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Fabrication of chitosan/TPP nanoparticles as a carrier towards the treatment of cancer

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Abstract

Cancer one of the deadliest disease and resistance of cancer cells has been the major issue in both the modern molecular targeted therapeutics as well as in the conventional chemotherapeutics. As on date chitosan has been the ideal choice as a carrier for delivering anticancer drugs like doxorubicin and cisplatin for targeted delivery. The present study focusses on the fabrication of chitosan nanoparticles using TPP by ionic gelation method loaded with 5-FU, an anticancer drug. The morphology of the nanoparticles synthesized were analysed using SEM and the size, zeta potential using particle size analyser. The mechanism of drug release was studied by fitting the release kinetics with Peppas model. Cellular uptake of the drug was analysed and using MTT the cytotoxicity & metabolic activity of the normal and the cancerous cells were evaluated. Results of the study showed that the synthesized nanoparticles possessed a positive charge and the release of the drug was through diffusion and degradation mediated. Also the cellular uptake of the drug showed the amount of drug uptake reduced with the cancerous cell as the time increase which was further confirmed using the MTT assay which showed the metabolic activity of the cancerous cell got reduced. The results of the study showed that the fabricated nanoparticles could potentially be used as a carrier towards the treatment of cancer.

Keywords: Chitosan, 5-FU, Cancer, MTT

Introduction

Cancer is an extremely heterogeneous complex disease characterized by indeterminate growth of cells with a cluster of disorders. The malady of cancer has troubled humans inspite of witnessing an imposing progress in the field of cancer biology [1]. There are several types of cancers that are still incurable or possess a severe adverse effect post treatment inspite of having attractive advances. Significant amount of efforts are being made to resolve the problem and enormous amount of research are being carried out towards the anticancer therapy and natural tissue healing, in particular towards targeted delivery of drugs [2]. Some of the drawbacks of the conventional chemotherapies include that of the nonspecific distribution of the drug in both the cancerous and normal cells. These nonspecific distributions not only affect the normal cells but limit the dosage of the drug on the target site resulting in a suboptimal treatment. Yet another method is the molecular targeted therapy which has attracted many scientist and researchers but it is limited by the lack in specificity compared to the conventional chemotherapeutic agents. However, the development of resistance in cancer cells has been the major issue in both the modern molecular targeted therapeutics as well as in the conventional chemotherapeutics [3-5].

The idea of creating tiny particles alias magic bullets by Ehrlich enabling the delivery of active molecules at a specific site of the body has s been the prime interest in the field of pharmaceutical sciences [6]. The fusion of nanotechnology and medicine so called nanomedicine is one of the most encouraging approaches in addressing diseases concomitant with conventional drug delivery methods. Many approach systems based on the polymeric nanoparticles (NPs) have been the keystone towards the progress in nanomedicine [7-8]. One of the important challenges over the last few decades has been the use of nanoparticles as a potential carrier of drug as their design enhances the pharmacological and therapeutic effects in addition to a reduction in toxic side effects. Nanoparticles decrease the drug doses and improve the patient compliance by allowing the continuous & controlled release of the therapeutic drugs within the desired level and also help in the targeted delivery of the drug to their intended tissues & cells [9-12]. Also it was observed that the therapeutic agents were much stable in the GIT when encapsulated inside a nanoparticle system. The carrier aids in improving the cellular uptake of the drug, deliver an elicited release at ease, even reveal in targeting when attached

with a targeting moiety and could control the physiochemical properties of the therapeutic agent. The nanoparticulate drug carriers are developed using either synthetic or natural polymer or by a combination of both. Amongst the various natural polymers that are available chitosan is one of the most widely used biopolymer for the preparation of nanoparticles and extensive amount of research has been reported so far. Chitosan is synthesized by the alkaline deacetylation of chitin, as it's made up of N-acetylglucosamine and glucosamine with varying viscosity, molecular weight and degree of deacetylation [13-16].

Chitosan is the polymer of choice for many researchers as they possess excellent biocompatibility, biodegradability, low cost, are mucus adhesive and so on which made them an ideal choice as a carrier for delivering anticancer drugs like doxorubicin and cisplatin for targeted delivery. Also the application of chitosan nanoparticles in the plasmid DNA delivery, siRNA, topical immunization and the characteristics like anti-microbial, wound healing, hypo-cholesterolemic & anti-ulcer activity comes as handy and makes it an ideal carrier for drug delivery [17, 18].

The present study focuses on the synthesis of chitosan nanoparticles using TPP solution by the ionic gelation method. The nanoparticles prepared were loaded with 5-FU anticancer drug and their *in vitro* release kinetics was analysed followed by fitting with *Peppas* model to study the mechanism behind the release kinetics. The cellular uptake of the drug and the biocompatibility of the prepared samples were evaluated.

Materials and methods

Chitosan and sodium tripolyphosphate (TPP) were purchased from Sigma Aldrich. Acetic acid was obtained from TCI chemicals and 5-FU was obtained from HI media. Molecular weight of chitosan was ~$1.2*10^5$ Da with >90% degree of acetylation.

Nanoparticles Preparation

Chitosan nanoparticles were prepared by the process of ionic gelation method. Initially chitosan (0.1% (w/v)) was dissolved in 0.05% aqueous acetic acid while the polyanionic solution was prepared by dissolving TPP (0.1% (w/v)) in deionised water. The polyanionic solution was added drop wise to the Chitosan solution under constant stirring and the nanoparticles were obtained spontaneously. The nanoparticles were centrifuged at 16,000 rpm for 30min, lyophilised and washed with distilled water. Nanoparticles were prepared with different concentrations (w/w) of TPP as given in table 1. The anticancer drug 5-FU (5 and 10wt%) was loaded onto the nanoparticles by adding it to the TPP solution at two different concentrations of 5 and 10wt%.

Table 1 shows the various concentrations of chitosan and TPP (w/w).

Model	Chitosan (mg)	TPP(mg)
1	10	1
2	10	2
3	10	3

Morphological Characterization

SEM (HITACHI S-3400model) analysis was carried out to study the morphological character of the prepared models. The average size of the particle and zeta potential for the stability of the nanoparticles were determined using dynamic light scattering method (Malvern ZEN3600).

In vitro Degradation

The *in vitro* degradation studies were adopted from Hou et al [19]. Briefly, the nanoparticles were incubated in 5mL of PBS containing lysozyme (0.1 mg/mL) at 37 ^oC with slow stirring. The degradation ratios were measured at varying times and the media was replaced once every week. The degradation ratios were expressed based on the difference in the initial weight of the nanoparticles to their weight after removal from the incubating media. The nanoparticles in PBS without lysozyme incorporation acted as control.

Drug Release Kinetics

The procedure adapted in release kinetics was as described in an earlier report [16]. Briefly, the drug loaded nanoparticles were immersed in 10mL PBS buffer at pH 6.2, followed by centrifugation. After centrifugation, the amount of drug released was determined spectrophotometrically at 266nm using UV spectrophotometer (PG Instruments) at a time interval of 5h for a period of 140 h. In order to study the mode of the release kinetics the obtained data were fitted in to the *Peppas* model [20-21].

Cellular Uptake of drug

The *in vitro* cellular uptake of the drug from the nanoparticles was analysed using normal fibroblast (L929) and cancerous cells (HeLa, MG-63, MCF-7) by absorption measurements. The 5% and 10% drug loaded nanoparticles were loaded onto 24 well plates with a cell concentration of 50,000 – 60,000 cells/well followed by incubation. After incubation, the cells were washed carefully with PBS followed by trypsinization. A sonicator was used to lyse the collected cells and the amount of drug uptake was quantified using the UV Visible spectrophotometer at 266nm. All the experiments were carried out in triplicates.

Biocompatibility Studies

MTT assay was used to evaluate the biocompatibility of the prepared biomaterials and also for analysing the metabolic activity of the tumour cells. Briefly, the cell lines (both normal and tumor cells) were cultured using Dulbecco's modified Eagle's medium supplemented with 1% penicillin–streptomycin, 5% fetal bovine serum followed by seeding onto a T75 tissue culture flasks. In order to supply the cells with fresh nutrients, the media was changed every 48 hours thereby enhancing the cell growth. Once the cells were confluent they were subjected to trypsinization i.e., removal of the adherent cells. The metabolically active cell mitochondria of the living cell cause the reduction of yellow tetrazolium salt that is MTT resulting in the formation of violet formazan crystal. Finally the absorbance at 575nm was measured

spectrophotometrically using ELISA reader which was used to study the cytotoxic nature of the sample and the metabolic activity of the cells were analysed [16, 22].

Statistical Analysis

The data obtained from the studies were analysed using one-way ANOVA test and the statistical significance was set at 0.05. Origin 8.0 version was used for the statistical analysis and the data were presented in the form of mean and variance.

Results and Discussion

Morphological Analysis

Chitosan nanoparticles capable of degradation & prolonged circulation were prepared successfully using TPP. The formation of the nanoparticles was due to the character of the chitosan to form a rapid gel on contact with the polyanions. The NH3+ in chitosan gets electrostatically attracted to the negatively charged TPP thereby forming the ionically cross-linked nanoparticles. As reported in previous studies the solution colour changed from clear to opalescent with all the models on the addition of TPP to the chitosan solution and was attributed to the formation of nanoparticles. In addition, the drug 5-FU may have electrostatic attraction with the chitosan as it is negatively charged.

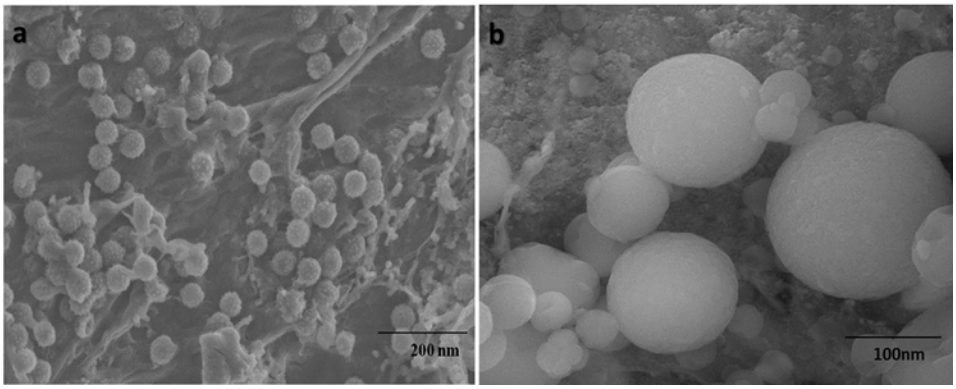

Figure 1 SEM images of the nanoparticles synthesized a) at lower magnification b) at higher magnification

Table 2 Average particle size and zeta potential values of the nanoparticles prepared

	Average Size in nm (5wt% 5-FU)	Zeta Potential in mV	Average Size in nm (10wt% 5-FU)	Zeta Potential in mV
Model 1	130 – 280	40 ± 0.7	120 – 210	39 ± 0.7
Model 2	100 – 270	35 ± 0.4	100 – 200	38 ± 0.5
Model 3	104 – 275	33 ± 0.4	90 – 195	36 ± 0.4

The nanoparticles prepared exhibited a mean diameter between 100nm to 272nm with the zeta potential values ranging between 40 mV to 32mV (Table 2). The values of the zeta potential were observed to be higher when the concentration of the chitosan was increased. The results of the study obtained from SEM were in agreement with the one obtained by Aydin et al [9] and the particles size ranges were comparatively lower. It should be noted that the prepared nanoparticles possess the advantage of escaping the vasculature by means of the leaky endothelial cells and could possibly accumulate in the solid tumour cells by enhanced permeation and retention effect (EPR). Being positively charged, these chitosan based nanoparticles might help in the passive targeting as the cationic group of chitosan may be attracted electrostatically to the negatively charged phospholipid head groups expressed on the tumour endothelial cells [23-25].

In vitro Degradation

Due to the hydrophilic nature of chitosan, it has the capability of absorbing enormous amount of aqueous solution, enduring degradation through hydrolysis, erosion and other biodegradation mechanisms which is consecutively governed by various other mechanisms. In the present study the *in vitro* degradation of chitosan in PBS and with PBS + lysozyme enzyme were evaluated. The results of the study were as anticipated in which the degradation of chitosan in PBS occurred at a slower rate in contrast to the rapid degradation observed with lysozyme. The ionic interaction of the polymer and its nature play a role in the degradation mechanisms. This was further confirmed by the degradation mechanisms observed for the three models wherein, the models 2 & 3 with higher amount of TPP showed dawdling phase of degradation as compared to model 1 which showed a degradation of 85% in 50h. This was 5-10% higher than the other two models. The *in vivo* degradation rate of chitosan was much

higher compared to the *in vitro* release as reported by *Lim et al* [26, 27]. The degradation results obtained in the present study were in agreement with the one observed by *Tomihata et al* [28]. There was no appreciable amount of degradation when chitosan was treated with PBS without enzyme at room temperature. The degree of deacetylation plays a vital role in the degradation of the polymer. The higher the deacetylation, lower is the rate of degradation.

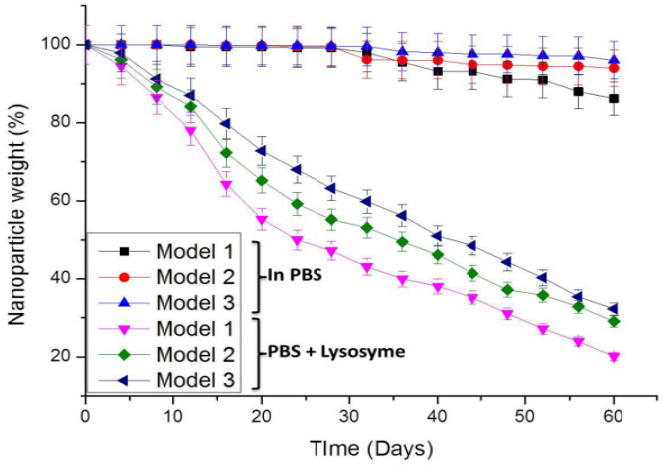

Figure. 2 *In vitro* degradation study on the nanoparticles synthesized

The degradation of chitosan in PBS with lysozyme was initially due to the binding of the hexameric site in the lysozyme with 3-4 or more acetylated units in the chitosan. The lack of consecutive N-acetyl residues then reduces the degradation rate to a greater extent [29]. Chitosan with degree of deacetylation greater than 90% was used in the study as previous reports showed that the chitosan with greater DD will have a longer residence time and strong cell adhesion with no inflammatory responses [26].

The SEM images of nanoparticles taken after immersion in PBS + lysozyme for one and two weeks are shown in figure 2. It showed that the particles lost the spherical morphology and started to degrade. Since all the models exhibited similar characteristics model 3 was chosen for further studies as the zeta potential values were on the higher side and the slow degradation compared to other models which might aid in the sustained release for this model.

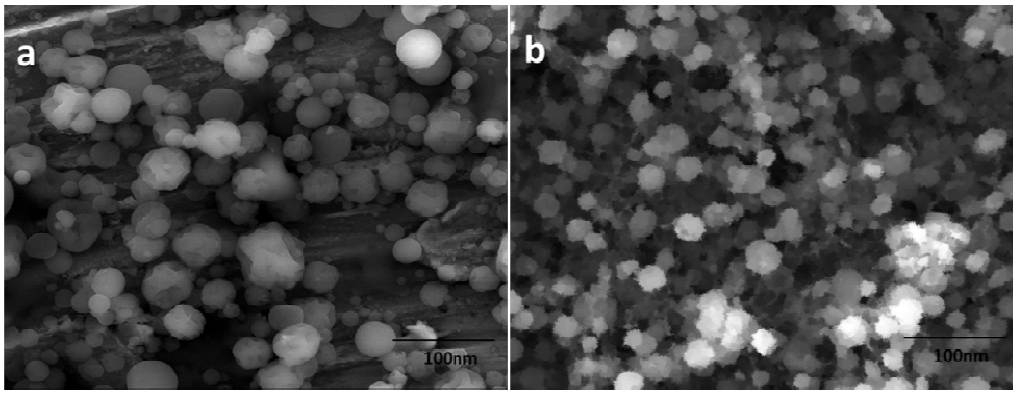

Figure. 3 SEM images of Chitosan nanoparticles after immersion in PBS + lysozyme. (a) After 7 days (b) After 14 days

Drug Release Kinetics

The drug release kinetics of the model 3 was evaluated by immersing the samples in PBS. *In vitro* release kinetics showed that 90% of the drug was released within the time period of 120h subsequent to the incubation in PBS. The release profile of 5-FU loaded nanoparticles exhibited an initial burst release of about 20% in 10 wt% drug loaded fibers followed by a slow and sustained release over a period of 120h. The aqueous solubility and the dissolution of the drug 5-FU that were present on the surface of the nanoparticles contributed to the initial burst release [16, 30].

The initial release was rapid and lasted for the first 10h following which the release became slower and was observed up to 130h. The release rate for a period from 10h to 50h was at a dawdling rate which may be due to the slower swelling rate of the polymer nanoparticle. The final stage of the release kinetics where around 50% of the drug release was observed was attributed to the degradation of the polymer matrix which was observed until 130h.

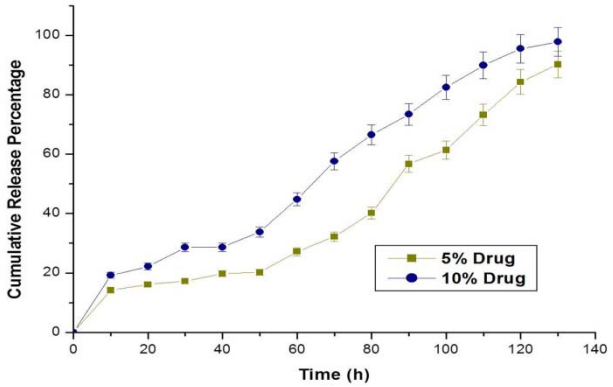

Figure. 4: Drug release profile of 5-FU from nanoparticles

The release kinetics suggested that the drug release was in a sustained manner and could possibly help in targeting and destruction of the tumor cells. In order to study the mechanism behind the release kinetics, the model was fitted on to the *Peppas* model (Figure 5). The slope of the release kinetics that was calculated from the log-log plot showed that the diffusion controlled power constant was close to 0.5. It was reported by *Peppas* et al that this kind of phenomenon is usually observed when the drug was released by the mechanism of diffusion and degradation [20, 21]. The high water uptake chitosan plays a vital role in the release kinetics. The increase in the drug release was due to the presence of water channels provided by chitosan to the drug for diffusion as well as assisting in the degradation of the polymer itself since the mode of degradation for chitosan is via hydrolytic process.

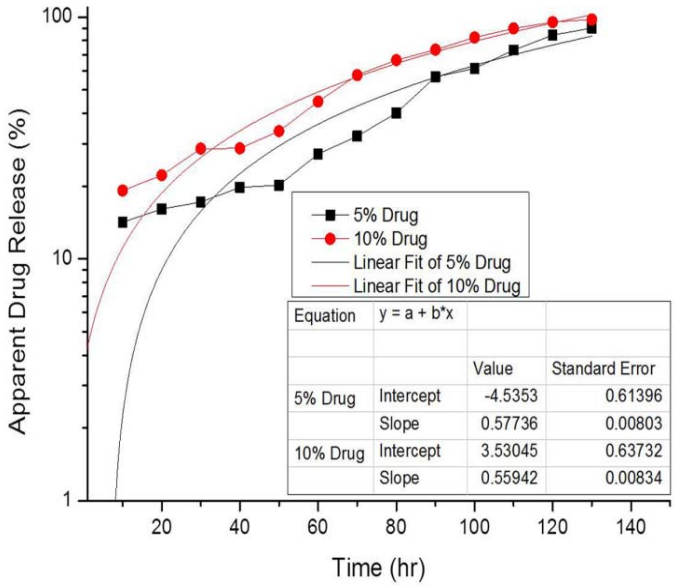

Figure. 5 Fitting of the release kinetics to *Peppas* model

Cellular Uptake

The cellular uptake of the drug by different cell lines, as shown in figure 6, showed that level of drug uptake in the cancerous cell lines were higher and over a period of time the amount of drug uptake declined which may be attributed to the death of the cancerous cells leading to the apparent lower amount of drug uptake. One of the important considerations for the nanoparticles for their biological application apart from the uptake profiles is their final subcellular location.

This is important particularly in drug delivery as it is important for the nanoparticles to reach the target site in the cell interior apart from delivering the drug to the target tissue and this was observed by the decrease in the number of cancerous cells. In addition to the cellular uptake of the drug, the metabolic activity of the cancerous cell also plays a vital role. The decrease in the metabolic activity was confirmed using the MTT assay.

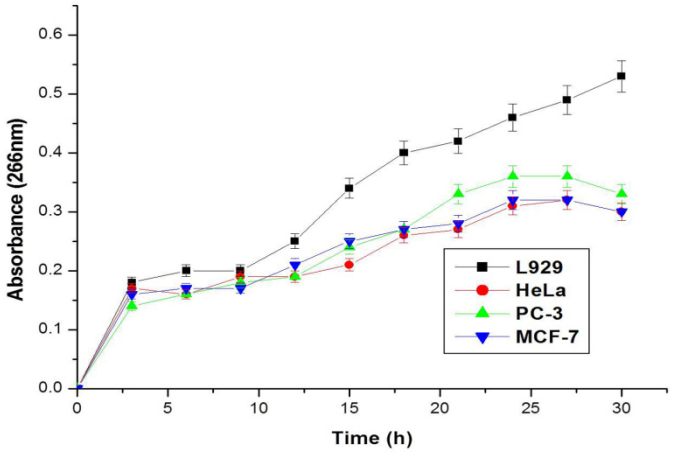

Figure. 6 Cellular uptake of 5-FU by normal and cancerous cell lines

Cytotoxicity Studies

The cytotoxicity studies of the samples showed that the viability of the normal cells was unaffected by both the bare nanoparticles as well as the drug loaded CS NPs. On the other hand, the MTT assay showed that the metabolic activities of the cancerous cells were greatly influenced by the drug loaded nanoparticles. The normal cells viability percentage was higher than that of the cancerous cells. From figure 7, the viability of the normal cells could be found to be between 80-85% in all the cases of nanoparticles with and without drug. Similarly the viability of the MCF-7 cell lines showed a decline gradually with increase in the drug concentration. Reports showed that the decrease in the cell viability corresponds to either the slow or no proliferation in the cells, thereby affecting the metabolic activity of the cancer cell line MCF-7[31]. Also other type of cancer cells showed a decline in their viability with the exposure to the nanoparticles with drug and the cell viability of cancerous cell lines got reduced as there was an increase in the drug concentration which was obvious.

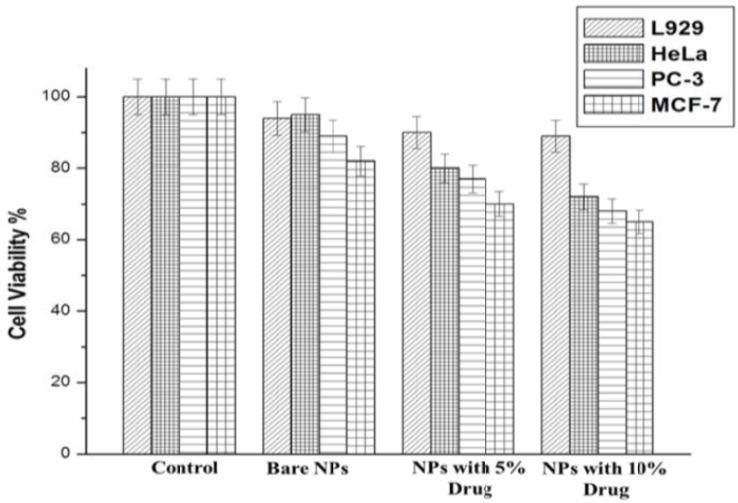

Figure. 7 *In vitro* cytotoxicity studies using MTT assay after 72h

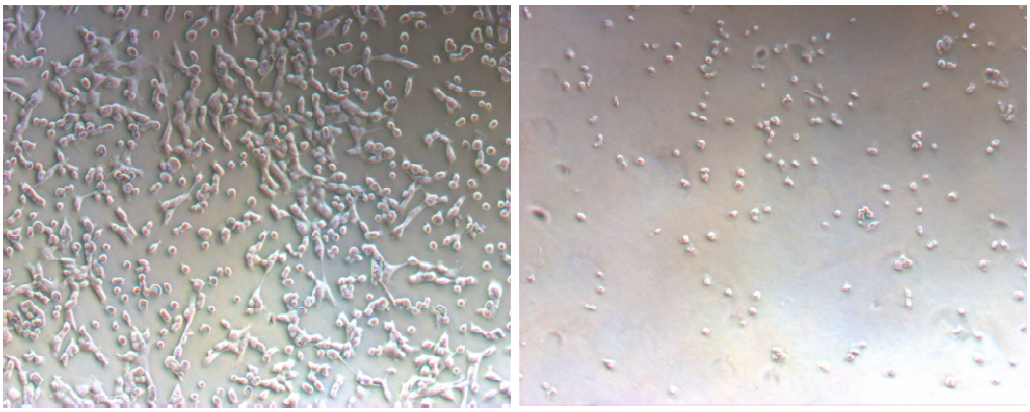

Figure. 8 Microscopic images of HeLa cell lines used before and after treatment with 10% drug nanoparticles

Conclusion

In the present study chitosan based nanoparticles carrying an anticancer drug 5-FU were fabricated and various *in vitro* studies performed. The nanoparticles synthesized showed a positive zeta potential with assize range of 100-200nm. Significantly, sustained *in vitro* release kinetics of the prepared models was observed and the mechanism of the release was diffusion and degradation mediated via peppas model. Cellular uptake studies showed the uptake of drugs by different cancer cell lines increased initially and then decreased with time where the later was attributed to higher mortality of the cells. Also the system showed more toxicity towards the cancerous cells compared to the normal cells. Thus the model prepared could potentially be used as a carrier in anticancer therapy.

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References

[1]. Jabir NR, Tabrez S, Ashraf GM, Shakil S, Damanhouri GA, Kamal MA. Nanotechnology-based approaches in anticancer research. International Journal of Nanomedicine. 2012; 7: 4391.

[2]. Talekar M, Kendall J, Denny W, Garg S. Targeting of nanoparticles in cancer:

drug delivery and diagnostics. Anti-Cancer Drugs. 2011; 22(10): 949.

[3]. Cho K, Wang X, Nie S, Shin DM. Therapeutic Nanoparticles for Drug Delivery in Cancer. Clinical Cancer Research. 2008; 14(5): 1310-1316.

[4]. Aravind A, Yoshida Y, Maekawa T, Kumar DS. Aptamer-conjugated polymeric nanoparticles for targeted cancer therapy. Drug Delivery and Translational Research. 2012; 2: 1-19.

[5]. Morgillo F, Lee HY. Resistance to epidermal growth factor receptor-targeted therapy. Drug resistance updates: reviews and commentaries in antimicrobial and anticancer chemotherapy. 2005; 8(5): 298.

[6]. Quintanar-Guerrero D, Allemann E, Fessi H, Doelker E. Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers. Drug development and industrial pharmacy. 1998; 24(12): 1113-1128.

[7]. Morrow KJ, Bawa R, Wei C. Recent Advances in Basic and Clinical Nanomedicine. Med Clin N Am. 2007; 91: 805–843.

[8]. Singh A, Dilnawaz F, Mewar S, Sharma U, Jagannathan NR, Sahoo SK. Composite polymeric magnetic nanoparticles for co-delivery of hydrophobic and hydrophilic anticancer drugs and MRI imaging for cancer therapy. ACS Appl Mater Interfaces. 2011; 3(3): 842-856.

[9]. Aydin RST, Pulat M. 5-Fluorouracil Encapsulated Chitosan Nanoparticles for pH-Stimulated Drug Delivery: Evaluation of Controlled Release Kinetics. Journal of Nanomaterials. 2012; Article ID 313961: 10 pages.

[10]. Feng SS. Nanoparticles of biodegradable polymers for new-concept chemotherapy. Expert Review of Medical Devices. 2004; 1(1): 115–125.

[11]. Brannon-Peppas L, Blanchette JO. Nanoparticle and targeted systems for cancer therapy. Advanced Drug Delivery Reviews. 2004; 56(11): 1649–1659.

[12]. Bhatia A, Shard P, Chopra D, Mishra T. Chitosan nanoparticles as Carrier of Immunorestoratory plant extract: synthesis, characterization and Immunorestoratory eficacy. International Journal of Drug Delivery. 2011; 3(2): 381-385.

[13]. Chaudhury A, Das S. Recent advancement of chitosan-based nanoparticles for oral controlled delivery of insulin and other therapeutic agents. AAPS Pharm Sci Tech. 2011; 12(1): 10-20.

[14]. Bowman, K.; Leong K. W. Chitosan nanoparticles for oral drug and gene delivery. Int J Nanomedicine. 2006; 1(2): 117–28.

[15]. Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J Control Release. 2004; 100(1): 5–28.

[16]. Shanmuga Sundar S, Sangeetha D. Fabrication and evaluation of electrospun collagen/poly(N-isopropyl acrylamide)/chitosan mat as blood-contacting biomaterials for drug delivery. J Mater Sci: Mater Med, 2012, 23:1421–1430. DOI 10.1007/s10856-012-4610-x.

[17]. Yue ZG, Wei W, Lv PP, Yue H, Wang LY, Su ZG, Ma GH. Surface Charge Affects Cellular Uptake and Intracellular Trafficking of Chitosan-Based Nanoparticles. Biomacromolecules. 2011; 12(7): 2440-2446.

[18]. Rajendran L, Kn Lker HJ, Simons K. Subcellular targeting strategies for drug design and delivery. Nat. Rev. Drug Discovery. 2010; 9: 29–42.

[19]. Hou Y, Hu J, Park H, Lee M. Chitosan-based nanoparticles as a sustained protein release carrier for tissue engineering applications. Journal of Biomedical Materials Research Part A. 2012; 100A (4): 939–947.

[20]. Ritger PL, Peppas NA. A Simple Equation for Description of Solute Release. I. Fickian and non-Fickian Release from Non-Swellable Devices in the Form of Slabs, Spheres, Cylinders or Discs. J Control Rel. 1987; 5: 23-36.

[21]. Ritger PL, Peppas NA. A Simple Equation for Description of Solute Release. II. Fickian and Anomalous Release from Swellable Devices. J Control Rel. 1987; 5: 37-42.

[22]. Shanmuga Sundar S, Sangeetha D. Investigation on sulphonated PEEK beads for drug delivery, bioactivity and tissue engineering applications. J Mater Sci. 2012; 47(6): 2736-2742. DOI 10.1007/s10853-011-6100-9

[23]. Fang J, Nakamura H, Maeda H. The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. Adv Drug Deliv Rev. 2011; 63(3): 136–151.

[24]. Torchilin V. Tumor delivery of macromolecular drugs based on the EPR effect. Drug Deliv Rev. 2011; 63(3): 131–135.

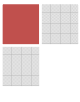
[25]. Gindy ME, Prud'homme RK. Multifunctional nanoparticles for imaging, delivery and targeting in cancer therapy. Expert Opin Drug Deliv. 2009; 6(8): 865–878

[26]. Gorgieva S, Kokol V. Preparation, characterization, and *in vitro* enzymatic degradation of chitosan-gelatine hydrogel scaffolds as potential biomaterials. Journal of Biomedical Materials Research Part A. 2012; DOI: 10.1002/jbm.a.34106.

[27]. Lim SM, Song DK, Oh SH, Lee-Yoon DS, Bae EH, Lee JH. *In vitro* and *in vivo* degradation behavior of acetylated chitosan porous beads. J Biomater Sci Polym E. 2008; 19: 453–466.

[28]. Tomihata K, Ikada Y. *In vitro* and *in vivo* degradation of films of chitin and its deacetylated derivatives. Biomaterials. 1997; 16: 567-575.

[29]. Freier T, Koh HS, Kazazian K, Shoichet MS. Controlling cell adhesion



and degradation of chitosan films by N-acetylation. Biomaterials. 2005; 26(29): 5872-5878.

[30]. Yassin AEB, Anwer MK, Mowafy HA, El-Bagory IM, Bayomi MA, Alsarra IA. Optimization of 5-fluorouracil solid-lipid nanoparticles: a preliminary study to treat colon cancer. International journal of medical sciences. 2010; 7(6): 398.

[31]. Zhang J, Du J, Yan M, Dhaliwal A, Wen J, Liu F, Segura T, Lu Y. Synthesis of protein nano-conjugates for cancer therapy. Nano Research. 2011; 4(5): 425-433.