

5.1. Summary

ABX is a chimeric monoclonal antibody reported for antithrombotic activity but their delivery remains challenging due to its poor stability in a biological system. The purpose of the first research work was to deliver ABX on the target efficiently using MSN. ABX-coated mesoporous silica nanoparticles (MSN-ABX) were formulated and analyzed for particle size, shape, zeta potential, surface morphology, and surface chemistry. XPS analysis confirmed the presence of ABX on the surface of amino-functionalized mesoporous silica nanoparticles (MSN-NH₂). The degree of ABX attachment was 67.53 ± 5.81 % which was demonstrated by the Bradford assay. Furthermore, the targeting efficiency of the targeted nanoparticles has been evaluated by capturing the fluorescent images *in-vitro* which showed the significant accumulation of the ABX-coated nanoparticles towards activated platelets. The significant ($P < 0.05$) increase in affinity of DiD dye-loaded nanoparticles towards the activated platelets was confirmed by using *in-vitro* imaging through photon imager optima. The hemolysis study of the nanoparticle formulations revealed that they were non-hemolytic for healthy human blood. The *in-vitro* antithrombotic effects of MSN-ABX were observed by blood clot assay and other *in-vivo* studies, revealing its superior antithrombotic activity over clinical injection of ABX and could be a promising carrier for improved ABX targeted delivery.

The targeting potential of ABX as a targeting peptide was further explored in combination with RUT. RUT is a flavonoid obtained from a natural source and is reported for antithrombotic potential, but its delivery remains challenging because of its poor solubility and bioavailability. In the second research work, we have fabricated novel RUT-loaded liposomes (RUT-LIPO, nontargeted), liposomes conjugated with RGD peptide (RGD-RUT-LIPO, targeted), and ABX (ABX-RUT-LIPO, targeted) by ethanol injection method. The particle size, ζ potential, and morphology of prepared liposomes were analyzed by

using DLS, SEM, and TEM techniques. The conjugation of targeting moiety on the surface of targeted liposomes was confirmed by XPS analysis and Bradford assay. *In-vitro* assessment such as blood clot assay, aPTT assay, PT assay, and platelet aggregation analysis was performed using human blood which showed the superior antithrombotic potential of ABX-RUT-LIPO and RGD-RUT-LIPO liposomes. The clot targeting efficiency was evaluated by *in-vitro* imaging and confocal laser scanning microscopy. A significant ($P < 0.05$) rise in the affinity of targeted liposomes toward activated platelets was demonstrated which revealed their remarkable potential in inhibiting thrombus formation. Furthermore, an *in-vivo* study executed on Sprague Dawley rats (FeCl_3 model) demonstrated improved antithrombotic activity of RGD-RUT-LIPO and ABX-RUT-LIPO compared with pure drug. The pharmacokinetic study performed on rats demonstrates the increase in bioavailability when administered as liposomal formulation as compared to RUT. Moreover, the tail bleeding assay and clotting time study (Swiss Albino mice) indicated a better antithrombotic efficacy of targeted liposomes than control formulations. Additionally, the biocompatibility of liposomal formulations was determined by an *in-vitro* hemolysis study and cytotoxicity assay, which showed that they were hemocompatible and safe for human use. A histopathology study on rats suggested no severe toxicity of prepared liposomal formulations. Thus, RUT-encapsulated nontargeted and targeted liposomes exhibited superior antithrombotic potential over RUT and could be used as a promising carrier for future use.

In summary, the ABX was efficiently delivered by using MSN as a nanocarrier and it was further explored as a targeting peptide for the site-specific delivery of RUT in the case of a thrombotic event. Here, we concluded, that the loading of ABX to the MSN can able to improve the stability, antithrombotic potential of ABX and simultaneously able to minimize the associated side effects. Moreover, the conjugation of ABX in RUT-LIPO as

a targeting moiety for the site-specific anti-thrombotic therapy showed superior *in-vitro* and *in-vivo* activity over the pure drug and nontargeted liposomes.

5.2. Conclusion

The objectives of the present research work include

- To develop ABX-coated mesoporous silica nanoparticles and to evaluate their physicochemical properties, *in-vitro* release, *in-vitro* activity-based assays, and *in-vivo* tail bleeding, clotting time, and FeCl₃-induced thrombosis studies in animals.
- To develop RUT-loaded targeted liposomes and to evaluate their physicochemical properties, *in-vitro* release characteristics, *in-vitro* activity-based assays and *in-vivo* pharmacokinetic study, tail bleeding, clotting time, FeCl₃-induced thrombosis and histopathology studies in animals.
- The particle size and polydispersity of both the nanoformulations were within acceptable limits.
- The acceptable range of zeta potentials of MSN-ABX and targeted liposomes were observed indicating their higher stability.
- The morphological assessment by SEM, TEM and SAED analysis showed that MSNs and liposomes were spherical and monodispersed.
- The XPS survey demonstrated the surface chemistry of MSN-ABX and targeted liposomes, the result supports the presence of ABX (in case of MSNs) and targeting ligand (RGD and ABX for liposomes) respectively on the surface of nanoformulations.
- The *in-vitro* drug release profile of ABX and RUT in case of MSNs and RUT-LIPO respectively showed sustain and prolonged release profile at pH 7.4.
- The *in-vitro* studies to evaluate antithrombotic property (blood clot assay) confirms the enhanced therapeutic potential of both the nanoformulation.

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- The DiD loaded nanoformulations showed enhanced platelet interaction as compared to pure DiD dye, and non-targeted formulations through *in-vitro* imaging studies.
 - Biosafety assessment of MSNs and liposomes using human blood confirms the biocompatibility and non-hemolytic nature of the nanoformulations.
 - In cytotoxicity evaluation the obtained results suggests that RUT elicit dose-dependent cytotoxicity, however the liposomal formulations possess no significant cytotoxicity and proven the cellular biocompatibility.
 - *In-vivo* (FeCl₃ induce thrombosis rat model) evaluations demonstrated that both the nanoformulations (MSNs and liposomes) maintained the antithrombotic function of ABX and RUT respectively, they showed to have a superior therapeutic benefit over ABX.
 - The pharmacokinetic evaluation of liposomal formulations was performed in Dawley rats, all the formulations exhibited improved pharmacokinetics than their respective pure drug form.
 - The *in-vivo* histopathological evaluations of liposomal formulations have demonstrated better safety in Sprague Dawley rats as compared to RUT.
 - The stability studies for 3 months at 4-8 °C was performed showing no significant physical changes in particle size and PDI values. The MSN formulation was found to be stable for 2 years in lyophilized form at room temperature. However, the further extended stability study of liposomes shown non-significant physical changes on long term storage. It was concluded that the use of suitable stabilizers and surfactant in combination of aseptic manufacturing can lead to long-term stability of nanomedicines.

5.3. Future perspective

To date, only a few USFDA approved anti-thrombotic drugs are available for the prevention and treatment of cardiovascular diseases like acute ischemic stroke, myocardial infarction, etc, despite their limitations in both efficacy and safety. Targeted anti-thrombotic therapy is a potential alternative to optimize the efficacy, safety and potency of therapy. Incorporating anti-thrombotic drugs within nanocarriers in combination with targeting structure can lengthen the residence time of the drug in the body, therefore, attain compelling thrombolysis at a reduced dose than standard; regulation of antithrombotic activity by controlling the unwanted release during the circulation thus decrease the risk of intracerebral hemorrhage; achieve sufficient local interaction of drugs with thrombus and allow targeted anti-thrombotic activity by customized nanocarriers decorated with targeting moieties or through US/magnetic force irradiation or by using shear force. Advanced antithrombotic nanomedicines developed by utilizing multifunctional strategies (incorporating targeted ligands or proteins, applying external stimuli like magnetic field and US, shear activated therapeutics, and adding imaging agents) will probably allow the development of numerous novel products for efficient antithrombotic therapy. Theranostic nanomedicines that contain both therapeutic agents and diagnostic substances can be served as a promising delivery system for personalized antithrombotic therapy (specific for each patient who suffers from thrombosis) to improve the specificity and efficacy of anti thrombotic drugs. The use of nano delivery systems to co-deliver thrombolytic drugs by utilizing targeting strategies shows a promising future in extending therapy with drugs in less period of time as well as provide a significant increase in therapeutic efficacy through synergistic effects. In further research we want to explore the targeted nanomedicines of other clinically used antithrombotic drugs in the combination of multiple targeting approaches for the specific management of thrombosis.

5.3.1. Translational potential

The ultimate objective of nanomedicine development has always been the generation of translational technologies that can improve existing treatments. Cardiovascular diseases in one of the primary target of nanomedicines since it affects the largest population worldwide and their clinical management is extremely tough due to life-threatening risk associated with available therapies. In this work, nanomedicine demonstrated the ability to improve drugs targeting via improving their pharmacokinetic properties and to provide a mechanism to establish novel therapeutic concepts utilizing physical interventions and biologics. However, in terms of commercial and practical feasibility, these nanomedicines use cheaper, readily available ingredients, and their large-scale production methods are well established which serves as an additional advantage for future clinical development.

Moreover, in this research we have performed initial preclinical examination of nano formulations. However, appropriate in-depth preclinical investigation studies, and the clinical trials must be carried out to confirm its potency in inhibition of platelet aggregation in individuals. The major concerns involved related to side effects like hemorrhage, biodegradability, toxicity should also be addressed prior to its regulatory approval for clinical usage. In addition the industrial scale manufacturing procedures has to be optimized for large scale production.

5.3.2. Challenges and limitations in the commercial production

Key challenges concerning the clinical development of these nanomedicine involve a number of factors including biological barriers, large-scale production, biocompatibility and safety, intellectual property, government restrictions, and overall cost-effectiveness in contrast to present medicines. These concerns can impose considerable barriers to the commercialization of nanomedicines, regardless of whether they are therapeutically helpful or not. Moreover, the main limitations of these nanomedicines involve the commercial

production. Conventional pharmaceutical industrial facilities are usually not well equipped for the manufacturing of nanomedicine products due to constraints in using organic solvents, capability to handle and process nanosized drugs/excipients due to environmental/operator/product safety concerns and requirement of sterile-manufacturing capability. Other than that, the clinical safety and efficacy estimation of these nanomedicines will be required before commercialization.