Development and Characterization of Polypeptide Based Nanocarrier(s) in Drug Delivery

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SUMMARY

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Nanocarriers offer several advantages in drug delivery including the ability to solubilize hydrophobic drug, increased retention time in the body, and the ability to target specific tissues. Nanocarriers are most commonly prepared by material of synthetic and natural origin. Synthetic materials have certain intrinsic limitations in terms of toxicity, high cost, non-renewable sources, and stimulation of chronic inflammatory reactions. The efficacy of nanocarrier as a drug delivery has been increased by taking advantage of novel protein based natural biomaterial.

Recent advances in material science has shown promising potential for protein based nanoparticles with subcellular size, non-toxicity and non-antigenicity, sustained release, stability and specific targeting at cellular or organ level. All these biomaterials (sericin, soy and whey protein) posses strong antioxidant activities, which are responsible for most of their biological effects. The antioxidant and free radical scavenging properties of biomaterials makes sericin, soy protein and whey protein as a material of choice for encapsulation of any drug for the prevention of oxidative stress associated diseases. Proteins are GRAS (generally regarded as safe) and in addition, show the possibility of less opsonization by the RES (reticuloendothelial system) through an aqueous barrier.

Atrorvastatin (Atr) was selected as a drug candidate because of its synergistic properties with our synthesized material (Soy-Whey crosslink, sericin). It was hypothesized that the encapsulation capabilities and cholesterol reducing properties of sericin, whey protein, soy protein may enhance the biological properties of Atr incorporated in nanoparticles. It was taken as a model drug for present study. However any drug other than Atr, having a mode of action having synergies with antioxidant potential or a disease which is fallout of free radical response, can utilize the proposed system for its probable synergistic action. Limitations like inadequate solubility, less absorption, less bioavailability, ineffectiveness in lowering of cholesterol levels, patient incompliance are noticed with Atr, which can be overcome by using protein based nanocarriers.

Cardiovascular disease is a major cause of disability and premature death throughout the world, and contributes substantially to the escalating costs of health care. The cardiovascular risk factors are tobacco use, an unhealthy diet and physical inactivity (which together result in obesity), elevated blood pressure (hypertension), abnormal blood lipids (dyslipidaemia) and elevated blood glucose (diabetes). One of the underlying risk factors for cardiovascular disease is hyperlipidemia, characterized by elevated blood levels of low-density lipoproteins (LDLs). Hence, Hyperlipidemia is a prevailing risk factor that leads to development and progression of atherosclerosis and consequently cardiovascular diseases.

The objectives were as follows:

- ✓ To extract and/or synthesize and characterize protein based material, which possess cholesterol lowering property
- ✓ To develop, characterize and optimize nanocarriers from the protein based material(s) containing cholesterol lowering drugs.
- ✓ To investigate the improved pharmacodynamic profile of prepared nanocarrier(s).

Preformulation Studies

- ✓ The drug identification studies were conducted which confirmed that drug (Atr) matches the standard monograph described for identity and purity as reported in literature. Atr was obtained as white to off-white crystalline, odorless powder. The melting point and partition coefficient of Atr was confirmed that drug (Atr) matches the standard monograph as reported in literature. Atr was found to be freely soluble in methanol. The value of λ_{max} was found to be 246 nm that is in accordance with the reports published in the literature. The IR spectrum of Atr showed characteristic peak of which confirmed the presence of different group.

- ✓ The calibration curve of the Atr was prepared using UV-absorption method at λ_{max} 246 nm in methanol and at λ_{max} 241 nm in PBS (pH 7.4). A straight line was obtained in all the cases in a concentration range of 2 to 20 µg/ml with R^2 value

0.998 and 0.994, respectively. This result reveals that Beer Lambert's law is followed in the used concentration range in UV spectroscopy.

- ✓ The validated HPLC analysis presented well resolved peak of Atr from the standard sample. The standard curve was drawn by plotting the peak area of Atr versus drug concentration in plasma. The chromatograms were recorded at 246 nm with the retention time of 4.6 for Atr and 6.7 for simvastatin. The calibration graph was linear in concentration range of 0.05-1.0 µg/ml with correlation coefficient of 0.995.

Development and Characterization of Soy-Whey based Atr Loaded Nanoparticles

- ✓ The objective of our study was to design and develop a new delivery approach for Atr by synthesizing the cross/SPI-WPC (crosslinked soy protein isolate with whey protein concentrate) using non toxic amine–amine crosslinker genipin (Gn). Further, cross/ SPI-WPC would be used as a bioactive material to encapsulate ATR by desolvation method. The *in-vivo* antihyperlipidemic activity of the optimized formulation was evaluated in rat model.

- ✓ Soy protein isolate and whey protein concentrate was crosslinked by genipin to combine the cholesterol lowering properties of SPI and WPC. The principle of the synthesis is based on Gn initiated crosslinking of $–NH_2$ groups in SPI and WPC. The appearance of cyan color after crosslinking demonstrated the reaction of Gn with amine groups of proteins. The ring opening reaction of Gn dihydropyran ring started with the nucleophilic attack of the amino group, followed by radical reaction of two amino-attached open rings. The result of FTIR and MS analysis indicated that Gn was successfully employed for crosslinking of SPI and WPC.

- ✓ Atr/SPI-WPC NPs (atorvastatin loaded soy-whey nanoparticles) were prepared from cross/SPI-WPC by desolvation method using ethanol as desolvating agent. The effect of amount of cross/SPI-WPC (%, w/v) and volume of DA (ml) on particle size, zeta potential, entrapment efficiency and drug loading was optimized. The optimized Atr/SPI-WPC NPs shows the particle size, zeta potential, EE and DL of 158.1±3.8nm, -33±2.6mV, 81.23±1.1% and 31.5±2.9% respectively.

- ✓ The optimized batches of Atr/SPI-WPC NPs were characterized for FTIR, XRD, shape and surface morphology. The less intense XRD peak of SPI, WPC, pm/SPI-WPC, cross/SPI-WPC did not show any characteristic crystalline peaks and there was no change in physical state of cross/SPI-WPC due to crosslinking. Atr/SPI WPC NPs-10 indicate that NPs are spherical, fairly smooth, and with uniform surface.

- ✓ The *in-vitro* drug release from Atr/SPI WPC NPs-10 showed sustained release pattern over 48 hr with no considerable burst release. This sustained release pattern of drug from NPs may be due to the Gn induced crosslinked SPI and WPC as a material for the preparation of NPs which possibly prevented quick dissolution of crosslinked polymer matrix.

- ✓ The highlight of this work is that Atr/SPI-WPC NPs group displayed lower level of lipids (SC=62.5±3.1, ST=56.7±3.8, LDL=16.2±4.6, VLDL=12.8±0.95) as compared to Atr group (SC=89.67±4.3, ST=65.67±4.10, LDL=25.6±3.1, VLDL=13±0.9) and triton treated group (SC=125±3.2, ST=110±3.2, LDL=75±2.9, VLDL=17.6±1.9). Therefore, it was concluded that Atr/SPI-WPC NPs showed potential hypolipidemic effect. The higher H/M ratio indicates a lower extent of cholesterol synthesis, which was observed in the Atr/SPI-WPC NPs treated group. In contrast triton administered group displayed a higher cholesterol synthesis (2.52) compared to control group (3.41). The liver sections of rats treated with Atr/SPI-WPC NPs-10 showed hepatic structure recovery.

- ✓ A cell viability study was performed in order to evaluate the cytocompatibility of the prepared SPI-WPC NPs and Atr/SPI-WPC NPs with J774 cells using MTT assay at incubation period of 24, 48 and 72 hr. The NPs were non-toxic, probably due to the use of naturally occurring dietary proteins (SPI, WPC) as well as natural crosslinker (Gn). SPI, WPC and Gn proved to be non toxic and thus it could be assumed that prepared NPs would also be biocompatible.

- ✓ The cellular uptake of FITC labeled Atr/SPI-WPC NPs showed that the majority of NPs co-localized in the cell cytoplasm and not in the nucleus of the cell, which was revealed by strong green fluorescent channel.

- ✓ The pH of all the nanoformulations stored at 40±3°C was found to decrease to some extent. On the other hand, the formulations stored at 4±2°C and 25±3°C did not show any change in pH. The change in color, consistency and particles size was not observed in case of Atr/SPIWPC NPs-10 formulations stored at 4±2°C and 25±3°C. It is evident that slight increase in average particle size was more pronounced when stored at 40±3°C in comparison to room temperature conditions. No significant change in PDI was observed when formulations (Atr/SPI-WPC NPs) were stored at 4±2°C and 25±3°C as compare to 40±3°C.

- ✓ It is evident that decrease in residual drug content was more pronounced when stored at 40±3°C in comparison to ambient temperature and refrigerated conditions. The data clearly suggested that refrigerated and room temperature conditions are suitable for the storage of nanoparticulate formulations after 6 months.

Development and Characterization of Sericin Based Atr Loaded Nanoparticles

- ✓ The objective of present work was to overcome the problems associated with synthetic polymers by developing non-toxic, genipin crosslinked sericin nanoparticles using desolvation method. It was hypothesized that the encapsulation capabilities and cholesterol reducing properties of sericin may enhance the biological properties of Atr incorporated in NPs.

- ✓ The extraction and characterization of sericin from silkworm cocoons of *Bombyx mori* was carried out. Extracted sericin was characterized for various parameters such as SDS-PAGE, IR and Bradford assay. The characteristic peak of sericin shows the absorption band at 1650 $cm^{-1}$is assigned to (amide I), 1524 cm^{-1} is assigned to (amide II), 1240 cm^{-1} is assigned to (amide III) and 543 cm^{-1} is assigned to (amide V). The extracted sericin was characterized by broad bands which indicate the wide range of molecular weight from 30 to 250 kDa. The calibration curve of the BSA was prepared using UV-absorption method at λ_{max} 595 nm in distilled water. A straight line was obtained in a concentration range of 10-100 µg/ml with R^2 value of 0.993.

- ✓ Desolvation technique followed by Gn intiated crosslinking was employed for the preparation of protein NPs. The influence of variables such as the effect of Gn concentration and crosslinking time on the particle size, crosslinking degree, entrapment efficiency and drug loading of the Seri-Atr NPs was optimized. In case of Seri-Atr NPs, the optimized batch shows the particle size, crosslinking degree, EE and DL of 166±0.30 nm, 35.1±2.10%, 91±0.69% and 50±1.1%, respectively.

- ✓ The optimized batches Seri-Atr NPs were characterized for FTIR, XRD, shape and surface morphology. In the FTIR spectra of Seri-Atr NPs, peaks of Atr were not distinguished reflecting the successful incorporation of Atr in the protein matrix.

Seri+Atr exhibited approximately similar peaks as compared to Seri, Atr with a negligible shift ascribed to no chemical modification of NPs with excipients. The vanishing of the Atr diffraction peaks in XRD pattern of Seri-Atr NPs revealed successful encapsulation of crystalline Atr into amorphous NPs. The prepared Seri-Atr NPs exhibited uniform spherical morphology with smooth surface as shown in TEM image.

- ✓ In case of Seri-Atr NPs, it was observed that drug release was slow and controlled with increase in Gn concentration. It was evident that Gn concentration as well as crosslinking time greatly influences the release of drug. The slow release of drug from NPs with high Gn content and longer incubation time might be attributed to the rigidity of the crosslinked protein matrix that slows down the penetration of water and leads to slow degradation, diffusion and erosion which finally retards the release of poorly water soluble Atr into the dissolution medium.

- ✓ *In-vivo* antihyperlipedimic activity showed that rats with high cholesterol levels treated with Seri-Atr NPs (equivalent to 10 mg/kg Atr) revealed a significant decrease in level of lipids (TG, TC, LDL, VLDL) and significant increase in HDL as compared to Atr (10 mg/kg) treated rats ($p<0.05$). The antihyperlipidemic effect of sericin results from its inhibition of cholesterol absorption in intestinal cells and its reduction of cholesterol solubility in lipid micelles. In Seri-Atr NPs treated groups, cholesterol synthesis in liver was lower (higher H/M ratio) as compared to Atr and Seri NP treated groups. The liver sections of rats treated with Seri-Atr NPs showed recovery of hepatic architecture with preserved parenchymal structures (darkly stained nucleus, no sinusoidal dilatation and congestion, no necrosis in hepatocytes).

- ✓ The results of pharmacokinetic study indicated that the Atr plasma concentration vs. time profile, obtained after administration of the Seri-Atr NPs was devoid of pronounced peak, suggesting that NPs reside in the body for prolonged period of

time. The data suggests long circulation time for the NPs which may be due to lower RES uptake of the particulate system consisting of naturally occurring protein such as sericin. The proteinaceous nature of sericin particles leads to formation of aqueous steric barrier around particle and hence lower opsonization and lesser degree of removal of particles from circulation.

- ✓ Non-toxicity and good biocompatibility of the Seri-Atr NPs towards cells attributed to the presence of genipin as natural crosslinker and sericin as a natural polymer, clearly demonstrated that the sericin provide nontoxic coating over drug.

- ✓ Confocal microscopy of Seri-Atr NPs clearly confirmed the qualitative uptake of nanoparticles within cells. The green channel revealed that Seri-Atr NPs were majorly co-localized in the cell cytoplasm. Internalization of NPs may be due to repulsive interactions which lead to binding of negatively charged NPs to cationic site of cell surface.

- ✓ The pH of all the nanoformulations stored at 40±3°C was found to be decrease to some extent. The formulations stored at 4±2°C and 25±3°C did not show any change in pH, color, consistency and particle size. However, a little change in color and consistency was observed in formulations stored at 40±3°C, may possibly due to degeneration of free traces of genipin, which resulted in the change in color (light blue) of the nanoformulations. It is evident that slight increase in average particle size was more pronounced when stored at 40±3°C in comparison to room temperature conditions. No significant change in PDI was observed when formulations (Seri-Atr NPs) were stored at 4±2°C and 25±3°C as compare to 40±3°C.

- ✓ It is evident that decrease in residual drug content was more pronounced when stored at 40±3°C in comparison to ambient temperature and refrigerated conditions.

The data clearly suggested that refrigerated and room temperature conditions are suitable for the storage of nanoparticulate formulations after 6 months.

- ✓ This is the first report of extracting and synthesizing novel biomaterial (sericin and crosslinked SPI-WPC) and preparing Atr loaded nanoparticles using these biomaterials for effective treatment of hyperlipedimia.