible for it.

The key findings have been summarized below.

- The current challenges associated with the targeted therapy is drug resistance, tumor heterogeneity and limitation in predicting the responsible biomarkers for TNBC. Our earlier work has reported the anticancer effect of combined DHA or Piperine loaded P4 NPs. However, the underlying molecular pathways and cellular genes responsible for inhibiting TNBC are not elucidated.
- Further, there is a scope to achieve improved targeting efficiency by modifying the NPs with receptors. In this line, we have conjugated the P4 NPs with folic acid to achieve maximum TNBC targeting efficiency through click chemistry method. Physicochemical properties were studied to ensure their successful synthesis followed by *in vitro* studies to check their TNBC efficiency. The prepared NFs exhibited an excellent pH dependent drug release through passive diffusion mechanism.

- The low IC_{50} value of Co-$NFs_{(8:2)}$, revealed the synergetic treatment efficiency of TNBC rather than antagonism or additive behaviour. The flow cytometry results depicted mitochondrial membrane potential mediated cell death in TNBC through G2-M phase arrest.
- Gelatin zymography study revealed the suppression of MMP-2/MMP-9 activity by focusing the anti-angiogenic non-invasive properties of CO-NFs. RT-PCR results confirms the anti TNBC nature of Co-NFs by demonstrating the changes in specific oncogenic markers such as DNMT3B, EGFR, Ki67, STAT3, Bcl2, CDK2 and Casp9 which further confirm the DNA-targeting, anti-proliferative and pro-apoptotic nature of Co-NFs.
- However, to comprehend the anti TNBC potential of the NFs and for successful clinical translation, *in vivo* studies are taken under consideration and will be reported as the advancement of this work.

Part 3: In this part, **"**A cross linker free and biocompatible copolymer, p(NAG-*co*-NAPA)$_{wc}$ NPs" has been synthesized to avoid the toxicity associated with the chemical cross linkers in the long run. The self-assembled structure of the NPs has been confirmed through MD Simulation. Further, this p(NAG-*co*-NAPA)$_{wc}$ NPs' potential use in wound healing and tissue regeneration application has been established in the rat model.

The key findings of this part are summarized below.

- We have successfully synthesized a novel self-assembled amino acid-based random di-block copolymer NPs of NAG and NAPA, i.e., p(NAG-*co*-NAPA)$_{wc}$.
- From various spectroscopy studies, it is confirmed that p(NAG-*co*-NAPA)$_{wc}$ was formed via mini emulsion radical polymerization without using any additional covalent cross-linker.

- The H-bonds caused by carboxylic acid groups of NAG and π-π stacking caused by the phenyl ring of NAPA triggered non-covalent cross-linking and accelerated the formation of self-assembled spherical p(NAG-*co*-NAPA)$_{wc}$ NPs and promoted the construction of a beautiful network-like structure.
- MD simulation studies also revealed that the p(NAG-*co*-NAPA)$_{wc}$ NPs formed aggregates in the temperature range of 298 to 315 K. In aggregates, hydrophobic and hydrophilic groups can make separate domains and show interactions in a proximity.
- As anticipated, the hemolysis study and in vitro cytotoxicity assay revealed that p(NAG-*co*-NAPA)$_{wc}$ NPs are hemocompatible and cytocompatible.
- The exciting hallmarks, angiogenesis, cell migration, and proliferation properties established the regenerative property of p(NAG-*co*-NAPA)$_{wc}$ NPs.
- Additionally, *in vivo* experiments demonstrated p(NAG-*co*-NAPA)$_{wc}$ NF's potential for use in regenerative medicine, i.e., acute wound healing without additional growth factors such as cytokines, cells, or genes, etc.

Part 4: To develop a gold standard method for the quantitative assessment of angiogenesis Gray Level Co-occurrence Matrix based Image Processing tool has been used. To establish this method, poly(*N*-acryloyl-glycine)-co-(*N*-acryloyl-*L*-phenylalanine methyl ester) NPs with a 1:1 composition is used as a model polymer.

The key findings are summarized below.

- The present work has developed an angiogenesis evaluation tool based on GLCM features that is straightforward and physiologically relevant.
- To establish this method, an amino acid-based copolymer p(NAG-*co*-NAPA) NPs, has been synthesized as a modelled polymer.
- As anticipated, hemolysis and *in vitro* viability assays with various cells, including HUVEC, revealed the biocompatible and proliferative nature of the NPs. The dose and

time-dependent angiogenic properties of p(NAG-*co*-NAPA) NPs have been computed from microscopic images obtained from *in ovo* model and tube formation assay using GLCM texture features based Image processing tool.

- As a comparison, further tube formation results are cross-verified with Angiotool analysis. Both tools depict that a 10μg dose of p(NAG-co-NAPA) NPs can be an optimum dose for promoting angiogenesis.
- These findings resemble that the application of Image processing tools in wet-lab provides an unbiased and high throughput analysis of various texture based features from 2D microscopic images over existing conventional tools.
- However, to comprehend the efficacies of p(NAG-*co*-NAPA) NPs and to make GLCM texture features based Image processing tool as a gold standard for the analysis of angiogenesis, more computational models and *in vivo* studies can be taken into consideration.

4.2. Future scopes of the present work

A library of copolymer NPs has been developed in this dissertation. All the NPs are biocompatible, hemocompatible and have been tested for their potential uses for various biomedical applications in cell-based studies.

As a future scope of this work, most importantly, *in vivo* toxicology studies for all the NPs can be considered for further validation of the library of copolymer NPs, along with cross linker free copolymer NPs. Further, to comprehend the efficacies of the NFs developed in Chapter 3 (Part I and II) and to progress towards future clinical trials, *in vivo* studies and gene-level expressions can be taken into consideration as a future scope of this work. Additionally, a probable mechanistic pathway can be established for the combination therapy of DHA with Piperine through amino acid-based copolymer nanocarriers. Furthermore, p(NAG-*co*-NAPA)$_{wc}$ NP's regenerative potential can be studied in higher animal models for the clearance of phase

trials (Chapter 3 (Part III)). Finally, Chapter 3 (Part IV) can be further validated through Machine Learning and Artificial Intelligence-based codes and can be translated into a user-friendly tool to analyse experimental angiogenic results with more precision and accuracy.

All the results will be reported as the continuation of the respective works.