Development and Optimization of Molecular Target Oriented Nanoparticles for Amelioration of Chronic Inflammatory Diseases(s)

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Summary

Inflammation is a protective response of the body's defence mechanism through the release of a variety of pre and pro-inflammatory mediators. When the body fails to deal with acute inflammation it will lead to chronic inflammation. To manage this chronic inflammation multiple drug regimens with different dosage forms are prescribed to treat as well as avoid such conditions.

Chronic inflammatory diseases are generally treated with systemic administration of immunosuppressant or anti-inflammatory or a combination of both drugs. Long-term administration of these drugs are associated with severe systemic side effects. A significant number of patients fail to respond to current regimens and none of them completely cure chronic inflammations.

The conventional dosage form delivers drugs to both targeted and non-targeted sites. The non-targeted sites drug delivery raises some issues like higher drug payload which produces undesirable side effects. Long-term use of these drugs is also associated with drug resistance or requiring large doses to elicit desired therapeutic effects and this enhanced drug payload is lead to affects vital body organs and physiological homeostasis. So, to deal with such types of issues, the need to develop newer therapeutic agents/dosage regimens, as well as advanced drug delivery systems for precise and effective treatments, arose.

To deal with such issues, the current study aimed to develop and characterize nano-carrier systems for targeted drug delivery in a controlled manner. Keeping in mind the present research scenario, the naturally occurring bioactive constituent thymoquinone (TQ) was chosen as a drug and its delivery was performed in a unique way using lipidic and pH-sensitive polymeric nano-carriers. Both carriers were designed, optimized, and evaluated for their drug loading and delivering ability. Further, the designed nano-carriers system was investigated for the amelioration of chronic inflammatory disease.

The selected drug (TQ) is reported to act as an anti-oxidant and an immunomodulator along with anti-inflammatory potential. Their anti-inflammatory and immune-modulating potential to ameliorate rheumatic inflammation are given in Figure 1.13 and chapter 1 sections 1.11.4 and 1.11.5. TQ ameliorates symptoms of chronic inflammation *via* modulation in the signaling of several pathways, including

toll-like receptor, NF-κB, MAPKs, Wnt/β-Catenin, and apoptosis-regulated signaling kinase 1(ASK1) signaling.

The pharmacological applications of TQ are limited due to its lower aqueous absorption and enzymatic degradation. So, it was thought meaningful, to incorporate it into the nano-particulate system to utilize its maximum therapeutic potential. The drug was procured and authenticated for purity by FT-IR, NMR, and UV spectroscopy, and the drug was found as per the given manufacturer specifications.

The calibration curve of the drug was established using UV and HPLC. In UV analysis the lower limit of detection of the drug (TQ) in phosphate buffer saline buffer pH 6.8 and methanol UV was found to be 5µgm/mL and the method shows linearity in the concentration ranges of 5-1250 µgm/mL with R^2 value 0.999. While via HPLC analysis, the intense peak of TQ was observed at 6.557 and the calibration curve showed linearity in the concentration ranges of 500- 10000ng/mL in methanol with an R^2 value of 0.9993.

The targeted delivery approach may deliver the bioactive molecules specifically to the inflammatory sites thereby increasing the effectiveness as well as reducing the dose of the drug. TQ was incorporated in the modified macromolecule (pH-sensitive grafted guar gum) and tamanu oil potentiated lipidic systems to improve its therapeutic application for the management of chronic inflammation like rheumatoid arthritis.

The pH-driven polymeric nanostructures are among the most explored stimuli-responsive nanocarriers due to variations in the physiological pH within the body. In pathological states like cancer, systemic infection, and inflammation, the extracellular pH is slightly lower than that of normal tissues and other biological fluids. Due to the wide range of pH deviation in human body systems, the use of pH-driven carriers widely explored for the molecularly targeted drug delivery to desired tissues, and specific organs, including intracellular compartments or microenvironments associated with certain inflammatory conditions.

In the polymeric nano-carriers, we utilized guar gum as a macromolecule which was grafted with monomers acrylic acid (AA) and acrylonitrile (ACN), using microwave irradiations to modulate its physiochemical properties. The % grafting was

optimized using a quality-by-design approach through multiple linear regression analysis and analyzed employing the second-order quadratic model. A total of 29 experimental trial batches were synthesized and validated for % grafting yield.

The optimized batch of gaur-g-(AA-co-ACN) was successfully grafted and it contained ACN (1.0 mol/L), and AA (0.03 mol/L) for 100 second reaction time with 2000 W microwave power. The effect of various parameters like monomer concentration, reaction time, and microwave power on grafting was studied. The optimized batch showed 82.84% of guar gum grafting whereas the predicted value was 80% which suggested consonance with the observed values.

The guar gum grafting through AA and ACN was characterized by various spectral and thermal techniques. The FTIR spectra of the gaur-g-(AA-co-ACN) displayed characteristic peaks at 2166 cm^{-1} (C≡N stretching of nitrile), 1644 cm^{-1} (C=O stretching of $CONH_2$-I), 1419.56 cm^{-1} (N-H bending of $CONH_2$-II), and 1222 cm^{-1} (C-N stretching of $CONH_2$-III) which demonstrates that the grafting of AA and ACN onto guar gum was successful.

Further grafting was validated through NMR experimentation. In the grafted guar gum, additional major peaks at 177.5 ppm and region 31-42 ppm were observed in grafted material. These peaks may be due to the CO group of $CONH_2$ and linear carbons ($-CH_2-CH_2-$) from the polyacrylic acid and polyacrylonitrile grafted on guar gum. A peak observed at 105.5 ppm may be attributed to the nitrile (-CN) carbon of acrylonitrile. The chemical shifts in characteristic peaks of guar gum and the appearance of new peaks confirm the successful grafting and cross-linking of AA and ACN onto guar gum.

Grafting of AA and ACN onto guar gum was also confirmed through thermal analysis. Grafting increases the thermal stability of guar-g-(AA-co-ACN) in comparison to native guar gum. In addition to thermal studies, a microscopic examination of both native guar gum and grafted guar gum was also performed and it was shown that grafting improved the porous, rough, and heterogeneous surface of guar gum into smooth and homogeneous architecture.

After confirmation of grafting onto guar gum, grafted material was processed for various evaluations like swelling behaviour and water hold capacity at different

pH of 3.5, 6.8, and 7.4. The grafted guar gum undergoes conformational changes on swelling through open to fully solvation coiling, and in the end, de-solvated globular conformations over an optimum pH range.

The study shows that the water uptake amount per mg of the gaur-g-(AA-co-ACN) enhance with the increase in time and maximum water uptake capacity was achieved with the pH-7.4 solution due to the formation of carboxylate groups by alkaline hydrolysis of cyanide of ACN and carboxamide groups of AA which causes enhancement in chain relaxation and thus, polymer chain expansion resulting in an increment in swelling capacity.

Further grafted guar gum was converted into a nano-particulate system for loading of the drug thymoquinone (TQ) and analyzed for their surface charge and size of the particles. Guar gum is almost neutrally charged, grafting with acrylonitrile and acrylic acid created a negative surface charge over particles. The zeta potential and particle size of NpTGC were found to be -21.74 mV and 166±10 nm respectively showing substantial utility in drug delivery applications.

Then, after TQ release from guar gum grafted nanoparticle (NpTGG) was analyzed in phosphate buffer pH 7.4 and 6.8 and higher TQ release at pH 7.4 (78%) compared with pH 6.8 (72%) which could be due to higher swelling of the polymer at higher pH(7.4) due to the formation of carboxylate groups by alkaline hydrolysis of cyanide of ACN and carboxamide groups of AA which increases polymer chain expansion followed by swelling ability as compared to lower pH 6.8.

The antioxidant potential was also explored through DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging. The % DPPH radical inhibition of thymoquinone loaded into nanoparticles of grafted guar gum was found to be enhanced significantly ($p<0.05$) from 45.02±3.5% to 60.80±5.3% due to the synergistic potential of thymoquinone with guar gum. Loading of TQ into polymeric matrix delayed drug release and prevention from premature degradation which also contributed to DPPH radical scavenging.

The gaur-g-(AA-co-ACN) was investigated *in silico* for anti-inflammatory potential using molecular docking with humans-PLA2. The molecular docking revealed a good interaction between ligand and assigned protein. The binding energy

between the grafted guar gum and assigned protein was found to be -7.37 kcal/mol, revealing that the grafted guar gum derivative possesses good anti-inflammatory activity.

Further hemocompatibility and cytocompatibility were assayed using goat blood and monkey normal kidney Vero cell lines respectively through SRB assay. Both naïve guar gum and grafted guar gum in the concentration range of 0.5-2mg/mL show less than 5 % hemolysis and are found hemocompatible as per the American Society of Testing of Material. Also, NpTGG ranging in size from 150-400 nm shows less than 5 % hemolysis indicating the comfortability of drug delivery in the bloodstream.

In the SRB assay, the cell viabilities were found to be more than 70 % for grafted guar gum (79.2±4.0%) and NpTGG (73.2±5.30%) at a dose of 10 µg/mL comparisons with the positive control (adriamycin). The cell growth studies of grafted guar gum and NpTGG appeared safe and non-toxic, probably due to naturally occurring guar gum as well as the loading of TQ. TQ proved to be nontoxic and thus it could be assumed that loading in grafted guar gum would be cytocompatible and can be regarded as a safe drug delivery carrier for pharmaceutical applications.

As summarized above the synthesis of the pH-sensitive crosslinked guar gum-g-poly-(acrylic acid-co acrylonitrile) [gaur-g-(AA-co-ACN)] was successfully achieved by using the microwave-assisted technique without using any initiator for the sustained release of thymoquinone. The synthesized gaur-g-poly(AA-co-ACN) and NpTGG were characterized by SEM, XRD, NMR, TGA, Zeta potential analyzer, and FT-IR spectroscopy. Grafting of acrylonitrile and acrylic acid improved the swelling, and drug loading, and sustained the drug release capacity of pure guar gum. Also, crosslinked guar gum-g-poly[AA-co-ACN)] was used to prepare the thymoquinone drug-loaded nanoparticles (NpTGG) which shows the maximum thymoquinone drug release from NpTGG at pH 7.4.

Cytotoxicity of gaur-g-(AA-co-ACN) and NpTGG was also determined by SRB assay. SRB assay confirmed that the gaur-g-(AA-co-ACN) and NpTGG were found to be safe. Moreover, thymoquinone-loaded nanoparticles grafted guar gum (NpTGG) exhibited hemocompatibility, with improved antioxidant potential and anti-inflammatory activities.

Overall, the synthesized grafted guar gum is safe and stimuli-responsive material that could be utilized for the molecular targeted drug delivery in response to the slight modulation in physiological pH of the targeted tissues, including intracellular compartments, specific organs or certain microenvironments associated with multiple inflammatory conditions.

In the second part of the experiment, lipid-based formulations, (nanostructured lipid carriers) were prepared using naturally occurring tamanu oil as liquid lipid and Precirol ATO5 as solid lipid. The tamanu oil has been reported for the management of various skin ailments and was utilized as a liquid lipid for the designing of nanostructured lipid carriers(NLCs).

The compatibility between tamanu oil and TQ was assayed through visual examinations and spectral examinations (UV and FT-IR) and TQ was found to be compatible with tamanu oil. The absorption maxima of TQ or tamanu oil or the physical mixture of both did not display any shifts in the UV spectrum. In addition, the IR spectrum of the components and the spectra on superimposition displayed all the characteristic peaks, indicating no interactions.

Surfactants and solid lipids (glyceryl monostearate, Precirol ATO5, and steric acid) were screened for their miscibility with tamanu oil. Precirol ATO5 was selected as solid lipid, and tween 20, and lauroglycol 90 as surfactants by preparing NLCs based on producing smaller size NLCs.

The blank and TQ-loaded NLCs (TQ-NLCs) were designed using the hot melt-emulsification method followed by sonication. The concentration of lipids, and surfactants and require sonication time to form the NLCs system were optimized based on the desired particle size and high %EE with drug loading using the QbD approach.

Further, as per the suggested design NLCs were prepared with a lipid content of 4.40 % w/w, a surfactant content of 5.52 % w/w, and a sonication time of 15 min. The particle size of the TQ-NLCs was found to be 153.9±0.52nm with a PDI of 0.136±0.0015. The % entrapment efficiency and drug content were found to be 85.6±0.50% and 16.75±0.52 (mg/g), respectively. The predicted value for optimized NLCs suggested a good agreement with observed values.

Further *ex-vivo* skin permeation studies of TQ-NLCs performed for the duration of 24 hr show improved TQ permeation for TQ dispersed in tamanu oil (TQT) and an aqueous suspension of TQ (TQS) in the order of TQ-NLCs> TQT > TQS. The TQ-NLCs (14.6 times) and TQT (7.1 times) reported significant ($p < 0.05$) increments in enhancement ratio (Er) compared to an aqueous suspension of TQ. The Er for TQ-NLCs was 2.06 times higher compared to TQ dispersed in tamanu oil (TQT).

These findings showed a significant enhancement in the skin penetration of TQ from TQ-NLCs compared to TQT and TQS. These findings were ascribed to the presence of fatty acids in the skin and tamanu oil which aids the solubility and permeability of tamanu oil along with the entrapped TQ.

The optimized formulation was processed for the stability studies as per the guideline issued by the central drugs standard control organisation and there was no significant variation in the particle size and drug content was found after freeze-drying and sorting at 4-8°C, while sorting at room temperature (23±2°C) affect the size of the NLCs as examined after 1 and 3 months.

After the fruitful finding from the stability studies, the lipidic formulation was assayed for its cytocompatible potential against normal kidney Vero cell lines using MTT assay. The blank NLCs formulation was found to be more than 78.50±0.15 cells viability in the dose of 10-80 µgm/mL for the duration of 24 h. This was attributed due to materials utilized to design the nano-lipidic system (NLCs) are enlisted in the generally regarded as safe category and were reported to be non-toxic as investigated in the human keratinocytes cells and normal human skin fibroblasts.

After *in-vitro* examination, the TQ-NLCs were incorporated into carbopol gel and preliminarily examined for their anti-inflammatory potential using the carrageenan-induced paw edema model in the albino Wistar rat. The % inhibition paw edema data showed that 1% w/w TQ loaded NLCs gel exerted a significant ($p<0.05$) reduction (51.48%) in the paw edema of TQ-NLCs gel treated group rats ($p<0.05$) when compared to blank NLCs and the aqueous dispersion of TQ on a topical application for transdermal delivery. These results demonstrated the significance of tamanu oil in the preparation of NLCs.

The controlled delivery of TQ from NLCs suggested greater inhibition due to increased TQ penetration, proving the ability of passive targeting through nanoformulations at an inflamed site in comparison with TQ aqueous suspension.

The synergistic potential of natural oil and drug was also evidenced in comparison with naïve TQ gel since the inhibition of paw oedema was significant. Additionally, incorporating the NLCs formulation into the NLCs gel system increased skin retention and contact time for transdermal drug delivery, enhancing its anti-inflammatory potential.

As per the favourable finding of TQ-NLCs in the acute inflammatory model the clinical aspects of tamanu oil-based NLCs were examined in the Freund complete adjuvant(FCA) induced rheumatic inflammation and cartilage destruction model. The formulations (TQ-NLCs, free TQ, and blank NLCs) were incorporated in a previously prepared 1% carbopol gel and were adequately spread over the skin surface of FCA-sensitized animals for transdermal delivery.

The treatment of TQ-NLCs gel and standard drug (diclofenac sodium gel) was initiated on the 8^{th} day of FCA induction and a significant reduction in the clinical score of arthritis was seen from day 15 to 27($P<0.05$ to $P<0.0001$), along with a reduction in edema/ redness in the arthritic group, displaying arthritis amelioration in comparison to TQ gel and blank NLCs gel.

After completion of the study, the right hind paw of the animals was exposed for X-ray examinations followed by the collection of blood for serum biomarker analysis. Thereafter, animals were sacrificed and the organs like the liver and spleen were excised to analyze their relative index.

Radiological images displayed numerous pathological alterations in periarticular soft tissue and bone erosion, a distinctive characteristic of FCA-induced arthritis. Rigorous swelling of soft tissue and bone erosion were depicted in the tibiotarsal joint region of the arthritic rats compared to the healthy rats. TQ has been proven to accelerate new bone formation in previous studies. Additionally, stem bark and seed extracts of tamanu (*Calophyllum inophyllum*) have also been reported to reduce bone destruction. Hence, to validate the combined protective nature of the TQ-NLCs gel on bone regeneration, joint erosion, and soft tissue swelling, an X-ray

examination of the ankle joints of the right hind paws were observed. Rigorous swelling of soft tissue and bone erosion were depicted in the tibiotarsal joint region of the arthritic rats compared to the healthy rats. Significantly ($p<0.05$) reduced bone erosion was depicted in the TQ-NLCs (Score 2.16 ± 0.40) gel and diclofenac gel-treated animals (Score 2.33±0.51) compared to the arthritic control group (Score 5.66±0.81).

Animals from the blank NLCs gel or arthritic control group showed enhanced liver and spleen indices. Conversely, these manifestations shifted towards normal control animals upon treatment with diclofenac gel or TQ-NLCs gel, ($P>0.05$ compared with the arthritis control group.

A significant ($p<0.0001$) enhanced expression level of TNF-α and IL-6 was demonstrated in the arthritic group and blank NLCs formulation group compared to the normal control group demonstrating the severity of arthritic inflammation. Thus blocking of TNF-α and other pro-inflammatory factors may decrease arthritis progression. Topical application for transdermal delivery of the standard diclofenac gel, TQ-loaded gel, and TQ-NLCs gel significantly ($p<0.05$) ameliorated serum TNF-α and IL-6 expression levels. Interestingly, the standard and TQ-NLCs gel displayed superior efficacy over the TQ–gel. The TQ-NLCs gel group displayed a significant ($p<0.0001$) reduction in serum TNF-α levels, compared to TQ-gel treated animals. Similar effects of TQ-NLCs and standard drug treatment were observed in the amelioration of serum TNF-α levels. The blocking of TNF-α and other pro-inflammatory factors decreases arthritis progression. When caspase-1 cleaves, it leads to an increase in pro-inflammatory markers: for example, IL-1β, IL-18, post-NLRP3 (NOD-like receptor family pyrin domain containing 3) inflammasome activation. TQ has been reported to block this cascade of events. The block of inflammatory markers was also supported by several published studies.

The nano lipidic formulation of TQ was found to ameliorate the clinical symptoms of arthritic inflammation as evidenced against both acute inflammation and Freund completed adjuvant(FCA) induced chronic inflammation. The physical parameter like edema and redness in the paw was ameliorated significantly in the formulation-treated group.

The outcome of the study was also supported by the down-regulation of molecular markers (TNF-α and IL-6) in the treatment group in comparison to the arthritic control group as evident in the FCA-induced chronic inflammatory model. The clinical effect of the formulation was driven by the efficient delivery of the drug at a molecular level using a passive targeting approach of enhanced permeation and retention at inflammatory sites.

The findings of both formulations showed efficient delivery of the selected drug in a defined manner against inflammation. The delivery of TQ from polymeric nanoparticles was pH-driven while the lipidic nanocarrier utilized a matrix system for controlled delivery of the drug thymoquinone. The polymeric nanoparticles were found to be cyto and hemocompatible and delivered the drug in a defined manner against inflammation.

The pH-sensitive cyto and hemocompatible polymeric nano-carriers of grafted gum were prepared for the delivery of bioactive constituent thymoquinone and assayed for their anti-inflammatory potential, while the tamanu oil-based novel nano-formulation was prepared to tackle poor permeation issues of bioactive compound TQ. The lipidic formulations (nanostructured lipid carriers) provided an opportunity for transdermal delivery of TQ as an assessable approach to circumvent hepatic degradation, thus improving antioxidant, and anti-inflammatory action in the joint synovium, to ameliorate rheumatic inflammation and relieving the symptoms of RA. Both formulations improved the pharmacokinetics of the drug thymoquinone and acted as suitable and efficient candidates for controlled delivery of the drug, thymoquinone. Nevertheless, further clinical trials are needed to establish the human delivery of the formulations.