www.amlett.org, www.amlett.com, DOI: 10.5185/amlett.2012.icnano.203
"ICNANO 2011" Special Issue Published online by the VBRI press in 2012

Low molecular weight palmitoyl chitosan: Synthesis, characterization and nanoparticle preparation

Yogesh M. Choudhari[1], Sachin V. Detane[1], Sushant S. Kulthe[1], Chandrakant C. Godhani[1], Nazma N. Inamdar[1], Seema M. Shirolikar[2], Lalit C. Borde[2], Vishnukant K. Mourya[1,*]

[1]*Government College of Pharmacy, Osmanpura, Aurangabad 431005 (M.S.) India*
[2]*Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai 400005 (M.S.) India*

*Corresponding author. Tel.: (+91) 9890183001; E-mail: vkmourya@gmail.com

ABSTRACT

Low molecular weight chitosan (LMWC) exhibits higher water solubility and produces nanoparticles of fairly low particle size. However, poor drug loading and shorter circulation time in body limits its application in preparation of nanoparticles. Acylation of LMWC ensures extended circulation of nanoparticles in body and hence enhanced bioavailability of the drug. We therefore synthesized the acylated LMWC using palmitoyl chloride and confirmed its synthesis by FTIR and NMR spectroscopy. The nanoparticles of LMWC and low molecular weight palmitoyl chitosan (LMWPC) were prepared by miniemulsion and chemical crosslinking method using glutaraldehyde and 5-fluorouracil (5FU) as a model drug. The nanoparticles were evaluated for particle size, zeta potential, morphology, drug loading and drug release. TEM analysis revealed nanosize and spherical nature of the particles. The palmitoyl chain of LMWPC increased particle size from 83.2±2.5 nm to 93.4±3.2 nm whereas zeta potential of nanoparticles decreased from 12.5±2.2 mV to 4.2±1.1 mV due to diminished amino groups of LMWPC as a result of acylation. The drug loading in nanoparticles was increased from 13.8±0.95% to 30.2±1.9%. LMWC showed 80±2.08% as maximum drug released in 10 h while only 52.3±2.14% was released in 24 h for LMWPC. Hence, LMWPC nanoparticles ensure increased drug loading capacity and sustained drug release profile without significant change in particle size.

Keywords: 5-Fluorouracil; crosslinking; low molecular weight palmitoyl chitosan; nanoparticles; *N*-acylation.

Yogesh. M. Choudhari has done his M. Pharm from Government college of Pharmacy, Aurangabad, India. Presently, he is pursuing Ph.D. under the guidance of Dr. V. K. Mourya. During his studies, he worked on the topical drug delivery to oral cavity and applications of chitosan in novel drug delivery systems.

Nazma N. Inamdar has done her M. Pharm from LM College of Pharmacy, Ahemedabad, India. She started her teaching career in NDMVP Samaj College of Pharmacy, Nashik. She is currently working as Assistant Professor at Allana College of Pharmacy, Pune. She teaches medicinal chemistry and analytical chemistry at graduate and post graduate level. She is pursuing Ph.D

V. K. Mourya is presently working as Principal at Government College of Pharmacy, Amaravati, (MS) India. He is engaged in the development of biomaterials and their application in drug delivery systems especially for the nano confined target. He has contributed in QSAR studies involving molecular modelling. He is pharmacy postgraduate from Dr. H. S. Gaur University, Sagar, India and Ph.D from SGSITS, Indore, India

Introduction

Biodegradable polymers are widely used in the nanoparticle preparation due to their ability to get degraded in the body [**1**]. Amongst biodegradable polymers, chitosan is widely used due to its non-toxicity, biocompatibility, biodegradability and ease of availability. Chitosan, a linear polymer of β-1, 4 linked N-acetyl-D-glucosamine and D-glucosamine units, possesses various properties suitable for nanoparticle preparation viz suitability for administration by any route, ability to control drug release, possibilities for chemical modification, mucoadhesivity due to its cationic nature and ability to cross link [**2, 3**].

However, the high solution viscosity and coiled nature of chitosan generates particles of large size. This problem can be addressed by depolymerization of chitosan to LMWC. LMWC produces aqueous solution with low viscosity and can be used to produce nanoparticles with size less than 100 nm, but only at the cost of premature drug release. The reason may be attributable to the shorter chains in LMWC which result in loose entrapment of drug and rapid erosion of LMWC due to its water solubility and enzymatic degradation consequences early drug release [**4**].

The existence of two hydroxyl groups and one amino group in the monomer of LMWC and its water solubility propose wide possibilities for chemical modification of LMWC and consequently for preparation of nanoparticles with small particle size for sustained drug delivery. N-acyl chitosan has shown its ability for longer retention in body and resistance to digestible enzymes like lysozyme and chitinase. Hirano *et al*. revealed that N-acyl chitosan is sparingly digestible by enzymes and is more biocompatible than native chitosan [**5**]. Chengyuan *et al*. showed that nanoparticles from succinyl chitosan have property of long term retention in the body resulting in sustained drug release [**6**]. Lin *et al*. acylated chitosan with caproyl chloride and prepared its nanoparticles with surface modification using glycyrrhizin to target hepatocyte. Nanoparticles from this acylated derivative offered more stability at physiological pH condition [**7**]. The findings of Tien *et al*. suggested that acylation of chitosan reduces the hydration of polymer and helps in sustaining the drug release with its poor diffusion through nanoparticles. This property is encouraged with increase in chain length of acyl group [**8**]. Also, results suggest that N-acylchitosans are more useful in tumor targeted drug delivery as they affect the selective aggregation of some cancer cells [**9**].

Hence, we acylated the LMWC with palmitoyl chloride and confirmed its synthesis by FTIR and NMR spectroscopy. Furthermore, the synthesized derivative was employed for preparation of nanoparticles by miniemulsion followed by cross linking with glutaraldehyde using 5FU as a model drug. The nanoparticles were characterized for particle size, polydispersity index (PDI), zeta potential, morphology, drug loading, entrapment efficiency and drug release characteristics.

Experimental

Materials

LMWC (2.5 kDa; DDA 75%) and palmitoyl chloride were synthesized in our laboratory and characterized as previously reported [**10**]. Sodium hydroxide was purchased from Merck Chemicals. 5FU was obtained as a gift sample from Khandelwal Laboratory (Mumbai, India). Glutaraldehyde was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Tween 80 and Span 80 were obtained from Research Lab. (Mumbai, India). All other reagents were of analytical grade and used without further purification.

Synthesis of LMWPC

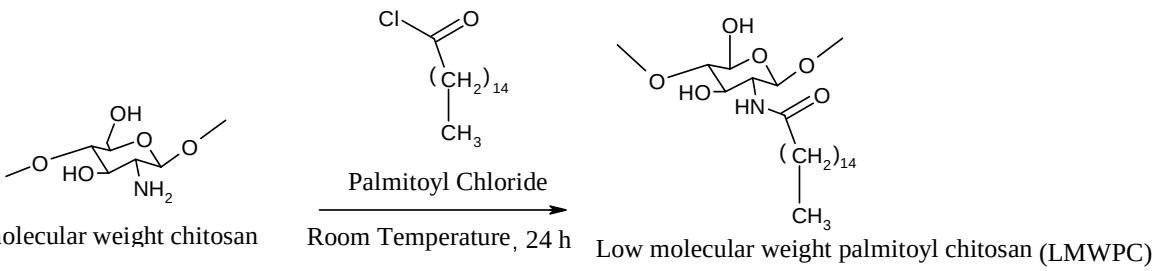
LMWC was acylated with use of acyl chloride. Briefly, LMWC (5 g) was dissolved in water and pH was adjusted to 7.2 with sodium hydroxide solution (0.1N). Palmitoyl chloride (prepared from the reaction of palmitic acid and thionyl chloride) was added drop wise (20 mL) in LMWC solution with continuous stirring and allowed to react for 24 h at room temperature (**Scheme 1**). The obtained product was precipitated with acetone and lyophilized.

Characterization of LMWPC by FTIR and NMR spectroscopy

FTIR spectrum of the obtained product was recorded from 400 to 4000 cm^{-1} with Shimadzu, Prestige 21 FTIR spectrometer. 1H NMR spectrum was recorded on a Bruker Avance 700 MHz NMR spectrometer against tetramethylsilane (TMS) as reference standard using D_2O as solvent.

Preparation of LMWC and LMWPC nanoparticles

LMWC and LMWPC nanoparticles were prepared by miniemulsion method followed by chemical crosslinking [**11**]. 5FU, 0.1 g of LMWC or LMWPC, 0.1 g of Tween 80 were dissolved in 10 mL of 0.5% w/v glacial acetic acid to obtain the aqueous phase. The oil phase comprised of



Low molecular weight chitosan — Low molecular weight palmitoyl chitosan (LMWPC)

Scheme 1. Synthesis of low molecular weight palmitoyl chitosan.

hexane (70 mL) and Span 80 (0.9 g). The pre-emulsion was prepared by adding aqueous phase into the oil phase with constant stirring at 400 rpm on magnetic stirrer for 20 min. The obtained pre-emulsion was probe sonicated at 500 W for 200 cycles (working sonication for 2 s following pause for 3 s) to get miniemulsion. The prepared miniemulsion was cross-linked with glutaraldehyde (1:10 molar number of chitosan) along with stirring for 4 h to get nanoparticles. The nanoparticles were washed with petroleum ether and centrifuged at 14000 rpm for 30 min to ensure complete removal of hexane and unreacted glutaraldehyde. The obtained nanoparticles were lyophilized.

Evaluation of LMWC and LMWPC nanoparticles

Determination of particle size, PDI, zeta potential and morphology: The samples for particle size, PDI and zeta potential were prepared by dispersing lyophilized nanoparticles in deionised water and analysed using dynamic light scattering (DLS) using Delsa Nano particle analyzer (Beckman Coulter, Inc., CA, USA) with a laser diode (30 mW) at 658 nm and 25°C. The morphology of nanoparticles was examined by Libra 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany). The sample was stained with 2% w/v phosphotungstic acid for 10 min, immobilized on copper grid coated with Formvar and dried for viewing by TEM.

Determination of % drug loading (DL %) and % entrapment efficiency (EE %): The 5FU loaded nanoparticles were redispersed into 10 mL of deionised water and vortexed for 5 min. The prepared dispersion was centrifuged at 14000 rpm for 30 min and supernatant was separated, filtered through 0.22 µm filter (Millipore™) and analyzed at 266 nm using UV-Visible 2501 PC spectrophotometer (Shimadzu Co., Kyoto, Japan).

DL % and EE % were calculated using expressions as described below [**12**]-

$$\text{DL \%} = \frac{\text{amount of 5FU added - amount of 5FU unentrapped}}{\text{amount of nanoparticles recovered}} \times 100$$

$$\text{EE \%} = \frac{\text{amount of 5FU added - amount of 5FU unentrapped}}{\text{amount of 5FU added}} \times 100$$

In vitro drug release study

The 5FU loaded nanoparticles (70 mg) were dispersed in 5 mL enzyme-free simulated gastric fluid (SGF) as per USP and were placed into cellulose dialysis bag (MWCO= 3.5 kDa, SpectraPor), presoaked in SGF. Dialysis was done against 60 mL of enzyme-free SGF for 2 h and further with simulated intestinal fluid (SIF) as per USP for 24 h with 75 rpm speed at 37°C on thermostatically controlled orbital shaker incubator (HMG India Lab Enterprises, Mumbai, India). Aliquots of 5 mL were withdrawn from the release media at predetermined time intervals of 0.25, 0.5, 1, 2, 3,…..12, 18 and 24 h and an equivalent amount of fresh SIF (prewarmed at 37°C) was added to the release medium after each collection of aliquot. The withdrawn aliquots were filtered through 0.22 µm filter and analyzed for content of 5FU by UV spectrophotometer.

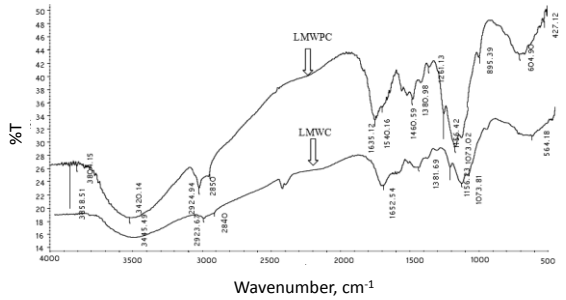


Fig. 1. FTIR spectrum of LMWC and LMWPC.

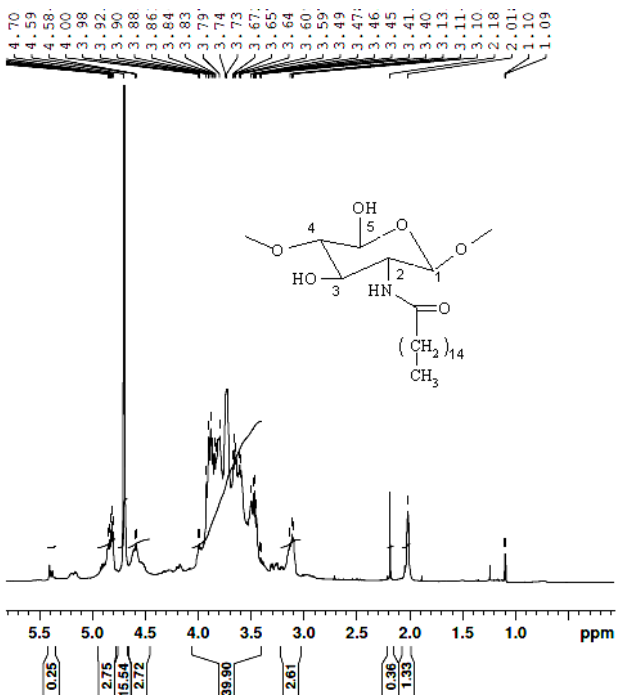


Fig. 2. NMR spectrum of LMWPC.

Results and discussion

Synthesis and characterization of LMWPC

The LMWC used in this study was synthesized in our laboratory using previously reported method. Briefly, high MW chitosan (300kDa) was depolymerised by nitrous acid treatment followed by precipitation of product with acetone and lyophilization [**13**]. The molecular weight (MW) of depolymerised chitosan was found to be 2.5 kDa which is further acylated with palmitoyl group. The highly reactive palmitoyl chloride was used to react with LMWC to produce LMWPC. The glucosamine residue of LMWC possess reactive amino group at C-2 position which reacts with palmitoyl chloride. After completion of reaction, the product was precipitated with acetone and dried by lyophilization for further use. Acylation of LMWC was confirmed by FTIR and NMR spectroscopy. The peaks in FTIR spectrum of LMWPC (**Fig. 1**) at 2924 cm^{-1} and 2850 cm^{-1} were attributed to C-H stretchings in alkyl chain of palmitoyl group. These bands are strong in LMWPC in comparison to LMWC. The band at 1156 cm^{-1} arising due to C–O stretching was also strengthened. While those

bands at 1635 cm^{-1} and 1540 cm^{-1} corresponded to amide bond C=O stretching and N-H bending vibrations, respectively. The peak at about 1740 cm-1 is an indicative of ester group formed by O-acylation. But, the aforesaid peak is absent in the synthesized product (**Fig. 1**) and hence confirmed that only N-acylation occurred during synthesis. These observations are similar with Hirano et al who studied the acylation of chitosan with different fatty acids. Author observed both N and O acylation with the lower chain fatty acids (acetyl, propionyl and butyryl chitosan) while only N-acylation occurred with higher chain fatty acids such as lauroyl, myristoyl, palmitoyl etc.[**13**] NMR spectrum showed signals at 1.0, 1.1 and 2.18 ppm indicating the presence of $–CH_3$,$–CH_2–$, and $–H_2C–C=O$ groups of LMWPC, respectively (**Fig. 2**). The signals near 3.5 to 4.0 were attributed to hydrogens of pyranose ring of LMWPC. The obtained observations are comparable with the Tien et al **[8]**.

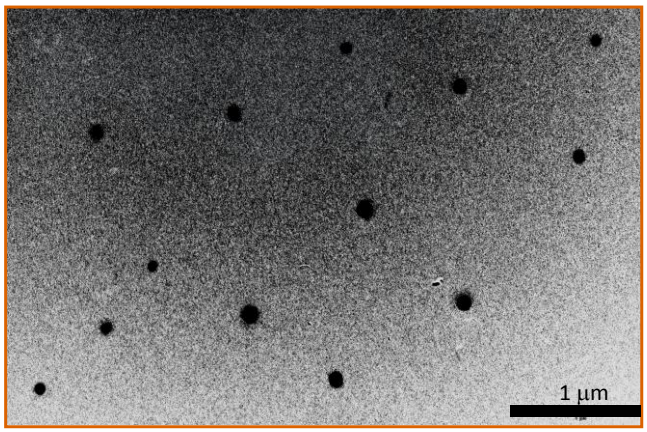


Fig. 3. TEM image of LMWPC nanoparticles.

Preparation of LMWC and LMWPC nanoparticles

In the present work, LMWC and LMWPC were used to prepare nanoparticles with a focus to achieve minimum particle size, enhanced DL and sustained release of 5FU. The reports reveal that, 5FU is more prone to degradation by enzymes such as dihydropyrimidine dehydrogenase or uracil reductase. Also, it is more susceptible to degradation on storage to cardiotoxic product. The entrapment of 5FU in the chitosan nanoparticles ensures protection from such degradation and justifies the rationale of drug selection [**14**]. The process of nanoparticles preparation included w/o miniemulsion followed by crosslinking with glutaraldehyde. Both, hydrophilic Tween80 and lipophilic Span80 were employed to ensure the stability of miniemulsion. The 5FU and LMWPC were dissolved in the water phase along with Tween80 which was further transferred to oil phase comprised of hexane and span80. Some preliminary experiments were carried out to ensure the optimimal stirring time, speed and sonication time for preparation of miniemulsion. The least globule size of miniemulsion was observed at 400 rpm for 20 min and sonication for 200 cycles. Thus, the same process parameters were implicated for further batches. The glutaraldehyde was added slowly in the prepared miniemulsion along with stirring for 4 h. The sufficient time was allowed for cross linking to occur as swelling of nanoparticles and rate of drug release is affected by the extent of crosslinking. During crosslinking, the protonated amino groups of chitosan reacts with aldehydic functional groups of glutaraldehyde in inter- and intramolecular fashion to form covalently cross-linked networks. Though there are different views about the use of glutaraldehyde as crosslinking agent, its safety and non-carcinogenicity is previously reported in literature and there are no reports suggesting its toxicity for concentration range used in our work [**15, 16**]. Furthermore, The C=N bond involved during the cross-linking reaction turn irreversibly into a stable C-N form, in vivo. Such a conversion would reduce probable side effects with higher concentration and justifies the potential use of glutaraldehyde. The unreacted glutaraldehyde and hexane were removed after washing with petroleum ether using centrifugation. The successful preparation of nanoparticles was confirmed by evaluating size, zeta potential and morphology.

The particle sizes of LMWC and LMWPC nanoparticles were found to be 83.2±2.5 nm and 93.4±3.2 nm, respectively. PDI for both LMWC and LMWPC nanoparticles were ranged below 0.3 which signified a fairly monodisperse pattern of size distribution. The slightly increased size of LMWPC could be due to its longer palmitoyl chain group. The nanosize and spherical nature of the nanoparticles was confirmed by TEM analysis (**Fig. 3**). The corresponding zeta potential values of LMWC and LMWPC nanoparticles were 12.5±2.2 mV and 4.2±1.1 mV. These results of zeta potential are comparable with the observations of Lin et al. Authors observed that surface treatment to N-caproyl chitosan nanoparticles decreases its zeta potential. Thus, he concluded that NH3+ density on the surface of nanoparticles is responsible for the measured positive zeta potential of chitosan nanoparticles [**17**]. The decrease in zeta potential of LMWPC nanoparticles could be a result of decreased free amino groups due to N-acylation of LMWC.

DL and EE

In nanoparticulate system, drug can be loaded either during the preparation of nanoparticles or after its preparation. In the present process, 5FU was loaded during nanoparticle preparation. The DL in chitosan nanoparticles is by hydrogen bonding and ionic interaction. The ionic interaction is increased in the case of LMWC due to shorter chitosan fragments which allow easy protonation of free amino groups, easy drug interaction and thus results in greater drug encapsulation efficiency. 5FU is an anionic drug which interacts with cationic LMWC and LMWPC to ensure maximum DL. The UV-Vis spectroscopic analysis was used to determine DL and EE. The DL and EE values were 13.8±0.95% and 69.04±2.5%, respectively for LMWC nanoparticle. LMWPC nanoparticles showed appreciably increased DL (30.2±1.9%) and EE (98.8% ±2.8%) as compared to LMWC nanoparticles. This could be due to increased hydrogen bonding by the palmitoyl chains which ensures strong entrapment of 5FU in nanoparticles. These results are analogous with Rathore *et al.* who determined DL of the N-lauroyl, palmitoyl and succinyl chitosan nanoparticles using albendazole as a

model drug. The obtained results suggest that N-acylation with longer alkyl chain results in increased DL in nanoparticles [**18**].

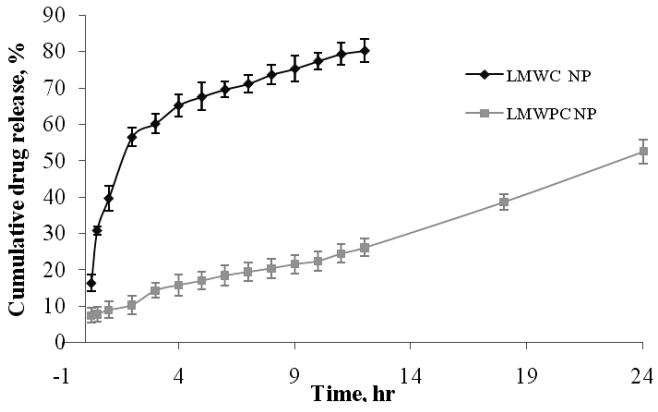


Fig. 4. 5FU release profile from LMWC and LMWPC nanoparticles.

Drug release

5FU possesses high rate of metabolism in body and demands continuous administration of higher dose to maintain therapeutic serum concentration which may lead to toxic effects [**14**]. The 5FU entrapped chitosan nanoparticles ensures protection of 5FU from metabolism and control on its release. The controlled 5FU release helps to maintain the therapeutic serum concentration and avoids toxicity related to higher drug dose. We have studied the *in vitro* release pattern of the prepared LMWC and LMWPC nanoparticles in SGF for first 2h and continued further in SIF. The observations of same are depicted in **Fig. 4**. There are three primary mechanisms such as erosion, diffusion, and swelling by which the release of drug molecules can be controlled in chitosan based nanoparticles and same is applicable for LMWC and LMWPC nanoparticles [**19**]. In first two hours, 56.4±1.98% and 10.3±0.73% of drug was released from LMWC and LMWPC nanoparticles, respectively. High release in first 2h with LMWC may be the consequence of release of adsorbed drug at and near the surface of nanoparticles. Also, higher penetration of water in nanoparticles results in relaxation of LMWC chains leading to erosion and bursting. Such kind of burst release was absent in LMWPC nanoparticles. The modulated solubility of LMWPC probably hinders the erosion effect by the aqueous environment. Agnihotri *et al*. observed that the penetration of water in the nanoparticles convert glassy polymer in to swollen rubbery matrix. As consequence drug starts diffusing proportionate to polymer swelling [**20**]. We observed that maximum drug released in 10h with LMWC nanoparticles was 80±2.08% while only 52.3±2.14% was released in 24h for LMWPC. This could be contributed to weak swelling and erosion of LMWPC nanoparticles due to hydrophobic palmitoyl chain. The extended release of the 5FU in LMWPC nanoparticles shows their higher stability at physiological pH and hence they may offer more time for accumulation of drug at tumor infected region.

Conclusion

The synthesis of LMWPC by acylation with palmitoyl chloride is easy and convenient method. The confirmation of acylation at N is done by IR and NMR spectroscopy. The preparation of nanoparticulate drug delivery by miniemulsion followed by crosslinking is possible with this hydrophobically modified LMWC. It produces spherical nanoparticles almost similar in size as the LMWC. Acylation of LMWC dramatically increases the DL and sustains the drug release. The sustained release of 5FU occurred as a consequence of the reduced swelling ability of LMWPC nanoparticles due to the palmitoyl chain. The sustained drug release by LMWPC nanoparticles ensures protection of 5FU for longer period and maintenance of therapeutic serum concentration for anticancer activity. The study of *in vitro* and *in vivo* anticancer activity of prepared 5FU loaded nanoparticles will be our future prospective. Nanoparticles prepared with LMWPC bestow higher DL, sustained release of drug and minimum particle size (below 100 nm) only at the cost of slightly decreased zeta potential. Hence, LMWPC nanoparticles can be proposed as prospective carriers for drugs as 5FU.

Acknowledgments

The authors are thankful to ICPA Health Products Ltd., Ankleshwar, Gujarat, India and Khandelwal Laboratories, Mumbai, Maharashtra, India for supporting the work

Reference

1. Soppimath, K.; Aminabhavi, T.; Kulkarni, A.; Rudzinski, W. *J. Controlled Release.* 2001, *70*, 1. **DOI:** 10.1016/S0168-3659(00)00339-4.
2. Janes, K.; Calvo, P.; Alonso, M. *Adv. Drug Delivery Rev.* 2001, *47*, 83. **DOI:** 10.1016/S0169-409X(00)00123-X.
3. Prabaharan, M.; Mano, J. *Drug Delivery*. 2005, *12*, 41. **DOI:** 10.1080/10717540590889781.
4. Santander, M.; Peula, J.; Goycoolea, F.; Ortega, J. *Colloids Surf., B.* 2011, *82*, 571.0 **DOI:** 10.1016/j.colsurfb.2010.10.019.
5. Hirano, S.; Yagi, Y. *Carbohydr. Res*. 1980, *83*, 103. **DOI:** 10.1016/S0008-6215(00)85369-0.
6. Yan, C.; Chen, D.; Gu, J.; Qin, J. *J. Pharm. Pharmacol.* 2006, *58*, 1177. **DOI:** 10.1211/jpp.58.9.0003.
7. Lin, A.; Chen, J.; Liu, Y.; Deng, S.; Wu, Z.; Huang, Y.; Ping, Q. *Drug Dev. Ind. Pharm.* 2009, *35*, 1348.
8. Tien, C.; Lacroix, M.; Ispas, P.; Mateescu, M. *J. Controlled Release.* 2003, *93*, 1. **DOI:** 10.1016/S0168-3659(03)00327-4.
9. Siiuca, A.; Wooden, R. *Fed. Proc.* 1970, *29*, 681; (b) Siiuca, A.; Wooden, R. *J. Natl. Cancer Inst.* 1971, *47*, 377.
10. Mourya, V.; Inamdar, N.; Choudhari, Y. *Polymer Sci. A.* 2011, *53*, 583. **DOI:** 10.1134/S0965545X11070066.
11. Du, Y.; Wang, L.; Dong, Y.; Yuan, H.; Hu, F. *Carbohydr. Polym.* 2010, *79*, 1034. **DOI:**10.1016/j.carbpol.2009.10.032.
12. Dudhani, A.; Kosaraju, S. *Carbohydr. Polym.* 2010, *81*, 243. **DOI:**10.1016/j.carbpol.2010.02.026.
13. Hirano, S.; Kondo, Y.; Fujii, K. *Carbohydr. Res*. 1985, *144*, 338. **DOI:**10.1016/S0008-6215(00)90682-7
14. 14. Arias, J. *Molecules.* 2008, *13*, 2340. **DOI:** 10.3390/molecules13102340
15. Mi, F.; Tan, Y.; Liang, H.; Sung, H. *J Bioact Compat Pol*. 2006, *21*, 351. **DOI:**10.1177/0883911506066930.
16. NTP. (1999). Toxicology and carcinogenesis studies of glutaraldehyde (CAS No. 111- 30-8) in F344/N rats and B6C3F1 mice

(inhalation studies). National Toxicology Programme Technical Report Series, *490*, 1–234.
17. Lin, A.; Chen, J.; Liu, Y.; Deng, S.; Wu, Z.; Huang, Y.; Ping, Q. *Drug Dev. Ind. Pharm.* 2009, *35*, 1348. **DOI:**10.3109/03639040902939197
18. Rathore, A.; Agrawal, S.; Manocha, N. *IJPRD*, 2011, *3*, 48.
19. Ko, J.; Park, H.; Hwang, S.; Park, J.; Lee, J. *Int. J. Pharm.* 2002, *249*, 165. **DOI:**10.1016/S0378-5173(02)00487-8
20. Agnihotri, S.; Nadagouda, N.; Mallikarjuna, T.; Aminabhavi, M. *J. Controlled. Release.* 2004, *100*, 5. **DOI:** 10.1016/j.jconrel.2004.08.010