International Journal of Pharmaceutics 371 (2009) 170–176

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Intravesical cationic nanoparticles of chitosan and polycaprolactone for the delivery of Mitomycin C to bladder tumors

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ARTICLE INFO

Article history:
Received 22 October 2008
Received in revised form 9 December 2008
Accepted 11 December 2008
Available online 24 December 2008

Keywords:
Mitomycin C
Chitosan
Poly-ε-caprolactone
Poly-L-lysine
Nanoparticle
Intravesical delivery

ABSTRACT

Cationic nanoparticles of chitosan (CS), poly-ε-caprolactone coated with chitosan (CS-PCL) and poly-ε-caprolactone coated with poly-L-lysine (PLL-PCL) were developed to encapsulate intravesical chemotherapeutic agent Mitomycin C (MMC) for longer residence time, higher local drug concentration and prevention of drug loss during bladder discharge. Nanoparticle diameters varied between 180 and 340 nm depending on polymer used for preparation and coating. Zeta potential values demonstrated positive charge expected from cationic nanoparticles. MMC encapsulation efficiency depended on hydrophilicity of polymers since MMC is water-soluble. Encapsulation was increased by 2-fold for CS-PCL and 3-fold for PLL-PCL as a consequence of hydrophilic coating. Complete drug release was obtained with only CS-PCL nanoparticles. On the other hand, CS and PLL-PCL nanoparticles did not completely liberate MMC due to strong polymer–drug interactions which were elucidated with DSC studies. As far as cellular interaction was concerned, CS-PCL was the most efficient formulation for uptake of fluorescent markers Nile Red and Rhodamine123 incorporated into nanoparticles. Especially, CS-PCL nanoparticles loaded with Rhodamine123 sharing hydrophilic properties with MMC were selectively incorporated by bladder cancer cell line, but not by normal bladder epithelial cells. CS-PCL nanoparticles seem to be promising for MMC delivery with respect to anticancer efficacy tested against MB49 bladder carcinoma cell line.

1. Introduction

Superficial bladder cancer is initially managed by transurethral resection (TUR) to allow accurate tumor staging and grading. However, the recurrence rate of superficial bladder transitional cell carcinoma is reported to be between 50 and 80% and has a 15% chance of progression after TUR (Highley et al., 1999; Burgues Gasion and Jimenez Cruz, 2006). Therefore, intravesical chemotherapy or immunotherapy is required. Most commonly employed intravesical agents in patients with superficial bladder cancer are Mitomycin C (MMC), thiotepa, etoglucid, anthracyclines such as doxorubicin, Bacillus Calmette-Guerin (BCG) and more recently paclitaxel and new Mitomycin C derivative KW-2149 (Farokhzad et al., 2006; Black et al., 2007). Intravesical chemotherapy and immunotherapy has a number of potential benefits including the maintenance of high drug concentrations at the tumor site, manipulation of exposure time, reduced systemic availability which minimises systemic toxicity.

However, intravesical drug delivery is challenged by the difficulty of establishment of a suitable and effective drug concentration because of periodical discharge of the bladder. This results in ineffective intravesical chemotherapy contributing to the high recurrence rate and progression associated with bladder tumors even after surgery (Parekh et al., 2006; Tyagi et al., 2006; Kaufman, 2006).

In this context, bioadhesive colloidal drug delivery systems have emerged as promising delivery systems for intravesical chemotherapeutic agents in the last decade. Microspheres of polymethylidene malonate (Le Visage et al., 2004) or eudragit RL and hydroxypropylmethylcellulose microspheres coated with mucoadhesive polymers (Bogataj et al., 1999), magnetic microparticles prepared from iron and activated carbon (Leakakos et al., 2003) along with chitosan rods (Eroğlu et al., 2002) and thermosensitive hydrogel prepared from PEG–PLGA–PEG triblock copolymer (Tyagi et al., 2004) have been designed to improve the efficacy of intravesical chemotherapy. Recently, gelatin nanoparticles loaded with paclitaxel were

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doi:10.1016/j.ijpharm.2008.12.015

reported for bladder cancer therapy initiating the application of nanoparticulate drug delivery agents for intravesical chemotherapy (Lu et al., 2004). Mitomycin C has previously been incorporated in colloidal delivery systems such as chitosan coated alginate microspheres (Mısırlı et al., 2005) and polybutylcyanoacrylate nanoparticles which were demonstrated to be effective carriers of MMC in rabbits bearing VX2-liver tumor (Xi-xiao et al., 2006).

In this study, the objective was to design and characterize a cationic nanoparticulate carrier system for Mitomycin C to obtain a high concentration of the drug at tumor site with prolonged residence at action site providing a controlled release profile in order to avoid drug loss during bladder discharge. For this reason three types of nanoparticles were designed; chitosan nanoparticles (CS), poly-ε-caprolactone nanoparticles coated with chitosan (CS-PCL) and poly-L-lysine coated poly-ε-caprolactone nanoparticles (PLL-PCL) all possessing positive charge but different physicochemical and biological properties. Coated PCL nanoparticles were demonstrated to be effective for mucosal drug delivery and were included in this study for the same goal. Poly-ε-caprolactone was selected because of its biocompatibility, lipophilicity to support passive uptake process and cost-effectiveness compared with other polyesters such as PLGA (Haas et al., 2005). These attributes combined with bioadhesive properties of chitosan or poly-L-lysine were believed to ameliorate the cellular interaction and drug uptake from mucosal tissues such as the bladder wall. This study aims to develop and optimize a nanoparticulate carrier based on cationic polymers for the encapsulation of MMC upon with vitro characterization techniques such as particle size, surface charge, encapsulation efficiency, controlled release, cellular interaction and anticancer efficacy.

2. Materials and methods

2.1. Materials

Chitosan (Protasan® Cl 113, Mw < 150 kDa, deacetylation degree 75–90%) (CS) was purchased from FMC Biopolymers, Norway. Tripolyphosphate (TPP), Rhodamine 123 and Nile Red were supplied by Sigma Chemicals Co. (USA) and Pluronic® F68 (PF68) was supplied by BASF (France). Poly-ε-caprolactone (PCL) (Mn: 42,500) was purchased from Aldrich (St. Louis, MO, USA) as well as poly-L-lysine (PLL) which was purchased as 0.1% (w/v) solution in water. Mitomycin C (MMC) was a kind gift of Onko Ecza (İstanbul, Turkey). Ultrapure water obtained from Millipore Simplicity 185 Ultrapure Water System (Millipore, France) was used in the preparation of nanoparticles. All other chemicals were of reagent grade and solvents were of HPLC grade and used without further purification in this study.

2.2. Preparation of cationic nanoparticles

2.2.1. Chitosan nanoparticles

MMC loaded bioadhesive chitosan nanoparticles were prepared according to the ionotropic gelation process (Calvo et al., 1997a). TPP aqueous solution (0.4 mg/mL) was added to CS aqueous solution (1.75 mg/mL) stirred at room temperature to obtain blank nanoparticles. The nanoparticles were believed to form due to the interaction of positive amino groups of chitosan and negative groups of TPP. To prepare the MMC-loaded CS nanoparticles, MMC was dissolved in TPP solution (20% of polymer weight) and then added to the CS solution with same technique described for the blank CS nanoparticles. Spontaneously formed nanoparticles were further separated by centrifugation at 13,500 rpm for 1 h and discarding of the supernatant.

2.2.2. Chitosan-coated poly-ε-caprolactone nanoparticles

Uncoated PCL nanoparticles were prepared by the nanoprecipitation technique following the method previously described (Fessi et al., 1988). In this method, 10 mg of PCL was dissolved in 2 mL acetone with mild heating. This polymeric solution was added to 4 mL of ultrapure water containing 10 mg of PF68 under magnetic stirring. Organic solvent was evaporated under vacuum to desired volume (4 mL) to obtain blank and uncoated PCL nanoparticles.

To manufacture the CS-coated and MMC-loaded PCL nanoparticles, CS (0.05% w/v) and MMC (10% of PCL weight) were dissolved in the aqueous phase. Coated and drug loaded nanoparticles formed spontaneously and were obtained in final form after removing of organic solvent under vacuum at 37 °C to the desired volume (4 mL).

2.2.3. Poly-L-lysine-coated poly-ε-caprolactone nanoparticles

To obtain PLL-PCL nanoparticles, drug loaded nanoparticles were first prepared with the nanoprecipitation technique (Fessi et al., 1988). MMC was dissolved (10% of polymer weight) in aqueous phase during nanoparticle preparation to achieve encapsulation of drug in nanoparticles. MMC-loaded nanoparticles were then incubated in PLL solution (0.01% v/v) in water for 30 min and centrifuged at 13,500 rpm to obtain coated nanoparticles after the discarding of the supernatant containing free drug and free PLL. PLL-PCL nanoparticles in the precipitate were then further diluted in ultrapure water to obtain final nanoparticle dispersion.

2.3. Characterization of cationic nanoparticles

2.3.1. Particle size distribution

Mean diameter (nm ± SD) and polydispersity index values of the CS, PLL-PCL and CS-PCL nanoparticles were determined by Quasi-elastic light scattering technique using Malvern NanoZS (Malvern Instruments, Worchestershire, UK). For the analysis, CS, PLL-PCL and CS-PCL nanoparticles were diluted to a volume ratio of 1:10 and analyses were performed in triplicate at 25 °C at a 90° angle.

2.3.2. Zeta potential

Surface charge of the nanoparticle formulations were determined by zeta potential measurements using Malvern NanoZS also in triplicate at 120° angle and 25 °C in ultrapure water.

2.3.3. Scanning electron microscopy (SEM)

SEM imaging of the nanoparticle formulations was performed with a JEOL SEM 6400 ASID-10 instrument (Tokyo, Japan). Nanoparticle samples were fixed on metal plates and sputtered with gold–palladium mixture at a thickness of 100 Å and observed at an accelerated voltage of 20 kV.

2.3.4. DSC analysis

Interaction of MMC with different nanoparticle excipients including PCL, PLL, CS and TPP and their mixtures were studied through their thermal behavior which was analyzed by Differential Scanning Calorimetry. Mixtures of MMC with polymers were prepared by repeating all nanoparticle preparation steps corresponding to the polymer in question without the presence of other excipients. Mixtures were obtained as dry powders prior to DSC analysis. Perkin Elmer Diamond DSC Instrument (Waltham, MA, USA) within the temperature range of 25–300 °C under nitrogen atmosphere. Samples weighing approximately 3 mg were heated in a hermetically sealed aluminum pan at a rate of 10 °C/min. DSC analysis was performed to elucidate the interaction of MMC and the polymers (CS, PCL, PLL) and excipients (TPP) used in the preparation of nanoparticles.

2.3.5. Encapsulation efficiency of nanoparticles

Determination of the entrapped drug quantity was realized by separation of nanoparticles from the aqueous suspension by centrifugation at 13,500 rpm for 1 h. The amount of free MMC in the supernatant was determined by HPLC analysis. Encapsulation efficiency (EE) values were calculated according to the following equation:

$$\text{EE}(\%) = \frac{\text{Total MMC} - \text{Free MMC}}{\text{Total MMC}} \times 100$$

Drug loss during preparation was confirmed by the quantitative analysis of all glassware used during preparation for MMC presence. HPLC method for the quantification of MMC consisted of an HP Agilent 1100 HPLC system with a reverse phase C18 column (150 mm × 4.6 mm, 5 μm, Capital Columns, USA), a mobile phase of water:acetonitrile (85:15 v/v) injected with a volume of 50 μL and a flow rate of 1.5 mL/min at ambient temperature. UV detector was set at 365 nm. HPLC method was analytically validated for MMC assay (r^2: 0.9998)

2.3.6. In vitro MMC release from nanoparticles

Different nanoparticle formulations of 100 μL were resuspended in capped tubes containing 2 mL of pH 6.0 phosphate buffer as release medium under sink conditions. For each time point, a separate tube was used. At the appropriate time intervals, the medium in the corresponding tube was filtered through a 0.22 μm membrane filter and the released MMC amount was determined by HPLC in the filtrate with the previously described method. The cumulative percent of released MMC from the nanoparticles was calculated.

2.4. Cellular uptake of fluorescent nanoparticles

2.4.1. Preparation of fluorescent nanoparticles

Rhodamine123 and Nile Red were used as fluorescent markers. Rhodamine is a very hydrophilic marker and was selected in this study to mimic the active ingredient MMC which possesses similar properties in terms of solubility. Nile Red on the other hand is a lipophilic marker practically insoluble in water. It was believed that Nile Red could mimic the properties of the insoluble particles, e.g. nanoparticles to a certain extent. Nile Red or Rhodamine123 loaded nanoparticles were prepared using the same techniques described above with the exception of incorporation of the marker instead of MMC in the preparation process. Nile Red control solution was prepared in castor oil while rhodamine solution was prepared in ultrapure water. The formation of fluorescent nanoparticles was confirmed by routine particle size, zeta potential, microscopic imaging, loading and release assays.

2.4.2. Cell culture

MB49 mouse urinary bladder carcinoma cell line was a kind gift from Dr. Sven Brandau (University Duisburg-Essen, Germany). HeLa, human cervix carcinoma; and G/G, mouse urinary bladder cell line (An1) was purchased from Cell Culture and Virus Bank, Foot-and-Mouth Disease Institute of Ministry of Agriculture and Rural Affairs of Turkey (Ankara, Turkey). L-929, mouse fibroblast; and MCF-7, human breast adenocarcinoma cell lines were obtained from the American Type Culture Collection (ATCC, LGC Promochem, Rockville, MD, USA). MB49, L-929 and MCF-7, HeLa cell lines were cultured in RPMI1640 medium and Dulbecco's MEM, respectively, supplemented with 10% foetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). G/G cells were cultured in a combined medium containing 45% Ham's F10, 45% Dulbecco's MEM, 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL), transferrin (5 μg/mL), insulin (5 μg/mL), selenite (5 ng/mL) and hydrocortisone (50 nM). The cultures were maintained at 37 °C in a humidified 5% CO_2 incubator. Otherwise specified, all reagents were obtained from Biochrom (Berlin, Germany).

2.4.3. Analysis of cellular uptake

The nanoparticles loaded with fluorescent markers were administered to the cells (2×10^5) after 1/8 dilution. Following the incubation for 1 h at 37 °C, the cells were harvested, washed, and counted on an EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA, USA). The excitations of Rhodamine123 and Nile Red fluorescence were determined at 488 and 512 nm, respectively. Untreated cells were used as autofluorescence controls. Cellular uptake of fluorescent dyes was calculated with the mean fluorescence intensity (MFI) ratio of the corresponding formulation vs. autofluorescence. Also, the photomicrographs of the cells were taken with a fluorescent microscope (Zeiss, Germany).

2.5. Anticancer efficacy of MMC-loaded cationic nanoparticles with MTT assay

The anticancer efficacy of MMC-loaded cationic nanoparticle formulations CS, CS-PCL and PLL-PCL were determined against MB49 cell line with MTT assay. MMC solution used in the cytotoxicity assays was prepared in ultrapure water. MMC concentrations in the nanoparticle formulations and aqueous solution were maintained in the same range throughout the cytotoxicity studies. MB49 cells were resuspended in complete medium, and seeded in 96-well tissue culture plates at a concentration of 2.5×10^3/50 μL per well. The cells were allowed to attach to the surface for 24 h and then exposed to 50 μL of diluted formulations ranging from 1:4 to 1:64. After 48 h of incubation, 25 μL of MTT solution (5 mg/mL) were added. The formazan crystals produced were solubilised by adding 80 μL lysing buffer (pH 4.7) composed of 23% SDS dissolved in a solution of 45% dimethylformamide. Optical densities (OD) were read at 570 nm using a microplate reader (Molecular Devices, USA). The cells incubated in culture medium alone served as a control for cell viability. All assays were performed in quadruplicate and mean OD values were used to estimate the cell viability.

3. Results and discussion

In this study, the potentiality of different cationic nanoparticles loaded with MMC was evaluated as intravesical drug delivery systems for the chemotherapy of superficial bladder cancer. Three different nanoparticle formulations of chitosan, chitosan-coated poly-ε-caprolactone and poly-L-lysine-coated poly-ε-caprolactone nanoparticles respectively were evaluated in terms of in vitro properties to ensure appropriate particle size distribution, positive surface charge, morphological properties, encapsulation efficiency and controlled release profiles, cellular interaction properties and anticancer efficacy of the nanoparticulate carrier system. PCL was used for its favorable biological interaction, biocompatibility and effective mucosal drug delivery properties as a nanoparticle excipient.

Mean diameter and polydispersity indices of the nanoparticles were found to be between 180 and 340 nm. Coating with hydrophilic polymers such as CS or PLL was demonstrated by the significant increase in particle size for the coated nanoparticles, CS-PCL in particular, as can be seen in Table 1. Smallest particles were obtained by PLL-PCL formulation but nevertheless all nanoparticle formulations were found to be smaller than 400 nm which can be considered to be promising in the enhanced permeation of nanoparticles in cancerous tissues of the bladder. On the other hand, for PLL-PCL formulations, the concentration of the PLL in the incubation solution was found to be effective on the parameters of particle size and zeta potential (data not shown). Higher

Table 1
Particle size distribution and zeta potential values of coated and uncoated nanoparticle formulations ($n = 3$).

Nanoparticle formulation	Mean diameter (nm) (±SD)	Polydispersity index	Zeta potential (mV)
CS	291 ± 8	0.360	38 ± 3
PCL	179 ± 0.5	0.088	−36 ± 2
PLL-PCL	190 ± 2	0.120	36 ± 11
CS-PCL	336 ± 5	0.200	22 ± 4

concentrations of PLL than 0.01% (w/v) resulted in higher particle sizes (above 400 nm) and zeta potentials (above +60). However incubation time in the PLL solution was not effective on the characterization parameters of the nanoparticles. Table 1 also represents the zeta potential of all coated and uncoated nanoparticles indicating positive surface charge and cationic property for CS, PLL-PCL and CS-PCL nanoparticles while the uncoated PCL nanoparticles exhibit a net negative charge prior to coating. Similar results were reported in the literature for ocular nanocapsules (Calvo et al., 1997b).

SEM photomicrographs were taken to elucidate the morphological properties of blank and MMC-loaded cationic nanoparticle formulations. Fig. 1A–F represents the SEM images of cationic

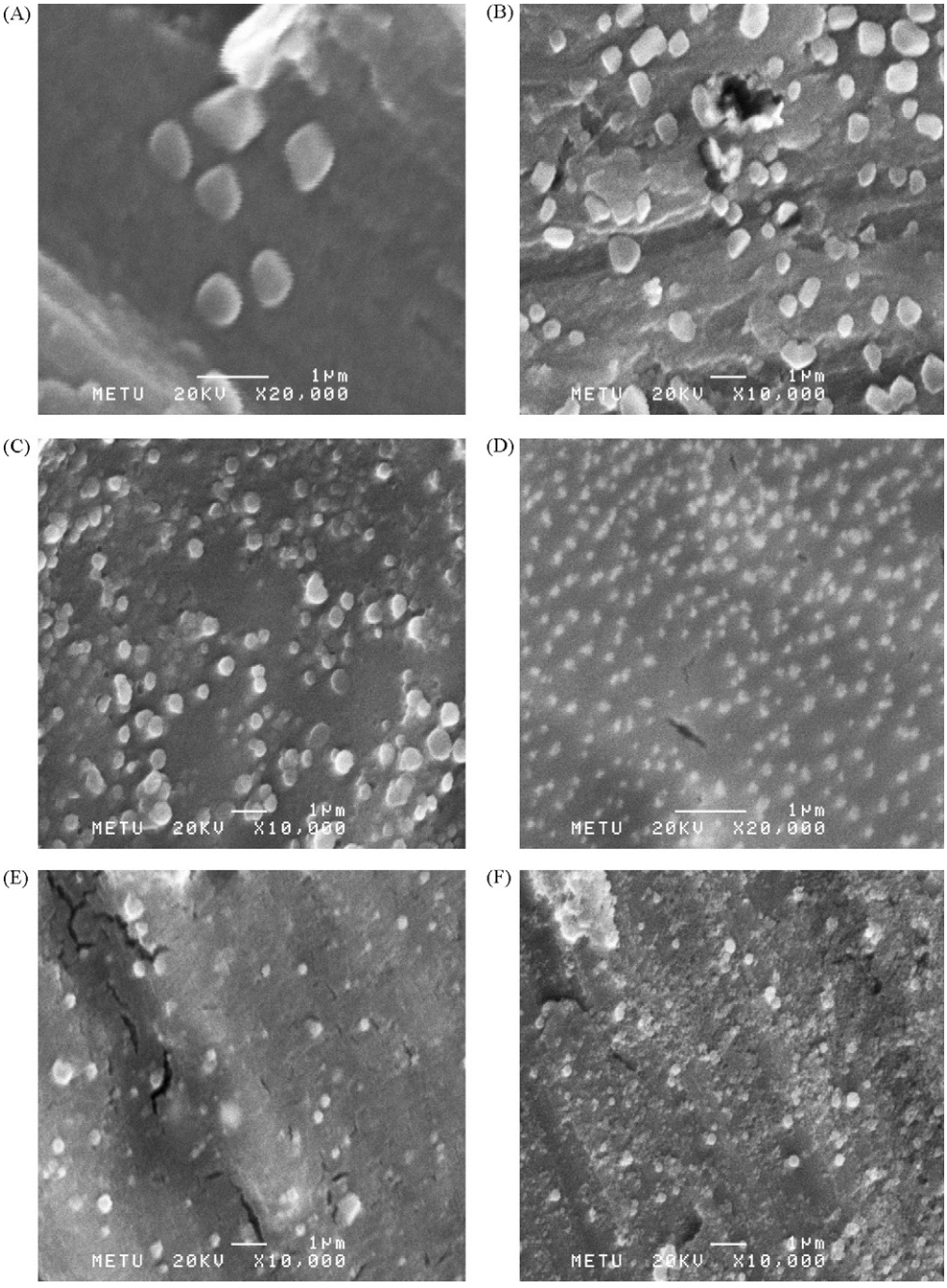

Fig. 1. SEM photomicrographs of cationic nanoparticles prior to and following MMC encapsulation; (A) blank CS nanoparticles, (B) MMC-loaded CS nanoparticles, (C) blank CS-PCL nanoparticles, (D) MMC-loaded CS-PCL nanoparticles, (E) blank PLL-PCL nanoparticles, (F) MMC-loaded PLL-PCL nanoparticles.

Table 2
Encapsulation efficiency (%) of MMC into CS, PCL, PLL-PCL and CS-PCL nanoparticles ($n = 3$).

Nanoparticle formulation	Encapsulation efficiency (%)
CS	21.1
PCL	7.9
PLL-PCL	21.4
CS-PCL	13.1

nanoparticles. These images indicate the smooth regular and spherical surfaces of all nanoparticle formulations prior to and following drug encapsulation. Unlike polymeric nanoparticles such as PLGA, PCL, PACA, etc., polysaccharide-based nanoparticles such as chitosan nanoparticles are known to shrink or loose shape under electron bombardment during SEM analysis. As seen in the photomicrographs, CS nanoparticles seem larger than the particle size determined by light scattering technique seen in Table 1.

Highest encapsulation efficiency was obtained with CS and PLL-PCL nanoparticles which both yielded approximately 21% loading for MMC. Uncoated PCL nanoparticles resulted in a somewhat poor loading property of 8%, however this value was increased by 2-fold when PCL nanoparticles were coated with CS and by 3-fold when PCL nanoparticles were coated with PLL. This fact can be explained by the hydrophilicity of the active ingredient MMC and its affinity to hydrophilic polymers. CS is a hydrophilic polymer and thus a better loading property is expected for MMC in CS. On the other hand, PCL is a very hydrophobic polymer and its interactions with and affinity to the hydrophilic MMC is limited which results in low encapsulation efficiency. When a hydrophilic coating polymer is introduced to the PCL nanoparticle system, encapsulation is significantly increased. Table 2 represents the encapsulation efficiency of MMC in different nanoparticle formulations prepared in this study.

In vitro release profiles of the cationic nanoparticles are seen in Fig. 2. The release profiles indicated that approximately 80% of MMC was released from CS-PCL nanoparticles. On the other hand, CS nanoparticles released only 10% and PLL-PCL nanoparticles released up to 20% of the active ingredient throughout the 6 h release period. When hydrophilic drugs like MMC are incorporated into polymeric nanoparticles, the drug has a higher affinity to the aqueous phase. Thus, the drug is believed to be situated at the surface of the nanoparticles adsorbed onto the nanoparticle (Ubrich et al., 2004). The burst effect followed by plateau seen in Fig. 2 in the release profiles confirms this hypothesis. This burst effect observed to a high extent for CS-PCL nanoparticles and somewhat lower for CS and PLL-PCL nanoparticles is not sufficient to predict the in vivo behavior of the drug-loaded nanoparticles. These data are needed to be confirmed or completed with cellular uptake and cytoxicity studies to further elucidate the drug release behavior of the cationic nanoparticles. In fact, many different release methods have been assayed to determine the release profiles of MMC from cationic nanoparticles and the tube technique was selected since it was the only technique providing sink conditions. Dialysis technique resulted in a relatively controlled release profile for all formulations (unpublished data) however the lack of sink conditions emerges as a major drawback for the interpretation of release data for nanoparticles.

DSC thermograms of MMC and nanoparticle components used in this study were also taken. Fig. 3 displays the thermal behavior of mixtures of active ingredient with polymers and mixtures of different polymers. As can be seen in DSC thermograms, MMC shows a strong interaction with TPP suggested by the disappearance of TPP melting endotherms around 110 °C. TPP is an excipient used in the ionotropic gelation process to obtain CS nanoparticles. It is noteworthy that MMC does not have the same type of interaction with CS since CS decomposition endotherm is still present when it is analyzed together with MMC. During the optimization studies of CS nanoparticles loaded with MMC, it was found that when the drug is dissolved in TPP aqueous solution drug loading is significantly higher than when the drug is dissolved CS solution (unpublished results). This confirms the fact that MMC interacts strongly with TPP. This strong interaction is believed to result in incomplete and slow release profile for CS nanoparticles seen in Fig. 3.

Since MMC is believed to be mainly adsorbed onto the nanoparticle surface, the interaction of MMC with nanoparticle surface material can be considered as the key parameter affecting drug release other than the water solubility of MMC. As seen in Fig. 3,

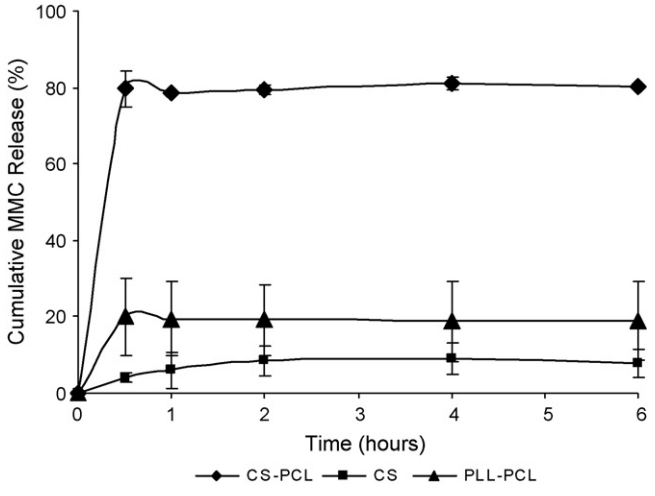

Fig. 2. In vitro release profiles of Mitomycin C from cationic nanoparticle formulations in pH 6.0 citrate buffer ($n = 3 \pm SD$).

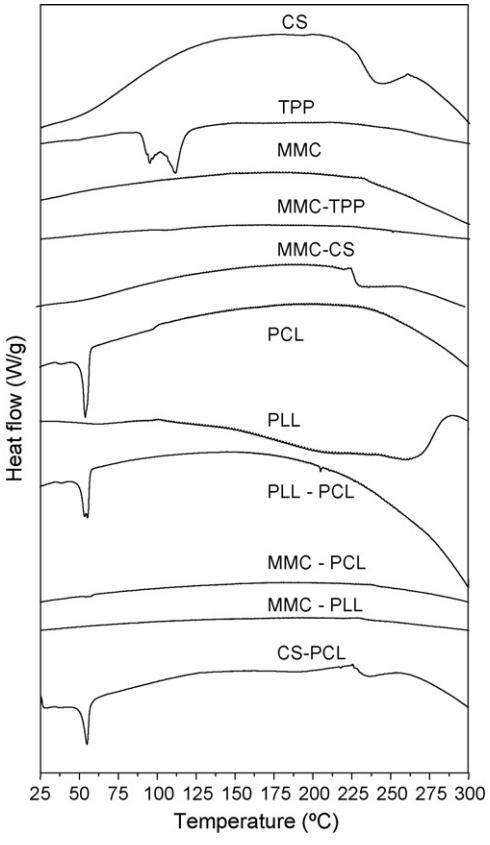

Fig. 3. DSC thermograms of active ingredient (MMC) and nanoparticle excipients (CS, PCL, PLL, TPP) and their mixtures used in the formulations.

MMC has changed the thermal behavior of both PCL and PLL. This explains the slow and incomplete release found in PLL-PCL nanoparticles, too. However, as far as CS-PCL nanoparticles are concerned, the release is largely affected by the relatively weaker interaction of MMC and CS resulting in the release of more than 80% of entrapped MMC from CS-PCL nanoparticles.

MMC is reported to be prone to degradation in acidic media (Beijnen and Underberg, 1985). In fact, one of the major limitations of intravesical chemotherapy with MMC is reported to be the acid lability of the drug leading to loss of therapeutic dosage. This may also affect the released drug into the pH 6.0 phosphate buffer used as release medium leading to degradation of released free drug resulting low cumulative release percentages for MMC.

Coating with cationic polymers CS and PLL was first reported by Calvo et al. (1997b) for ocular nanocapsules. It was found that CS or PLL coating increased particle size and resulted in a net positive charge confirming our findings. Coatings had no effect on release profiles but significantly affected ocular penetration which was believed to be a result of chitosan's penetration enhancer properties since PLL had no enhancing effect on ocular permeation of model drug. PLL coating has also been applied to polystyrene nanoparticles for the enhancement of DNA vaccine delivery (Minigo et al., 2007) as well as to PLGA microparticles (Cui and Schwendemann, 2001). It was also reported that drug release from PLL-coated nanoparticles as well as CS-coated analogues was significantly dependent on and linearly proportional to the sodium chloride concentration in the release medium (De and Robinson, 2003). On the other hand, blending of polycaprolactone with chitosan was reported to result in increased storage properties of PCL but decrease in biological activity of chitosan (Sarasam et al., 2006). The effect of chitosan on urinary bladder wall in terms of permeation was studied by Grabnar et al. (2003). Apparent permeability of urinary bladder wall was increased by 3-fold in the presence of chitosan supporting the applications of chitosan in the development of mucoadhesive intravesical drug delivery systems (Grabnar et al., 2003).

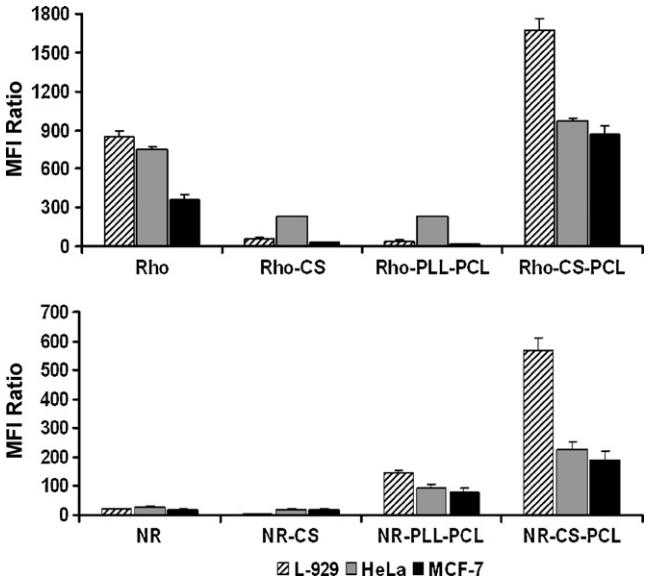

Fig. 4. The uptake of Rhodamine123 (Rho; upper panel) and Nile Red (NR; lower panel) loaded nanoparticles in L-929, HeLa and MCF-7 cells. Mean fluorescence intensity (MFI) values were obtained by flow cytometric analysis. Cellular uptake of fluorescent dyes was calculated with the mean fluorescence intensity (MFI) ratio of the corresponding formulation vs. autofluorescence of untreated cells.

CS-PCL was the most efficient formulation both for the uptake of hydrophilic and hydrophobic fluorescent markers for all of the cell lines as seen in Fig. 4. The formulations were tested against a range of cell lines including normal and cancer cells, L929, MCF7, HeLa, G/G and MB49 cells. Especially, CS-PCL nanoparticles loaded with the marker Rhodamine123 sharing hydrophilic properties with MMC were selectively incorporated by the MB49 bladder cancer cell line, but not by the G/G normal bladder epithe-

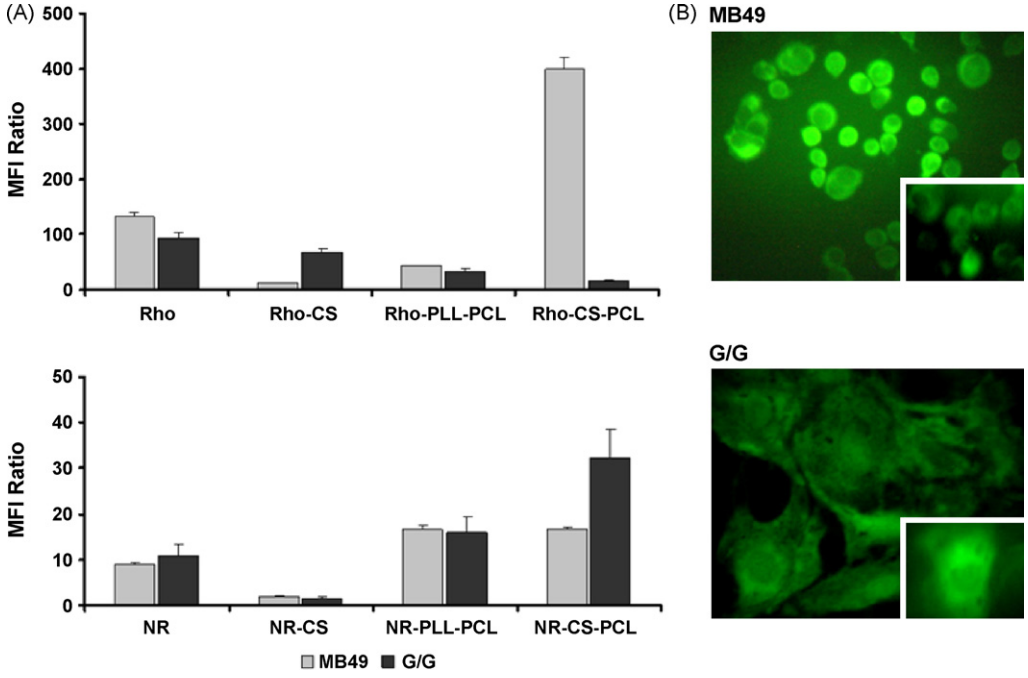

Fig. 5. The uptake of nanoparticles in normal epithelial and malignant bladder cell lines. (A) Mean fluorescence intensity (MFI) values were obtained by flow cytometric analysis of mouse bladder cancer (MB49) and normal epithelial (G/G) cell lines treated with Rhodamine123 (Rho; upper panel) and Nile Red (NR; lower panel) loaded nanoparticles. Cellular uptake of fluorescent dyes was calculated with the mean fluorescence intensity (MFI) ratio of the corresponding formulation vs. autofluorescence of untreated cells. (B) Photomicrographs of the MB49 and G/G cells treated with Rho-CS-PCL nanoparticles (×1000). The photographs of the control cells treated with Rhodamine123 solution only can be seen on the lower-right corners.

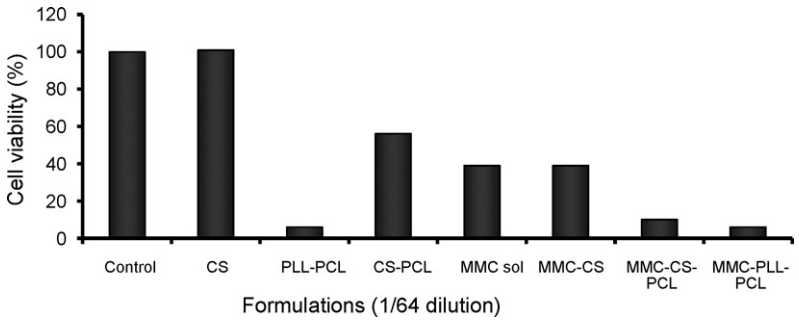

Fig. 6. The effect of MMC solution and blank and MMC-loaded CS, CS-PCL and PLL-PCL nanoparticles on viability of MB49 bladder cancer cells. All assays were performed in quadruplicate and mean OD values were used to calculate the cell viability.

lial cells as can be observed in Fig. 5. On the other hand, CS-PCL loaded with the hydrophobic Nile Red dye was preferentially uptaken by G/G cell line. Nile Red stains the hydrophobic compartments such as the lipid membranes and intracellular lipid droplets (Greenspan et al., 1985). Therefore, the type of the cell used in the delivery of hydrophobic molecules is a limiting factor for determination of the uptake efficacy. The selective delivery capacity of the Rhodamine123-loaded CS-PCL particles in bladder cancer cells may be implicated in the targeted therapy approaches for superficial bladder tumors.

Anticancer efficacy of the nanoparticles loaded with MMC was evaluated on MB49 cells as reported in Fig. 6. Blank CS-nanoparticles were non-toxic while MMC-loaded CS-nanoparticles displayed comparable cytotoxicity with MMC solution. Notably, MMC-loaded CS-PCL nanoparticles exerted higher toxicity compared to MMC solution. On the other hand, the cell viability was also impaired with blank CS-PCL nanoparticles. Finally, for PLL-PCL formulations, both blank and MMC-loaded nanoparticles showed extreme non-specific toxicity within the study range. Even though, blank CS-PCL particles were more toxic than CS nanoparticles, PCL nanoparticles coated with CS seem to be a better formulation for MMC delivery for intravesical chemotherapy.

4. Conclusion

Different cationic nanoparticles have been developed for the effective delivery of MMC in bladder cancer. It was observed that coating PCL nanoparticles with bioadhesive polymer CS resulted in favorable drug loading and release profiles as well as good cellular interaction and anticancer efficacy. Chitosan emerges as a coating material for bioadhesive intravesical drug delivery systems as well as a nanoparticle material itself. Further studies are planned to elucidate the in vivo behavior of cationic nanoparticles in animal model for a better optimization of the intravesical delivery system.

Acknowledgement

Authors wish to acknowledge that this study was financially supported by Turkish Council of Scientific and Technical Research TUBITAK Scientific Research Project SBAG-HD-235 (107S247).

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