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ORIGINAL ARTICLE

Lectin Conjugated Hydrogel Nanoparticles Using Supercritical Fluid Technology

Atul Baravkar[1*], Velhal M. K.[2], Avinash Tupe[3], Ram Dhanaji Nikhate[4] and Himanshu Shewale[5]
[1*]Agricultural Development Trust's School of Pharmacy & Research Centre, Baramati, Pune
[2]KJ's Trinity College of Pharmacy, Pune
[3]SVPM's College of Pharmacy, Malegaon Bk, Baramati, Pune
[4]SND College of pharmacy, Yeola, Nashik
[5]Dr. Babasaheb Ambedkar technological university, Lonere, Raigad
***Corresponding Author**: Atul Baravkar
Email: atul200678@gmail.com

ABSTRACT
Lectin conjugated drug deliveries have been explored in targeting various organs in human body due to lectin's mucoadhesion, cytoadhesion and cytoinvasion properties. In these studies, Soybean lectin was covalently conjugated to hydroxyl-propyl-methyl cellulose K-100M encapsulated retinol acetate nanoparticles by green, single step modified rapid expansion of supercritical solution with mixing and sonication process. Influences of processing parameters on obtained nanoparticles were successfully studied. Retention of biorecognitive activity of lectin after the covalent coupling procedure was confirmed by haemagglutination test along with FTIR spectroscopy, C-13NMR spectroscopy and by UV-Visible spectroscopic method. These lectin conjugated nanoparticles were evaluated by various in-process tests, like particle size distribution, drug content (%), encapsulation efficiency (%). Biological activity evaluations were done by mucin adsorption, mucoadhesive strength, COLO-205 cell line cytotoxicity studies. These tests results have proved potential of prepared lectin conjugated hydrogel nanoparticles in colon cancer therapy.
Keywords*: Retinol acetate, RESS, Hydrogel, Nanoparticles, Mucoadhesion, Conjugation, Lectin, Evaluation.*

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INTRODUCTION

Intravenous administration of the most chemotherapeutic drugs for colon cancer therapy produces severe systemic side-effects due to its cytotoxic effect on normal cells. Therefore, significant efforts have been made to develop novel oral formulations for colon-specific delivery. In tumor pathology, aberrant glycosylation is a common attribute of neoplastic growth [1]. Various conventional methods to prepare nanoparticles have failed to yield particles of suitable size distribution. In addition, some of these methods are subject labile molecules to high thermal and mechanical stress as indicated by Agnieszka Z. *et al.* [2]. Widely known techniques for conjugation of lectin to nanoparticles includes multistep chemical reactions like carbodiimide reaction, maleimide mediated, disulphide coupling reactions or covalent bonding formation. All these methods are time consuming, multistep processes with involvement of several organic solvents [3]. Supercritical fluid technology (SFT) is an emerging, an efficient, alternative technology. It is environmentally friendly technology having better control over process and resultant nanoparticles. Due to use of supercritical fluid (SCF) mainly carbon dioxide (CO_2), due to its inexpensiveness, noninflammability, nontoxic nature with excellent solvent strength and diffusibility used as a solvent in supercritical processing's. This SCF CO_2 has zero surface tension, adjustable density, viscosity with compressibility and gas like transport capacity. Due to its inert nature used for processing with thermolabile compounds, these advantages were noted by Pasquali I. *et al.* [4], Zhang Q. *et al.* [13] In rapid expansion of supercritical solution (RESS), solutes dissolved in supercritical fluid CO_2 (carbon dioxide) are de-pressurized to obtain micro/nanoparticles. There is limitation of RESS process for polar,

high molecular weight compounds due to it's slight solubility in supercritical carbon dioxide. As well as agglomeration of nanoparticles occurs after collection due to adhesiveness [5]. Hence, we have devised, patented and used a modified technique involving a combined effect of ultrasonication with mixing and supercritical fluid technology to remove limitations of RESS process and instrumentations. "Sonication Assisted Particle production using Supercritical Solution (SAPSS®)," was used throughout this formulation studies due to better process and product control [6]. Retinol acetate is a naturally occurring fatty acid ester form of retinol (Vitamin-A) with potential antineoplastic and chemopreventive activities. It binds to and activates retinoid receptors which are rich in colon epithelial cell layers by inducing cell differentiation and decreasing cell proliferation, tumor growth [7]. Hydrogels are crosslinked polymer networks that absorb substantial amounts of aqueous solutions. Hydroxy propyl methyl cellulose K-100M (HPMCK-100M), methocel cellulose ethers polymer was selected owing to its hydrophilicity, good gelling and moisture retention property. HPMCK-100M has molecular weight of about 150000 Daltons with a biological half life 4 to 6 hours [7]. HPMCK-100M is non-ionic polymer, so they minimize interaction problems when used in acidic, basic or other electrolytic systems. HPMC polymers work well with soluble and insoluble drugs and at high and low dosage levels. Generally, methocel K grade products exhibit a higher tolerance for salt solution because of the difference in the organic substitution. HPMCK-100M is considered Generally Regarded as Safe (GRAS) and is also referenced in many of the Food and Drug Administration (FDA) approved drug products lists, owing to its biodegradability and biocompatibility [8].

Lectins are non-immunoglobulin protein macromolecules that are highly specific for sugar moieties usually of plant or animal origin. Their main function is glycoprotein synthesis, cell adhesion. Lectins are capable of specific recognition and reversible binding to carbohydrate moieties of complex glycoconjugates and glycans from normal and cancer cells without altering the covalent structure of any of the recognized glycosyl ligands. [9, 10] Soybean (*Glycine-Max*) lectin is basic, tetramer, galactose / N-acetylgalactosamine binding molecule in sodium salt form, mol weight 120 KiloDaltons with isoelectric point 9.0 [11].

Lectin-mediated delivery exploits lectin-cell interactions to increase the uptake and internalisation within cells. The lectin-coated nanoparticles could bind to intestinal epithelial cells which may be sufficient to stimulate enterocytes to endocytes and transport the particles across the cell and into blood circulation. Therefore, lectins combine two functions in anticancer drug delivery systems, targets to cancer cells and inducing anticancer effect [12].

Mucoadhesive lectin conjugated polymeric nanoparticles for colon targeting are designed due to their advantages like termination of drug action by avoiding first pass mechanism and prolonged contact time at the site. These nanoparticles have controlled and sustained-release properties with enhanced therapeutic effectiveness due to their subcellular size. These nanoparticles have biocompatibility with tissue and cells. Due to smaller size and higher drug loading nanoparticles are responsible for improvement of drug bioavailability and reduction of the dosing frequency with long shelf life [13, 14].

In the present investigation, an attempt has been made to formulate Soybean lectin conjugated functionalized HPMCK-100M encapsulated retinol acetate nanoparticles by using patented technology, "Sonication Assisted Particle production using Supercritical Solution (SAPSS®)." The resultant nanoparticles were characterized in terms of particle size distribution, polydispersivity index (P.I.), zeta potential (ZP), particle morphology, encapsulation efficiency (%E.E.), drug content (%), percent yield (%w/w), haemagglutination test, differential scanning calorimetry (DSC), powdered X-ray diffraction (PXRD) and Fourier transform infrared (FTIR) spectroscopy, C-13 N.M.R. spectroscopy, mucin absorption and mucoadhesion studies. The *in vitro* drug release studies along with *in vitro* COLO-205 cell lines cytotoxicity studies of the resultant nanoparticles were also studied.

The novelty of this work lays in the fact that functionalization of surface of HPMCK-100M encapsulated retinol acetate nanoparticles with a Soybean lectin conjugation was first time physically applied by covalent hydrogen bonding formation in SFT. Importance of the conjugated system is that the lectin provides target specificity and overcomes disadvantages of conventional therapy due to its higher specificity.

MATERIAL AND METHODS

Retinol acetate®325 GFP, HPMC K-100 M (Methocel K-100M premium), doxorubicin were obtained as a gift sample from BASF Ltd India, Colorcon, India and Cipla Ltd., India respectively. Soybean lectin (*Glycine-Max*, L1395) was purchased from Sigma-Aldrich Germany. The following laboratory excipient and analytical grade reagents were purchased from S.D. Fine Chemicals Ltd., Mumbai, India; tween-80 (Tw-80), sodium hydroxide, potassium chloride, sodium acetate, concentrated hydrochloric acid, acetic acid,

potassium dihydrogen orthophosphate, periodic acid/Schiff reagent (PAS). Mucin (M3895) from bovine sub maxillary gland of sheep was purchased from Sigma Aldrich, Germany. Sheep mucosa was purchased from a local slaughter house from Mumbai, India. COLO-205 cell lines were procured from National Centre for Cell Sciences, Pune, India. Foetal bovine serum (catalogue number 10432), RPMI media (catalogue number AT162-IL) were purchased from Himedia Ltd., India. Food grade carbon dioxide (purity of 99.9%) was purchased from Rakhangi Ltd., Mumbai. Double distilled water from Millipore system USA was used throughout the studies.

Experimental Methodology

Based on the results of compatibility studies results in preformultaion compatibility studies as per stability guidelines all drug, excipients were selected by. Literature searches of supercritical fluid processes nanoparticles generation, previous studies in our lab and solubility data of compounds in SCF CO_2 were also considered [15, 16].

The Supercritical fluid extractor® 500MR from Thar Ltd, USA with a sonicator bath (Model-PH350EL) from iUltrasonic Ltd., USA and magnetic stirrer was used. This patented process instrumentation as, "Sonication Assisted Particle production using Supercritical Solution (SAPSS®)," was used for the formulation purpose. The reactor was loaded with a previously weighed mass of drug with polymer (8g batch, ratio 1:1:1, w/w of drug: HPMC K-100M: Soybean lectin) and then with SCF-CO_2. Pressure and temperature of the extraction vessel were regulated and monitored using the inbuilt equipment software. Initially, abbreviated back pressure was kept at 80 bars, temperature of extraction collection vessel was maintained at 35◦C and process was operated. Further pressure was gradually increased from 95 bars, 200 bars to 290 bars, temperature of reactor collection vessel was increased from 45, 60 to 75◦C and the process was studied for 45 minutes of reaction time. The mixture was agitated using an impeller operated at 60 revolutions per minute (rpm) to ensure complete mixing of the solids with the SCF-CO_2. Process was carried out at the optimized conditions of pressure 290 bars, at temperature of reactor collection vessel was increased from 45, 60 and 75◦C for 45 minutes of reaction time with mixing speed 60 rpm.

The supercritical solution was then depressurized in an expansion vessel by means of a nozzle over the surface of the aqueous solution or stabilizing solution. Before initiating the next batch, the reactor was cleaned by passing SCF-CO_2 through the reactor so as to sweep out the remaining solid particles in the line or reactor. The resultant dispersion was mixed with sonicated for 10 minutes. Nanoparticles were collected in double distilled water of 25 ml volume and with tween-80, 1%w/v same volume in the collection vessel. These composition, pressure, temperature and time were taken for the studies by considering previous trials and optimization studies results we have performed.

Evaluation of nanoparticles

Obtained nanoparticles were studied for physicochemical parameters and biological activities characterizations of *in-process* and optimized nanoparticles obtained are given below.

Haemagglutination test

Presence of lectin conjugated to the nanoparticle was confirmed by hemagglutination test. Red blood cells (RBC) suspension was prepared by centrifugation of mixture of Albino *rat's* blood and saline containing EDTA, at 10000 rpm. The sediment so obtained was dispersed in double distilled water. 96 well microtitre round bottom plate from Biorad Ltd., India was used with the first well placed 100 µL saline solution with 100 µL RBC. And to the second well to third well 100µL RBC with nanoparticles dispersion sample same volume were put and incubated. After an hour by taking a drop from each well on glass slide and by covering with cover slip seen under 300X magnification of compound microscope from Scientific India Ltd., Mumbai for hemagglutination.

Particle size distribution, Polydispersity index (P.I.)

Particle size distribution and polydispersity index was determined using Zeta Sizer ZS® Nano analyzer (Malvern Ltd., Worcestershire U.K.) at back scattering angle of 173°. Prior to analysis, samples were suitably diluted using 0.45µm filtered, double distilled water (DDW) (1:10, sample: DDW).

Drug content (%)

1ml of the dispersion was transferred to a 10 ml of volumetric flask and the volume was adjusted to 10 ml with 50: 50 % v/v of ethanol: water. This stock solution was sonicated for 15 minutes and suitably diluted, filtered through a 0.45 µm membrane. Drug content was determined by analyzing this solution using UV spectroscopy double beam spectrophotometer (Model-V-670) Jasco, Japan at λ_{max} of 326 nm by comparing with the standard calibration graph of retinol acetate.

Encapsulation efficiency (E.E. %)

For determination of encapsulation efficiency, 1ml of the dispersion was transferred to 10 ml volumetric flask and the volume was adjusted using 50:50 % v/v of ethanol: water. The system was shaken for 5 minutes and the system was centrifuged for 15 minutes at 20000 rpm. Thereafter, the aqueous phase was

withdrawn and filtered through a 0.45µm membrane filters from Biorad Ltd., India. The filtrate was diluted appropriately with 50:50 (%v/v) of ethanol: water and analyzed by UV-Visible spectrophotometrically at 326 nm. The entrapment/encapsulation efficiency was calculated by the following equation,

$$\text{Percentage entrapment efficiency} = \left[\frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}}\right] x\,100$$

Where, "$W_{initial\ drug}$" was the mass of initial drug content and the "$W_{free\ drug}$" was the mass of free drug detected in the filtrate of an aqueous dispersion.

Percentage yield (%w/w) of nanoparticles

For the collected nanoparticles, the percentage yield (%w/w) was calculated by using the initial quantity of drug and excipients taken in the reactor of RESS SFT and quantity of powder remaining in the reactor after processing. % w/w yield was calculated by using the formula,

$$\text{Percentage (\% w/w) yield} = \left[\frac{\text{Quantity of nanopowder collected in the vessel}}{\text{Initial quantity of drug, excipients taken in the reactor}}\right] x\,100$$

Lectin conjugated (%)

Standard sample of Soybean lectin was dissolved in phosphate buffer of pH 6.8. Collected nanoparticle dispersion was centrifuged at 20,000 rpm and Surprenant solution was separated. Absorbance of each standard and test solutions were measured at 234 nm using phosphate buffer of pH 6.8 as a blank by UV-Visible double beam spectrophotometer. Lectin conjugated (%) was determined using following formula,

$$\text{Percentage lectin conjugated} = \left[\frac{\text{Lectin content standard} - \text{Unbound lectin}}{\text{Lectin content standard}}\right] x\,100$$

Measurement of zeta potential

Measurement of zeta potential was done using the Malvern Zetasizer Nano ZS®, UK. The samples were suitably diluted using 0.45 µm filtered, double distilled water (DDW) (1:10, sample: DD water), free of any particulate matter.

Fourier Transform Infrared spectroscopy (FTIR study)

1mg of collected nanoparticles powder was triturated with 0.1g of potassium bromide (KBr). The pellet was prepared in a KBr Press was used as a reference sample. And FTIR spectrum was recorded in the range between 4000 and 400 cm^{-1}, for 4 mm/s with a resolution of 2 cm^{-1} on FTIR Spectrum RXI spectrometer. (Model -LM 500) Perkin Elmer Ltd., Germany.

C-13 N.M.R. spectroscopy

4/5 mg of lectin conjugated nanoparticles sample collected was dissolved in deturated chloroform and filtered through Pasteur pipette. The NMR spectra of each sample were taken using 400 MHz Agilent NMR spectrophotometer, Germany with trimethylsilane (TMS).as an internal standard The time between two pulses for complete relaxation of the 13C nuclei was kept for 5 seconds.

Differential Scanning Calorimetry (DSC) Thermogram

DSC thermoanalytical measurements of liquid / solid samples were carried out on Themo Fischer PVT. Ltd., India with an empty standard aluminium pan as a reference sample. Thermograms were obtained with a heating rate of 10° C / minute, over the temperature range of 30°C to 300°C. For nanocarriers, the dispersion was introduced in the pan and allowed to
dry under vacuum overnight at room temperature and then subjected to thermal analysis.

X ray Diffraction (XRD)

It was carried out for nanoparticles powder 3 to 5 mg, in order to clearly elucidate solid state of nanoparticles, using a Bruker D-8 advanced diffractometer made by Bruker Biosciences Ltd., Spain. The X-ray diffractogram pattern was scanned with diffraction angle 2Ø range of 20 to 80° at a scan rate of 0.02°/min. and angle with a steep angle 0.02° and count time 1 second at a constant room temperature 25°C.

Particle morphology by scanning electron microscopy (SEM)

The liquid sample was spread on a double adhesive tape previously adhered to SEM aluminium stubs and then sputtered with platinum in an ion sputter for 300 seconds. Images were collected at an acceleration voltage of 15 kV using a back scattered electron detector on Joel JSM 6360®SEM at 25± 2°C.

***In-vitro* drug release study at different pH**

1 ml of optimized nanoparticles dispersion was placed in each test tube in four sets. To each test tube 9 ml of buffer of pH 1.2, pH 4.5, pH 6.8, pH 7.4 were added respectively. Those test tubes were kept in a

constant temperature shaking water bath at 37◦C at 100 rpm. Sampling was done at a fixed interval and replenishment of sample quantity was done with the respective fresh buffer. Triplicate readings were taken using UV spectroscopy at 326 nm and the concentration of released drug was determined. This resultant drug release values obtained were put in the different kinetics equations. And the mathematical modelling release kinetics equation applicable to each system was determined with the help of resultant reaction constant value (R^2) obtained.

Mucin adsorption studies (Mucin glycoprotein assay)

A colorimetric method, as described by Mantle and Allen *et al.*1978, using periodic acid Schiff reagent (PAS) was used for the determination of free mucin concentration and adsorption of mucin on nanoparticles. Schiff reagent contains 100 ml of 1 % v/v basic fuschin (Parasoaniline) aqueous solution. 20 ml of 1M HCl, sodium bisulphite (0.1g) was added to every 6 ml of Schiff reagent before use and resultant solution was incubated at 37◦C until it was turned to colourless or pale yellow. Periodic acid solution was freshly prepared by adding 100 µl or 50 % v/v of periodic solution to 7 ml of 7 % v/v acetic acid solution. Standard calibration was done with mucin standard solution of 0.25, 0.5, 0.7 and 1.0 mg/ ml. Nanoparticles solution 100 µl was incubated with mucin aqueous solution at 37◦C for 5 to 60 minutes in pH 7.4. After adding 0.2 ml periodic acid reagent, samples were incubated at 37◦C for 2 hours. Then 0.2 ml of Schiff's reagent was added at room temperature. After 30 minutes, absorbance was recorded at 555 nm using UV spectrophotometer in triplicate. Mucin adsorption content was determined from the standard calibration curve.

Mucoadhesive strength measurement

The mucoadhesive performance of nanoparticulate formulation was determined using Brookfield texture analyzer (CT-3), Germany. A cylindrical plastic probe (1.33 cm^2 surface area) with a 2 ml of nanoparticles formulation was brought into contact with sheep gastric mucosa for 60 seconds and withdrawn at a speed of 0.1 mm / second. For each formulation and blank (gastric mucosa), mucoadhesive strength readings were taken in triplicate.

Cell cytotoxicity study

To evaluate anticancer activity effect, COLO-205 cell lines were used. The effect of retinol acetate encapsulated nanoparticles on the tumor cell proliferation was determined using an MTT (Tetrazolium dye, 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetetrazolim bromide) assay. To the each well cell count 10^6 (100µl) was taken. Cells were incubated for a day. On the second day different concentrations of drug 100, 200, 300 µg / ml were put to incubated cells. To this 10 µL of dye (1mg / ml MTT) with 0.04 M HCl and isopropanol were added. This was further incubated for 4 hours by placing in aluminium foil. Cell viability was measured by ELISA plate reader (Model- xMark™) Biorad Ltd., India at 570 nm in triplicate. For standard sample of nanoparticles, doxorubicin, polymer, drug dilutions same procedure was repeated.

RESULT AND DISCUSSION

Feasibility studies

The key mechanisms of nanoparticles generation in modified process were rapid expansion of supercritical solution (RESS) with particles generation by saturated supercritical solution (PGSS). By this methodology both soluble and insoluble components are get processed to have nanoparticulate size. The components explored with SCF get diffuses, melting point reduction with plasticization effect. Simultaneously in SCF, solubilisation, nucleation, encapsulation, mass transfer of the components occurs. Lectins due to polar glycoprotein nature not soluble in supercritical fluid (SCF) CO_2. With inert SCF, lectin has weakened its structure with braking of bonds with formation of smaller particles without degradation. Due to presence of amine, amide bonds in lectin, it gets physically conjugated to polymer encapsulated polymeric drug nanoparticles simultaneously by formation of covalent or hydrogen bonding in SFT process. Similar effects and mechanisms of SCF CO_2 were noted by Zhang Z. *et al.*[13] and Pasquali et *al.*[4].

Haemagglutination test

Since lectin exhibits positive haemagglutination test, presence of lectin in the drug lectin polymeric nanoparticles were confirmed. With conjugated lectin, hemaagglutination was observed under microscope as clots with damaged RBCs aggregates. Lectin and the lectin conjugated nanoparticles with and without stabilizer tween-80 have demonstrated haemagglutination activities. Whereas there was found absence of agglutination in saline solution, similar studies were noted by Lam S.K. *et al.* [17]. This has provided that the conjugation of lectin was feasible with the polymeric drug nanoparticles using this modified supercritical fluid process.

Effect of temperature/ pressure and stabilizer tween-80 on particle size and polydispersity index of nanoparticles

At low temperature and high pressure near the supercritical region, SCF-CO_2 has higher density and as a consequence, better solubility of components. However, due to relatively more polar nature, SCF-CO_2 has failed to solubilise nonpolar biopolymer lectin as compared to drug. Thus, on depressurization of supercritical solution, the drop in density of SCF-CO_2 contributed to precipitation of drug in the reactor vessel and on the surface of the formed polymer particles leading to encapsulation of drug with further conjugation of lectin simultaneously. Increase in temperature and pressure were resulted in reducing density with viscosity of SCF CO_2 and solubilising components thus increasing solvent power with enhancing solubilisation. It was revealed that; at higher temperature with pressure due to higher supersaturation with higher nucleation rate on expansion of SCF, lower particles size and narrower particle size distribution were obtained. Similar results were obtained in experimentation by Turk *et al*. [18]. Lectin conjugated on polymer itself acted as a stabilizer, so in presence of tween-80, 1 %w/v, decrease in particle size was not observed. Similar observations were seen to I. Linear *et al*. [19]. Lectins are "natively unfolded" proteins with conformational flexibility related to their surfactant and micelle-forming properties. Thus, lectin has provided a stable, biocompatible, protective environment by adsorption and ionic interaction with nanoparticles. Hence lower particle size was obtained as shown in the figure 1.0. Results obtained were agreed with the study's results by Rabia *et al.* [20] and C.B.Fernandes *et al*.[32].

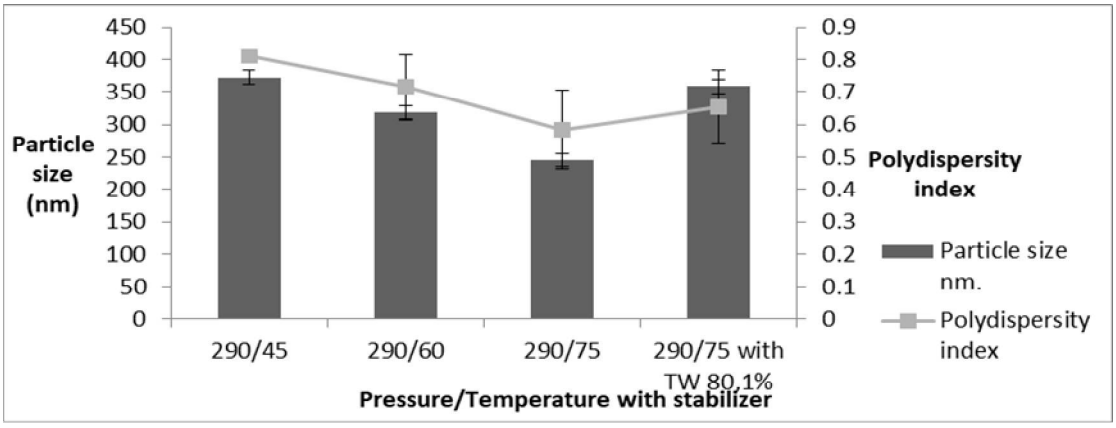

Figure 1.0: Effect of pressure/temperature and stabilizer on particle size and P.I. for lectin conjugated nanoparticles

Effect of temperature/ pressure and stabilizer on lectin conjugation (%) and yield (% w/w) for lectin conjugated drug polymeric nanoparticles

Lectin conjugation (%) and the yield (% w/w) of lectin conjugated polymeric nanoparticles were found high for polymeric nanoparticles obtained at pressure of 290 bars and temperature of 75^{o}C is shown in the figure 2.0.

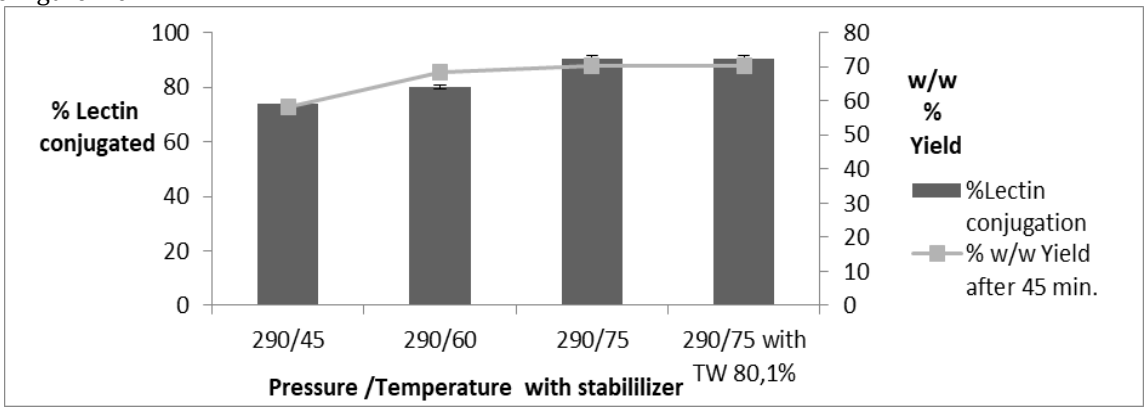

Figure 2.0: Effect of pressure/temperature and stabilizer on % lectin conjugation and yield of lectin conjugated nanoparticles

From observations and graph, it was observed that at increased pressure and temperature, drug content and yield were maximum. This can be attributed due to more solubilising and mass transfer of

components with increased pressure and temperature, such observations were also seen by P.Pathak *et al.*1999.[21] At these conditions of higher pressure and temperature, flexibility of polymer chain was optimum and that lead to higher percentage of drug that was encapsulated due to solubilisation with higher solvent strength of SCF with higher supersaturation ratio, uniform mixing, nucleation were achieved in the SFT process. Due to equilibrium in the system with optimum, lower viscosity and density of drug and polymers higher lectin conjugation to encapsulated polymeric nanoparticles and lower particle size were obtained. The results were in sync with observations by M.Meziani *et al*. [5].

Effect of temperature/ pressure and stabilizer on encapsulation efficiency (%) and drug content (%) for lectin conjugated drug polymeric nanoparticles

With increase in pressure and temperature due to solubilising of the components in SCF at higher supersaturation, nucleation and higher solvent strength lower particle size with higher encapsulation efficiency were obtained. It was observed that at lower pressures interaction between drug and polymer was reduced, resulting in lower encapsulation efficiency. Also flexibility of polymer chain was optimum at higher pressure and temperature. So higher drug encapsulation was anticipated due to uniform mixing, solubilisation, optimum viscosity of solution. Based on these results, it was believed that high pressure for enhanced solvent strength of $SCFCO_2$ along with lower density/ viscosity of the system were essential for the interaction of polymer, lectin and drug. These observations and results were in sync with resultss by Turk *et al.*2009[18].

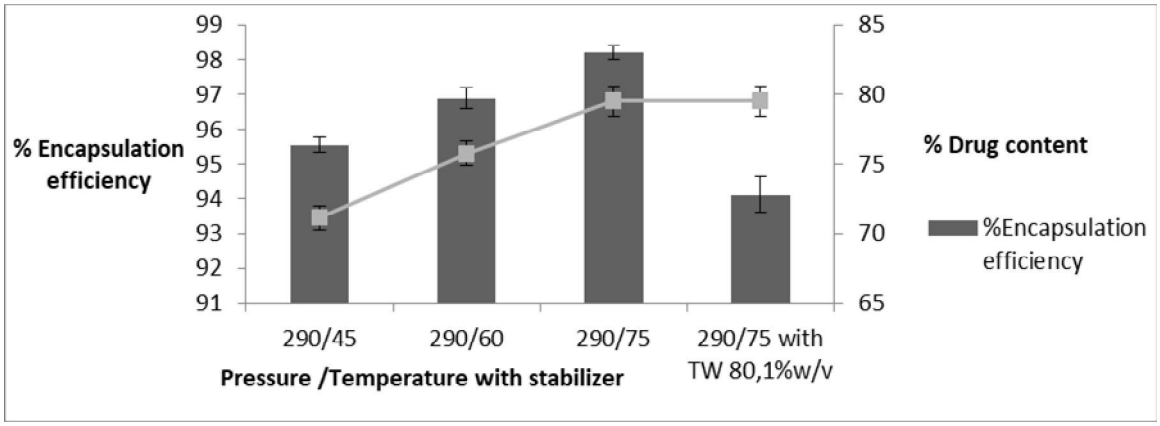

Figure 3. 0: Effect of pressure/temperature and stabilizer on % encapsulation efficiency, % drug content of nanoparticles

As preformulation studies results has showed that drug was soluble in tween-80, there was a chance of removal of loosely bound drug from encapsulated nanoparticles by tween-80 by forming micelles. So encapsulation efficiency (%) was reduced in presence of tween-80. Similar results were obtained were described by A.A.Date *et al* [22] in their review also in the research experimentation by Amporn S. *et al.* [15] and C.B.Fernandes *et al.* [16].

Zeta potential

Zeta potential of hydroxypropyl methyl cellulose encapsulated retinol acetate nanoparticles (before lectin conjugation) was found -14.9 ± 2.0 mev. While zeta potential of lectin conjugated hydroxypropyl methyl cellulose encapsulated retinol acetate nanoparticles was found -14.5 ± 1.2 mev. Reduction in zeta potential of lectin conjugated drug polymeric nanoparticles can be attributed due to stabilization of particles by lectin by decreasing electrostatic interaction with increase in the charge density. All lectin conjugated nanoparticles have shielding effect on the superficial charge by diffusion of lectin, so negative zeta potential value with lowered zeta potential was obtained. This has suggested that encapsulation of drug by polymer and conjugation of lectin to nanoparticles. These results obtained were agreed with Xiaoling Gaoa *et al.*2006[1] experiments results.

Particle morphology by scanning electron microscopy (SEM)

As observed in the SEM images (figure: 4) of drug retinol acetate: HPMC K-100M optimized nanoparticles and lectin conjugated nanoparticles were almost spherical shaped. There was homogeneity of shape and obtained dispersion was found viscous in nature. Particle size was in the desired for range targeted delivery which was below 350 nm size. Particles size and polydispersity index for retinol acetate: HPMCK-100 M nanoparticles before and after lectin conjugation was 138.6 ± 1nm; 0.583 ± 0.03 and 244.8 ±10.2

nm; 0.583 ± 0.12 respectively. Lectin conjugated nanoparticles have fuzzy morphology with brighter sides.

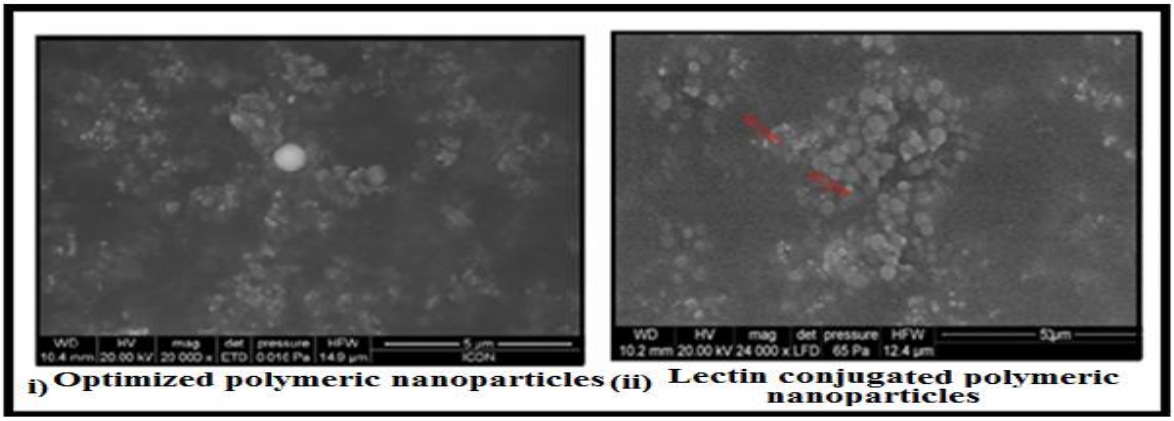

Figure 4.0: SEM images of retinol acetate: HPMC K-100 M nanoparticles before and after lectin (1:1:1 w/w) conjugation

Overall results of optimized lectin conjugated nanoparticles characterizations is given in the table no :1 below.

Table 1.0: Overall result of optimized lectin conjugated nanoparticles

Nanoparticles system at 290 bars and75^{o}C for 45 minutes	Particle size (nm) with std. dev.	Polydispersity index (P.I.) with std. dev.	Zeta potential (mev) with std. dev.	E.E. (%) with std. dev.	Lectin Conjugated (%) with std. dev.	Drug content (%) with std. dev.	Yield (%w/w) with std. dev.
Drug /HPMCK-100M/ Lectin 1/1/1, w/w	244.8 ± 10.2	0.583 ± 0.12	-14.5 ± 1.2	98.2 ± 0.2	90.65 ± 1.02	79.5 ± 1.1	70.2 ±1.0

Fourier transform infrared spectroscopy (FTIR) studies

In FTIR spectras (Figure:5) majority of drug, HPMCK-100M and lectin spectral peaks of functional groups were observed in lectin conjugated nanoparticles with slight change in intensity and change in spectrum wave number of major functional groups. Characteristics of retinol acetate, B-ionone ring 2873.94 cm^{-1} changed to 2918.23 cm^{-1}, 2914.66 cm^{-1} alkyl group from HPMCK-10M was seen at 2918.63 cm^{-1} in lectin conjugated nanoparticles. Characteristic of amine /amide bonds at 1630.18 cm^{-1} in lectin were seen in nanoparticles at 1635.57 cm^{-1} wave-number frequency with broadening and change in the spectral peak position. These all findings have indicated that modified RESS process with mixing and sonication was able to encapsulate drug and conjugate lectin without affecting their characteristics.

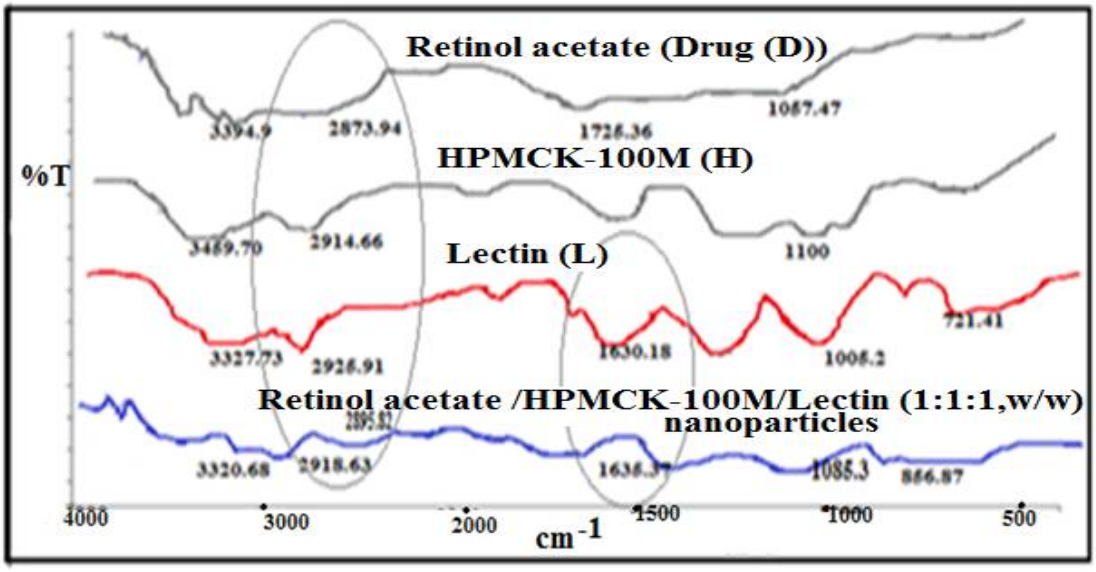

Figure 5.0: FTIR spectra's of drug, excipient and nanoparticles

Characteristics of ether / ester functional groups of drug and polymer, lectin in between 1000-1200 cm^{-1} were seen in nanoparticles with broadening of peak. In conjugation of lectin by SFT, there was formation of covalent bonding with hydrogen bonding. Thus, preparation of lectin grafted micro/nano-particles was initiated by activation of carboxyl groups of the bioerodible polymer with nitrogen /amide /amine bonds of lectin, similar studies were also performed by Xiaoling Gao *et al.*2006.[1]

C-13 N.M.R. spectroscopy

Major changes in the peak chemical shift positions of functional groups, peak broadening and encapsulation, conjugation are encircled in all optimized lectin conjugated nanoparticles in the figure 6. Characteristic of retinol acetate B-ionone ring at 120 PPM chemical shift has been changed to 135.1 PPM in nanoparticles. Characteristic pyranose ring at 120 PPM for lectin and 122.2 PPM for HPMCK-100M were seen in lectin conjugated HPMCK-100M encapsulated retinol acetate nanoparticles NMR at 130 PPM, 124 PPM respectively. Characteristics of nitrogen bonding amide or amine from lectin were seen at 165 PPM changed position to 173 PPM. C-13 NMR spectra of lectin cojugated drug retinol acetate polymeric nanoparticles obtained with HPMCK-100M, has also shown major functional groups those were characteristics of drug, lectin and the polymer. Nanoparticles have also shown amide group. This has indicated that the lectin was conjugated with nanoparticles and encapsulation of drug by polymer such studies were also done by Yamaguchi *et al.*2006.[23]

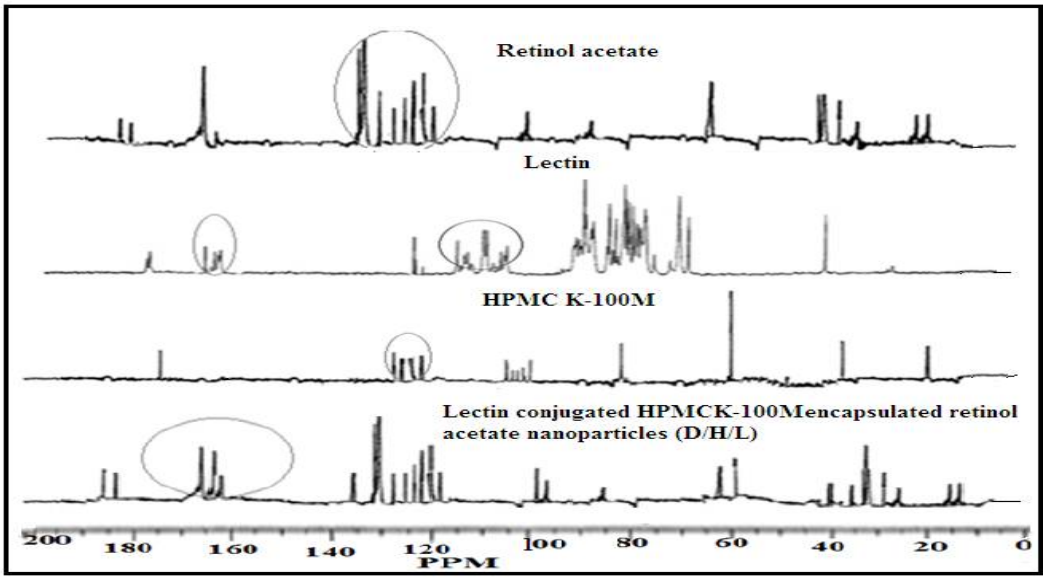

Figure 6.0: C-13 N.M.R. spectra of lectin conjugated nanoparticles

X-ray diffraction (XRD) study

XRD pattern of HPMC K-100M polymeric (1:1:1, w/w) exhibited amorphous state. Retinol acetate XRD study exhibited several peaks suggesting crystalline -amorphous. While lectin XRD study exhibited amorphous state. In lectin conjugated drug polymeric nanoparticles, the Prominent, characteristic crystalline peaks of drug substance around 10-20 Ø were found reduced due to encapsulation of drug in polymer and conjugation of lectin.

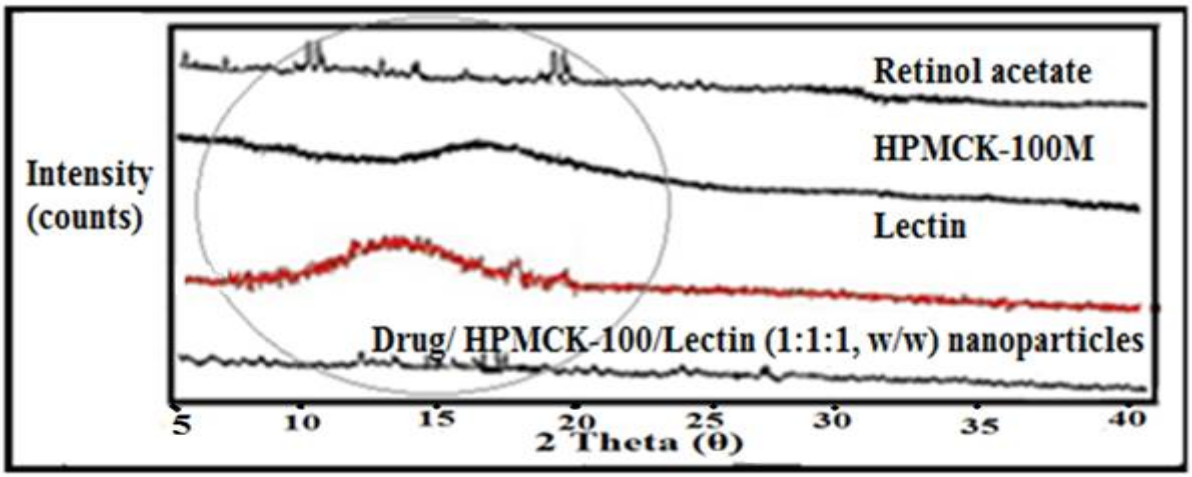

Figure 7.0: XRD patterns of drug, polymer, lectin and lectin conjugated nanoparticles

Obtained nanoparticles were amorphous due to increased imperfections due to rapid change in the environment in SFT process as reported by Pratik S.*et al*.2013[24] these all changes have shown by encircled in the XRD figure 9.

***In-vitro* drug release study**

As shown in the figure: 8, in pH 1.2, 4.5, 6.8 initial drug releases were found about 20 % , further no release was observed till 4 hours and then complete release was observed for a period of about 8-9 hours thus showing biphasic release, slower and faster burst release. In pH7.4, slow release was observed (about 20% release over period of 5 hours) for initial period of 5 to 6 hours and then gradual rise in release was observed till 10 hours. However, after 10 hours release was stagnant and complete release was not observed till the tested time point of 13 hours.

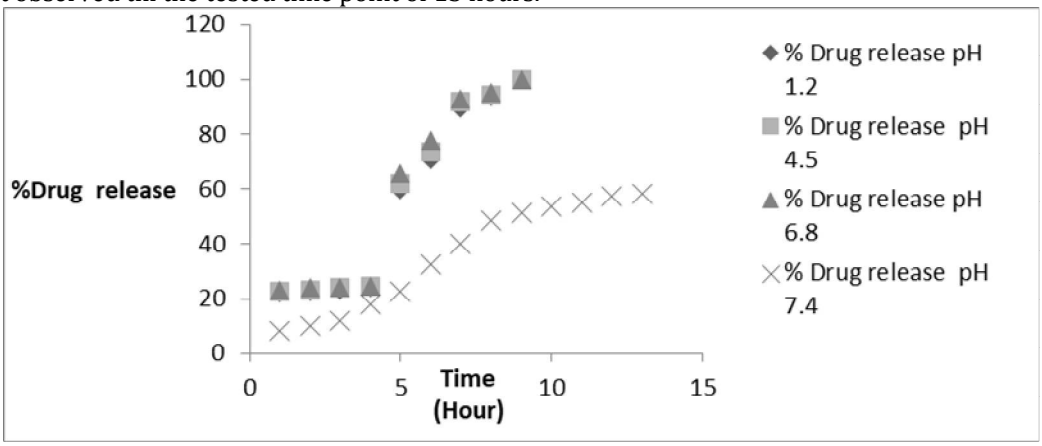

Figure 8.0: *Invitro* drug release study of lectin conjugated nanoparticles in different pH solutions

The release characteristics of these nanoparticles could be attributed due to profound swelling of HPMC K-100M and solubility limitations of lectin, is shown in the figure. The initial fast rate of release was commonly ascribed due to drug detachment from nanoparticles surface while the later slow release results from sustained drug release from the inner surface. This has indicated that lectin conjugation has influenced the slightly retarding drug release profile in various studied media due to stabilizing nature of lectin, as compared to unconjugated naoparticles. Lectins stabilizing nature with prolonging release of drug in formulations was also indicated by Yamaguchi *et al.* 2010.[23]

For the release of drug in various pH with time, the release characteristics were studied with the standard kinetic equations. As lectin acted as stabilizer drug release with time was found definite, slow without

change in area and disaggregation of particles. Lectin was found to be responsible for prolonged activity and suspended drug release from nanoparticles as compared to unconjugated nanoparticles. Similar results were obtained to Paulo Costa *et al*. 2001.[25] As R^2 value for all the studied pH range was observed to 0.952 to 0.972 was complying with the criteria of zero order release mechanism of drug. (in the table 2.0; bold lettered equations suits the release pattern)

Table 2.0: Kinetic *in-vitro* drug release equations for lectin conjugated nanoparticles

Nanoparticles with buffer solution pH	Zero order release	First order release	Hixon-crowell release	Higuchi release	Korsmeyer Peppas
D/H /L, 1.2	**Y=10.78X+2.1 R^2 = 0.963**	Y= - 0.13X +2.093 R^2 = 0.89	Y= 0.5X -0.468 R^2 = 0.883	Y= 31.90X -9.821 R^2 = 0.872	Y= 0.753X +1.239 R^2 = 0.817
D/H/L, 4.5	**Y=10.8X+2.81 R^2 = 0.961**	Y= - 0.148X +2.124 R^2 = 0.866	Y= 0.498X - 0.465 R^2 = 0.877	Y= 32. 9X -9.444 R^2 = 0.878	Y= 0.745X +1.254 R^2 = 0.816
D/H /L , 6.8	**Y=10.87X+3.5 R^2 = 0.952**	Y= - 0.163X +2.115 R^2 = 0.918	Y= 0.502X - 0.442 R^2 = 0.895	Y= 33.96X -9.038 R^2 = 0.878	Y= 0.741X + 1.265 R^2 = 0.811
D/H /L, 7.4	**Y= 5.735X + 0.1 R^2 = 0.972**	Y= - 0.35X + 2.011 R^2 = 0.970	Y= 0.11X -0.023 R^2 = 0.972	Y= 19.61X -6.201 R^2 = 0.875	Y= 0.85X + 0.826 R^2 = 0.903

Mucin adsorption and Mucoadhesive strength studies

Lectin conjugated nanoparticles was also found to attach to glycocalyx of mucosa, as well as they have higher diffusion and penetration activity. So obtained more mucin adsorption and higher mucoadhesive strength as shown in the figure 9 and 10 respectively. Time, molecular composition, pH, molecule conjugated, particle size, concentration were found effective factors in mucin adsorption. These results were also agreed to observations and the reports of S.B.Patil *et al*.2006[26] and S.E.Harding *et al*.2003. Higher mucin adsorption and mucoadhesive strengths are necessary to prolong residence time of drug at the site of action, thus increases concentration gradient to have improvement of bioavailability of drug delivery systems as indicated by Lis, H.*et al*.1998.[27]

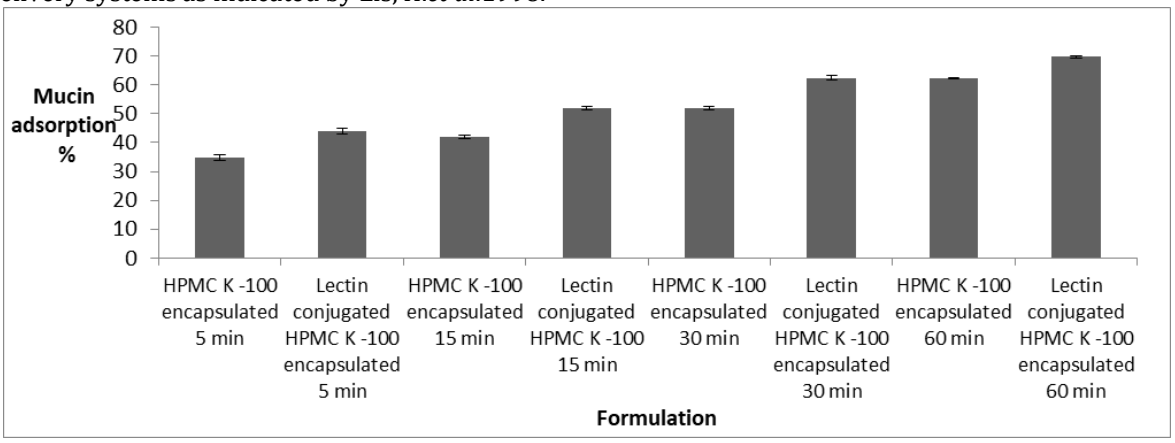

Figure 9.0: Mucin adsorption (%) with time for nanoparticles

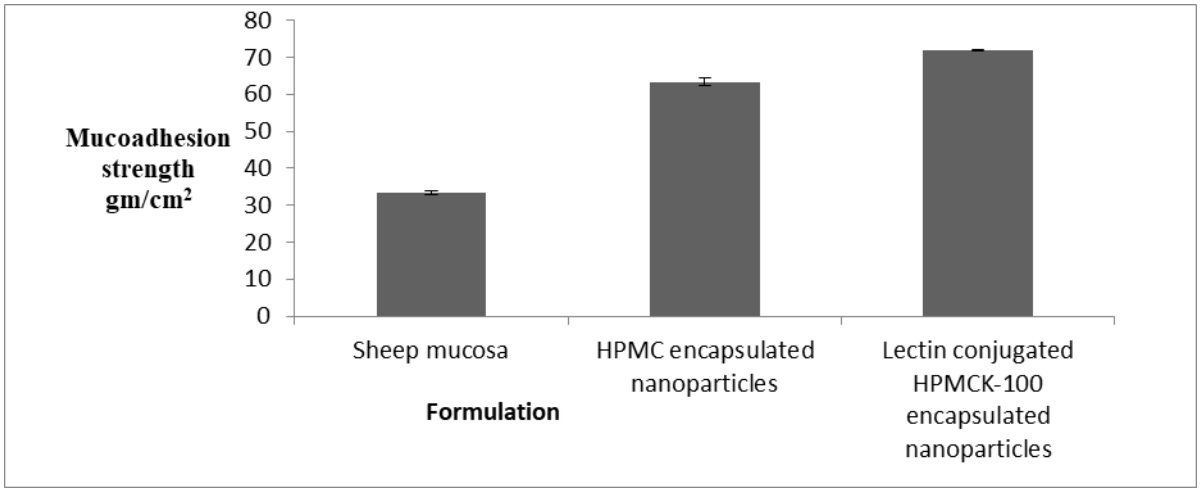

Figure 10.0: Mucoadhesion strength for nanoparticles

Long polymer chain compounds with anionic group (eg. carboxyl groups) charge have higher flexibility and entanglement of chain penetrate mucus easily by adsorption and elelectrostatic interactions. Hence higher mucin adsorption with mucoadhesive strength were obtained for lectin conjugated polymeric nanoparticles. Mucoadhesive strength and mucin adsorption of lectin conjugated nanoparticles were found more due to close surface contact with mucosa due to stronger electrostatic attractions. Similar observations were reported by Clark M.A. *et al.*2000[28] and S. E. Harding *et al.*2003.[29]

Cell cytotoxicity studies

Lectin was found responsible for improved cell cytotoxicity, cytoadhesiveness with more intracellular retention of nanoparticles. These lectin conjugated nanoparticles were taken by COLO-205 cell-lines by active transcytosis with vesicular receptor mediated transport. Hence higher have also proved potential for the COLO-205 cell line as a useful tool to evaluate lectin conjugated as well as unconjugated nanoparticles colon cancer studies. Similar studies were also done by Wirth M. *et al.*2002[30], Qingxiang Song *et al.*2012[31] in the colon carcinoma cell lines for screening anticancer activity of formulations.

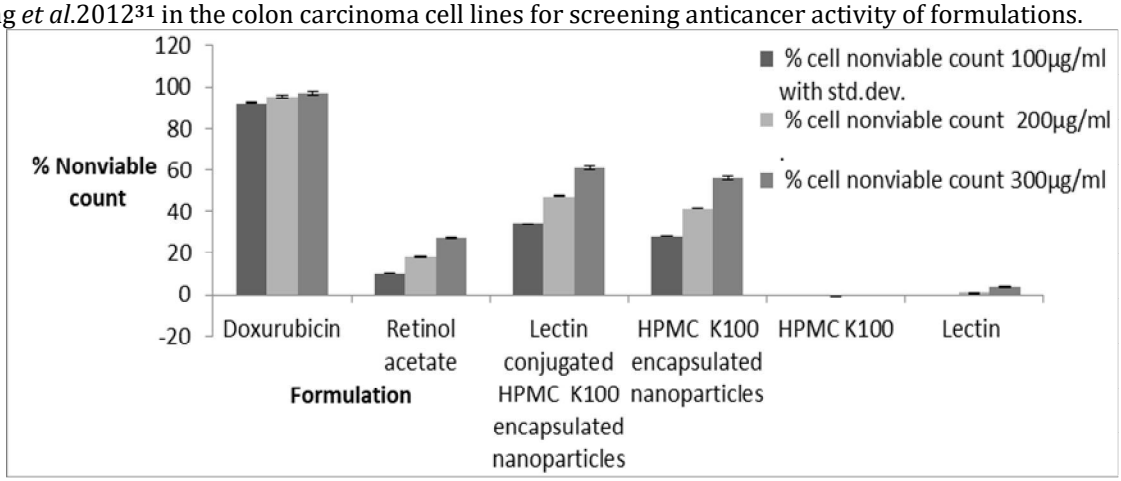

Figure 11.0: MTT cell cytotoxicity studies for components and nanoparticles

Cell cytotoxicity results were shown in the experiments results shown in the figure: 11.

CONCLUSIONS

Modified RESS process (SAPSS®) with mixing and sonication was found to be effective, single step, simple, green, feasible technology for conjugation of lectin with nanoparticles. For all nanoparticles system, at higher process pressure and temperature (290 bars, 75°C, for 45 minutes) good yield was obtained. Also at these conditions the best results were obtained for encapsulation efficiency (%), lectin conjugation (%) and drug content (%). These parameters were considered as an optimization condition. Ultra-low surface tension of SCF-CO_2 has allowed facile penetration, increasing contact efficiency, better adsorption of lectin on surface, increasing overall yield, solubility adjustment of lectin. Lectin itself acted as stabilizer /surfactant so nanoparticles particles size and polydispersity index were found smaller and

narrower as compared to collection with tween-80. Presence of lectin in nanoparticles system were ensured by FTIR spectroscopy, hemagglutination test, UV-Visible method for conjugation quantity determination and C-13NMR spectroscopy. All nanoparticles were found spherical in shape with particles size below 350 nm. Lectin conjugated nanoparticles has shown comparatively more mucoadhesive strength, mucin adsorption and cell cytotoxicity. These studies have confirmed that lectin-conjugated nanoparticles offer an effective, potential and promising drug delivery system to target the colon to provide
necessary therapeutic effect of the drug substance.

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DECLARATION
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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