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Original investigation

Comparison of Magnetically Responsive Trimethyl Chitosan and Chitosan Nanoparticles for Gemcitabine Delivery With in Vitro Studies

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ABSTRACT

Purpose: Gemcitabine is nucleoside analogue and used for various carcinomas like non-small cell lung cancer. Nanoparticle-based therapeutic agents have been developed for use in cancer therapy. Trimethyl chitosan (TMC) is methylated derivative of chitosan. TMC can be preferable because of the limited solubility of chitosan. Magnetic nanoparticles can be concentrated at cancerous tissue which provide targeted cancer therapy. In this study, we tried to develop and compare magnetically targeted trimethyl chitosan and chitosan nanoparticles for gemcitabine delivery in lung cancer therapy.

Methods: Chitosan was trimethylated using methyl iodide. Magnetic nanoparticles were synthesized using co-precipitation method. TMC and chitosan nanoparticles were prepared by cross-linking method with tripolyphosphate. Gemcitabine was loaded onto nanoparticles via adsorption technique. After that characterization studies were performed and in vitro drug release tests were carried out. In order to determine cytotoxicites against A549-luc-C8 and CRL5809 cell lines, MTT assays were performed.

Results and conclusion: Trimethylation of chitosan was verified with FTIR analysis. Gemcitabine was loaded with 54.7 and 30.3% on magnetic TMC nanoparticles and chitosan nanoparticles, respectively. According to drug release experiments, both carrier system had controlled drug release profile. IC_{50} values of gemcitabine loaded magnetic TMC nanoparticles were lower than that of magnetic chitosan nanoparticles. In conclusion, it was suggested that trimethyl chitosan nanoparticles had greater potential than chitosan nanoparticles for further analysis as a magnetically targeted therapy agent for lung cancer.

Keywords: chitosan, trimethyl chitosan, gemcitabine, magnetic nanoparticle, drug delivery system, lung cancer

INTRODUCTION

Chitosan (Ch) is a native polysaccharide, which is compose of repeated unit of β-(1-4)-2-amino-2-deoxy-D-glucose, and can be obtained by partial deacetylation of chitin. Chitosan is biodegradable and non-toxic polymer for this reason it is widely used in pharmaceutical, and non-pharmaceutical application (1). However, chitosan has poor aqueous solubility and loss of penetration-enhancing activity above pH 6 so it is major problem for its use at physiological conditions. For solving this problem, chitosan derivatives are widely used. Chitosan derivatives is constituted by quaternization of chitosan's primary amine groups. Quaternized chitosan derivatives are soluble at physiological conditions (2).

Trimethylchitosan (TMC) is hydrophilic chitosan derivative which is synthesized by trimethylation of chitosan (3). TMC has high solubility, very low toxicity and is also completely biodegradable, biocompatible, and also high bio-adhesive in comparison with chitosan so, TMC can be used as a nano-carrier system for pharmaceutical applications. TMC has cationic charge and soluble in neutral or alkaline media (4). TMC can open tight junctions, hence simplify the paracellular diffusion of peptide drugs. The process has an advantage, being reversible after removal of the polymer, leading to the resealing of the tight junctions (1).

Gemcitabine (2', 2'-difluoro 2'deoxycytidine, Gem) is a hydrophilic cancer drug in the anti-metabolite class. It interferes with DNA synthesis and also indirectly interferes with DNA replication. Gem is used as treatment in various types of solid tumours including pancreatic, non-small cell lung cancer (NSCLC), breast cancer, and some blood cancers (5). Pharmacokinetics pathway of gemcitabine is similar to other deoxycytidine analogs. Gemcitabine is generally well-tolerated, but haematological toxicities are commonly reported. Furthermore, certain percentage of patients experience serious and life-threatening complications after the administration of gemcitabine.

In bioprocesses, magnetic nanoparticles provide many advantages. These magnetic nanoparticles are moved in magnetic field. They could get extra properties and biocompatibility by coating with some molecules such as methylmethacrylate and chitosan (6).

Here, we developed gemcitabine loaded magnetic trimethyl chitosan nanoparticles (GMTMC) and gemcitabine loaded magnetic chitosan nanoparticles (GMC) for lung cancer treatment. Novelty of this work is preparing of gemcitabine containing magnetically targeted trimethyl chitosan nanocarriers. Additionally, it was purposed to investigate the differences and similarities of these two carrier systems.

EXPERIMENTAL

Materials

In this study, chitosan (deacetylated chitin) was purchased from Marine Bio Resources. 1-methyl-2-pyrrolidone, iodomethane (methyl iodide), diethyl ether, sodium hydrochloride, acetic acid and sodium chloride were supplied from Sigma Aldrich. Tripolyphosphate was purchased from Merck. Gemcitabine was used as Gemzar® (Lily Co.). RPMI 1640, DMEM, physiological buffered solution (PBS) were bought from Lonza; 1% penicillin-streptomycin, L-glutamine, Tripsin-EDTA were provided from Gibco. 3-(4.5-dimethyl thiazol-2-yl) 2.5-diphenyltetrazolium bromide (MTT) was supplied from Amresco. All other chemicals were of analytical grade. As non-small cell lung cancer cell lines A549-luc-C8 was bought from Perkin Elmer and CRL-5807 was provided from ATCC.

Synthesis of Trimethyl Chitosan

Trimethyl chitosan (TMC) was synthesized by methylation of chitosan in alkaline medium (1, 7–9). Chitosan was treated with sodium iodide at 60°C for 20 min in the presence of sodium hydroxide (NaOH) and N-methyl-2-pyrrolidone (NMP) with stirring. After that, methyl iodide (CH_3I) was added the solution and stirred at constant rate under relux. Then, CH_3I and NaOH were added into the mixture again and stirred at constant rate under reflux. Diethyl ether was added for precipitation of the polymer, and it was dissolved by sodium chloride solution. Obtained TMC was dialysed against pure water for 2 days and dried. TMC was characterized with Fourier transform infrared spectrometer (FTIR, IRTracer-100, Shimadzu).

Preparation of Gemcitabine Loaded Magnetic TMC and Chitosan Nanoparticles

Optimization of TPP amount

TMC nanoparticles (TMCN) and chitosan nanoparticles (CN) were prepared through ionic gelation method which was previously described (10–12). TMC (2 mg/mL) was dissolved in d-water and 10 N (NaOH) was added into it until the pH was reached to 4–5. Chitosan (2 mg/mL) was dissolved in acetic acid and also 10 N NaOH was added into it or adjusting the pH 4–5 as similar. Different volumes of 1 mg/mL tripolyphosphate (TPP) solution (2–8 mL) were added by dropwise into the reaction mixtures and stirred at a constant rate, 480 rpm for 20 min. These solutions were centrifugated at 13000 rpm and pellets were washed with d-water. Optimum amount of TPP was determined by hydrodynamic size analysis with zeta-sizer (Malvern Zetasizer Nano ZS).

Optimization of magnetite concentration

Magnetite was previously synthesized in our lab according to the co-precipitation method (13) and verified with FTIR. Various concentrations of magnetite dispersion (2–6 mg/mL) were added into TMC and chitosan solution. Then, 4 mL of TPP solution was added drop by drop into reaction mixture and centrifugation step was performed. Then, magnetic TMC nanoparticles (MTMC) and magnetic chitosan nanoparticles (MC) were obtained. Appropriate magnetite concentration was also identified by hydrodynamic size analysis.

Optimization of gemcitabine concentration

Gemcitabine was linked to both MTMC and MC by adsorption technique. Varying concentrations of gemcitabine (1–3.5 mg/mL) solutions were added into the prepared nanoparticles at optimum conditions. The mixtures were incubated at 37°C for 15 h. Unbound drug was removed from nanoparticles by centrifugation at 13000 rpm. Nanoparticles were also washed with d-water. At the end, gemcitabine loaded magnetic TMC nanoparticles (GMTMC) and gemcitabine loaded magnetic chitosan nanoparticles (GMC) were obtained. Drug loading efficiency was determined via spectrophotometric drug determination in supernatants at 268 nm using UV-Visible Spectrophotometer (Perkin Elmer Lambda35). FTIR, hydrodynamic size analyses and transmission electron microscopy (TEM) (JEOL TEM-1400-EDX) examinations were carried out for optimization.

In vitro Drug Release

Drug release characteristics of GMTMC and GMC were determined in 10 mM pH 7.4 phosphate and pH 6 phosphate buffer. Nanoparticles were taken in a dialysis membrane (MW: 14000 Da, Sigma Aldrich) and placed at a water bath at 37°C. The dialysis media were changed for certain periods and replaced with fresh buffer. Free gemcitabine formulation was also used for release profile. The released drug amounts were calculated as follows;

Cumulative release (%)= (Amount of released gemcitabine (µg))/ (Initial amount of gemcitabine (µg))×100

and release profiles were evaluated (14, 15).

Cytotoxicity Tests

A549-luc-C8 and CRL5807 cell lines were cultured at 10% FBS, Penicillin-Streptomycin, L-glutamine containing RPMI 1640 medium and incubated at 37°C under 5% CO_2 environment. Cytotoxicity analyses were performed with MTT assay (n=3).

For all cell lines, 5 x10^3 cells per well were seeded in 96 well-plate and incubated for 24 h. After cell attaching, 100 µL of drug groups (GMTMC, GMC at 0.625–40 µg gemcitabine/mL and free drug at 0.16–5 µg gemcitabine/mL concentration) were added on the cells and incubated at 37°C under 5% CO_2 environment for 72 h (16–18). After incubation, media were discarded and medium: MTT solution (10:1) (v: v) mixture was added to each well. Cells were incubated for 4 h and formed formazan crystals were dissolved in DMSO. Absorbance were recorded at micro plate reader (Polarstar Omega) at 540 nm. IC_{50} values were calculated using GraphPad Prism 8 software, and the data were expressed as means ± standard deviation. Furthermore, the data were analyzed and compared by one-way ANOVA test using SPSS Statistics v25 program.

RESULTS AND DISCUSSION

Synthesis of Trimethyl Chitosan

TMC has higher solubility in water than the chitosan at wider pH and concentration range and higher stability over a wide range of ionic conditions. Moreover, TMC is more protective against hydroxyl radicals than other chitosan derivatives and is more prone to adsorption. For this reason, the use of TMC has been preferred. In this work, chitosan was trimethylated by reacting with excess of iodomethane in N-methyl-2-pyrrolidone and using sodium iodide. All these reactions produced permanently positive charged sites in TMC structure (19).

As seen from Figure 1a, chitosan has N-H stretching peak at 2850 cm^{-1} but this peak was not observed in the TMC structure. Moreover, TMC structure has C-H peak at 1450 cm^{-1}, which is belong to methyl group but this peak cannot be seen in chitosan structure. This showed that chitosan was successfully methylated. Similarly, in an another work, asymmetric angular deformation of C–H bonds of methyl groups at 1475 cm^{-1} in the spectrum of TMC is absent in the spectrum of chitosan (20). Also, angular deformation of N–H bonds of amino groups arise in both chitosan and TMC, at 1577 cm^{-1} for chitosan and at 1559 cm^{-1} for TMC, but it is weaker or disappears due to the occurrence of N-methylation. Moreover, new peak appears at high wave number 1630–1660 cm^{-1}, assigned to the quaternary ammonium salt.

In a study, there were peaks at about 1415–1430 cm^{-1}, which were assigned to the characteristic absorption of N–CH_3 (8). In our work, C-H bending peak at 1369 cm^{-1} and also stretching anhydrous band at 1039 cm^{-1} were easily seen in FTIR spectrum. In an another work, chitosan had broad peak between 3350 and 3270 cm^{-1} was attributed to a combination of stretching modes of O-H and N-H bonds (21).

Preparation of Gemcitabine Loaded Magnetic TMC and Chitosan Nanoparticles

Chitosan nanoparticles are formed by intramolecular and intermolecular crosslinks by anionic molecules. This method is called ionic gelation. Spherical shaped nanoparticles could be formed spontaneously by entering into electrostatic interactions with TPP, a polyanion that acts as a chitosan cross-linker. This method is one of the most important advantages of ionic gelation method in forming nanoparticles at room temperature and mild conditions. TMCN could also be prepared like chitosan nanoparticles (22). Magnetite is a biocompatible and FDA approved magnetic structure and is clinically used as an MRI contrast agent with commercial forms such as Endorem™, Feridex®, and Resovist® (23), for this reason, it is preferable to use.

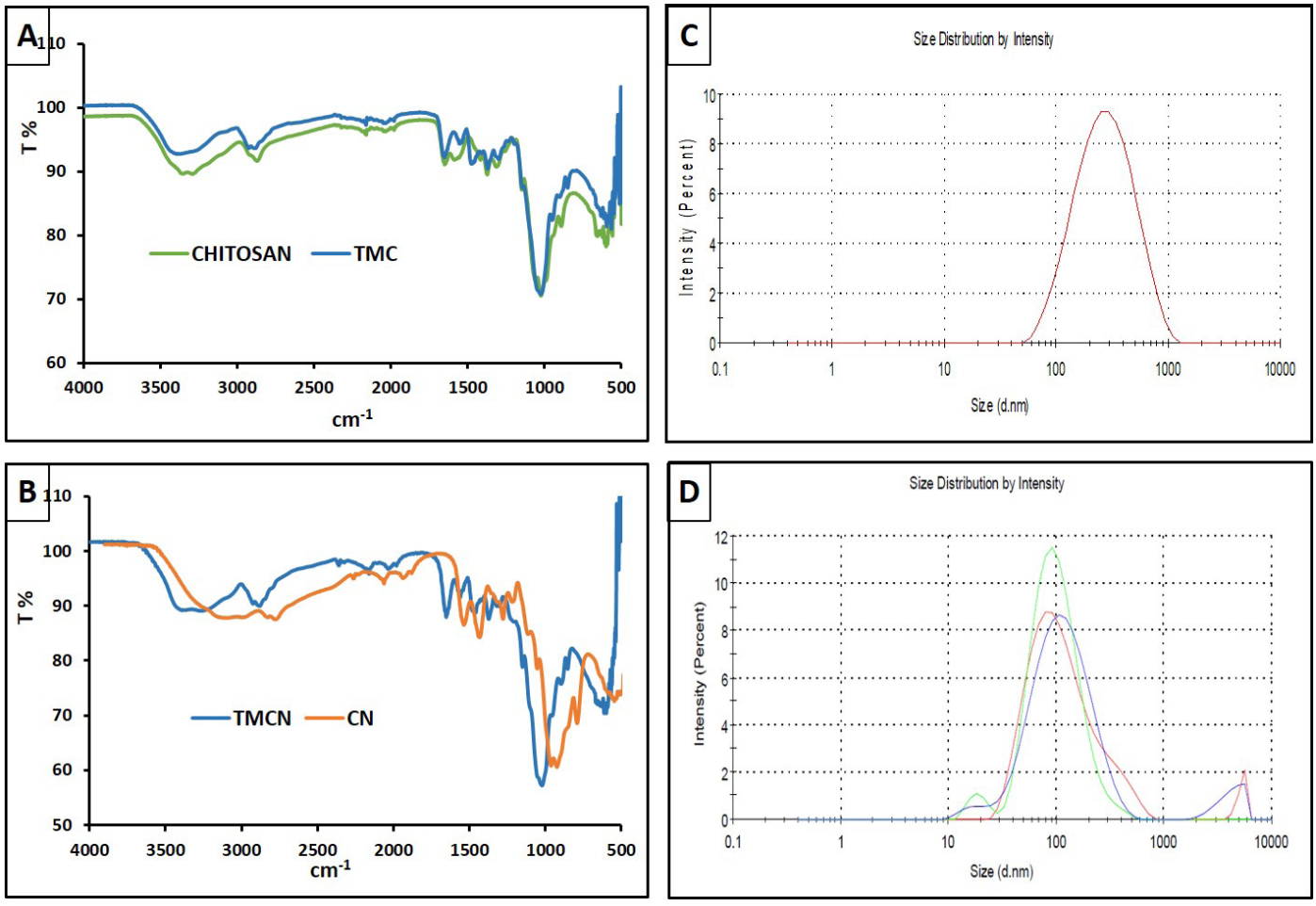

Figure 1. a–d. FTIR spectra of; TMC and chitosan (a), TMCN (trimethyl chitosan nanoparticles) and CN (chitosan nanoparticles) (b). Hydrodynamic size distributions for; TMCN (c), CN (d).

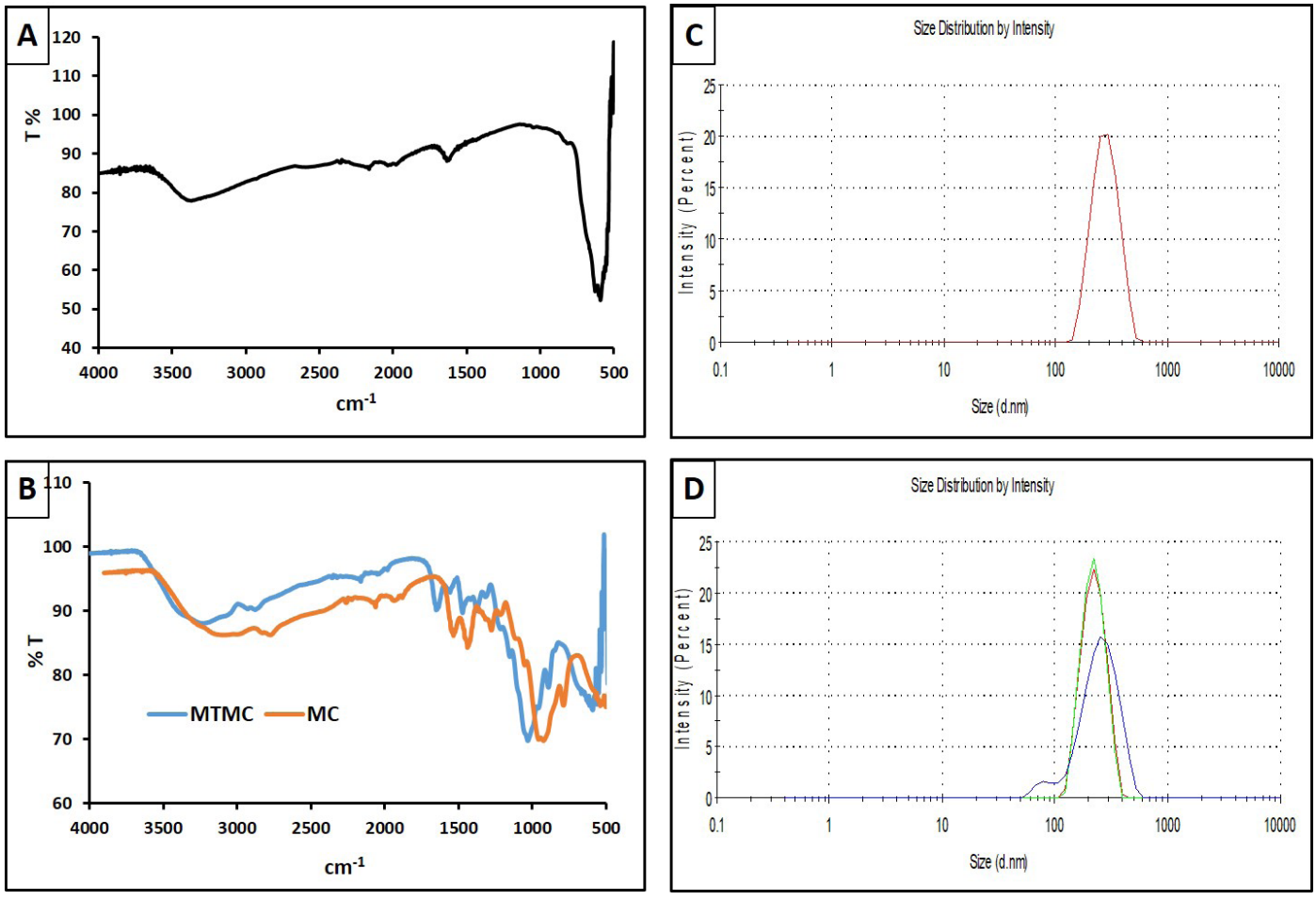

Figure 2. a–d. FTIR spectra of; magnetite **(a)** MTMC (magnetic trimethyl chitosan nanoparticles) and MC (magnetic chitosan nanoparticles) **(b)**. Hydrodynamic size distributions for; MTMC **(c)** and MC **(d)**.

Table 1. Hydrodynamic size results of TMC and chitosan and nanoparticles prepared with different concentration of TPP

Volume of TPP (mL)	Size of TMCN (nm)	PDI of TMCN	Size of CN (nm)	PDI of CN
4	294.1±18.85	0.246	226.2±53.56	0.341
5	406.3±55.62	0.241	728.5±119.2	0.539
6	432.60±23.26	0.254	703.5±255.6	0.592
7	662.9±44.12	0.380	802.7±154.4	0.713

TMCN: trimethyl chitosan nanoparticles; **CN:** chitosan nanoparticles.

Optimization of TPP amount

Both nano-carrying systems were not formed in reaction media containing 2 and 3 mL of TPP, besides, undesired pellet formations were observed in 8 mL TPP containing. However, optimum TPP amount was found as 4 mL of TPP according to zeta size analysis for both system (Table 1). For TMCN, polydispersity index (PDI) and particle size results was detected as 0.246 and 294.1±18.85 nm, respectively (Figure 1c). Similarly, in a study by Zhen et al. (2009), the mean diameters were found as 184.3±8.3 nm and PDI was also found 0.17±0.05 for TMC nanoparticles (24). For CN, PDI and particle size were detected as 0.341 and 226.2±53.56 nm in turn (Figure 1d). Just as, data was checked by another work (9, 21, 22). For this reason, optimum amount of TPP was determined as 4 mL in both system and this volume was used in further studies.

FTIR analyses showed that N-H bending peak at 1640 cm^{-1} indicated chitosan structure. This peak was clearly visible in chitosan, whereas, for chitosan nanoparticles, this peak shifted to 1520 cm^{-1} (Figure 1b). This demonstrated that NH_2 groups were crosslinked with TPP. Moreover, P-O stretching peak at 786 cm^{-1} and also P=O peaks between 1200 and 1270 cm^{-1} verified that chitosan nanoparticles were composed of TPP. Besides, P=O peak between 1116 and 1216 cm^{-1} clearly confirmed TMC nano-particulate structure with TPP. C=C streaching peak at 1651 cm^{-1} and N-O peak at 1550 cm^{-1} were observed TMCN structure in FTIR spectrum. Those data were in convenient with a previous work (8, 21).

Optimization of magnetite concentration

Magnetic nanoparticles provide drug accumulation in the tumour region with magnetic gradient, thereby anticancer effect of the

Table 2. Hydrodynamic size results of magnetic TMC and chitosan nanoparticles prepared with different concentration of magnetite dispersion

Concentration of magnetite (mg/mL)	Size of MTMC (nm)	PDI of MTMC	Size of MC (nm)	PDI of MC
3	276.9±5.09	0.433	265.4±87.19	0.337
4	279.1±8.91	0.300	888.1±614.6	0.520
5	330.9±17.33	0.265	750.6±181.7	0.483
6	337.5±17.05	0.316	-	-

MTMC: magnetic trimethyl chitosan nanoparticles; **MC:** magnetic chitosan nanoparticles.

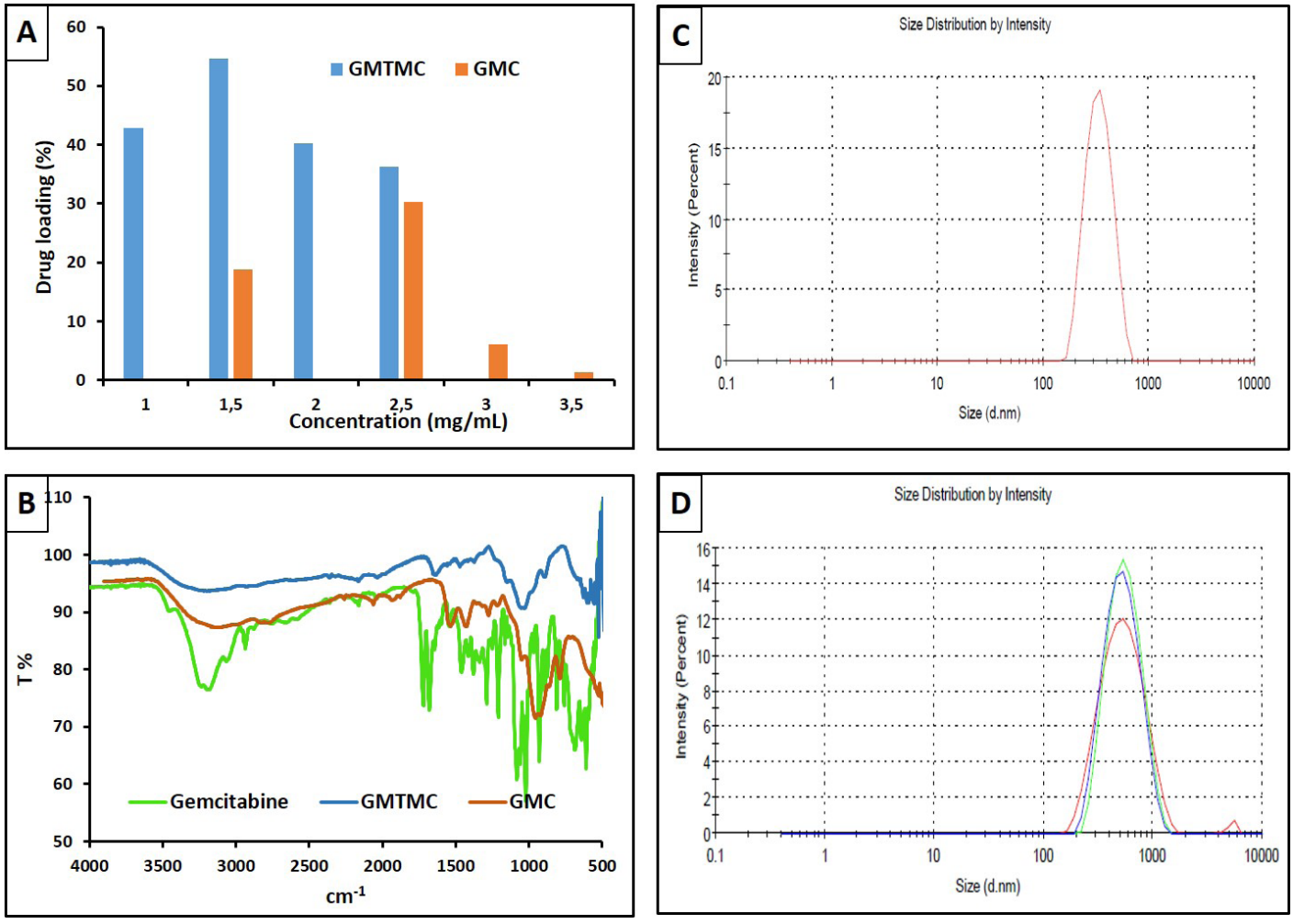

Figure 3. a–d. Drug loading efficiency of both nano-particular system: GMTMC (Gemcitabine Loaded Magnetic TMC Nanoparticles) and GMC (Gemcitabine Loaded Magnetic Chitosan Nanoparticles) **(a)**. FTIR spectra of gemcitabine, GMTMC and GMC **(b)**. Hydrodynamic size distributions for; GMTMC **(c)**, GMC **(d)**.

chemotherapeutic agent can increase and systemic toxicity of the agent can reduce to acceptable levels (25). In this work, magnetite nanostructures characterized with FTIR for determination of chemical structure. According to FTIR spectrum, sharp peak at 550–600 cm^{-1} was characteristic band for Fe-O (Figure 2a). This data confirmed the structure of magnetite and was also consistent with the previous works (22, 26). For TMC and chitosan nanoparticles, magnetite concentration of 4 mg/mL and 3 mg/mL were chosen to be optimum, respectively, based on the minimal PDI value and size (Table 2). Particle size distributions of MTMC and MC which prepared with optimum magnetite concentration are given in Figure 2c and Figure 2d. In addition, according to FTIR spectra, sharp peak at 558 cm^{-1} in MTMC and 550 cm^{-1} in MC were characteristic bands for Fe-O (Figure 2b), showing those nano-carrying systems were encapsulated with magnetic nanoparticles.

Optimization of gemcitabine concentration

Gemcitabine was loaded on the both magnetic nanoparticle systems with weak interactions. As seen in Figure 3a, maximum drug loading occurred with the initial concentration of 1.5 and 2.5 mg/mL concentration of gemcitabine (with adsorption efficiencies of 54.7 and 30.3%) for MTMC and MC, in turn. and it was found

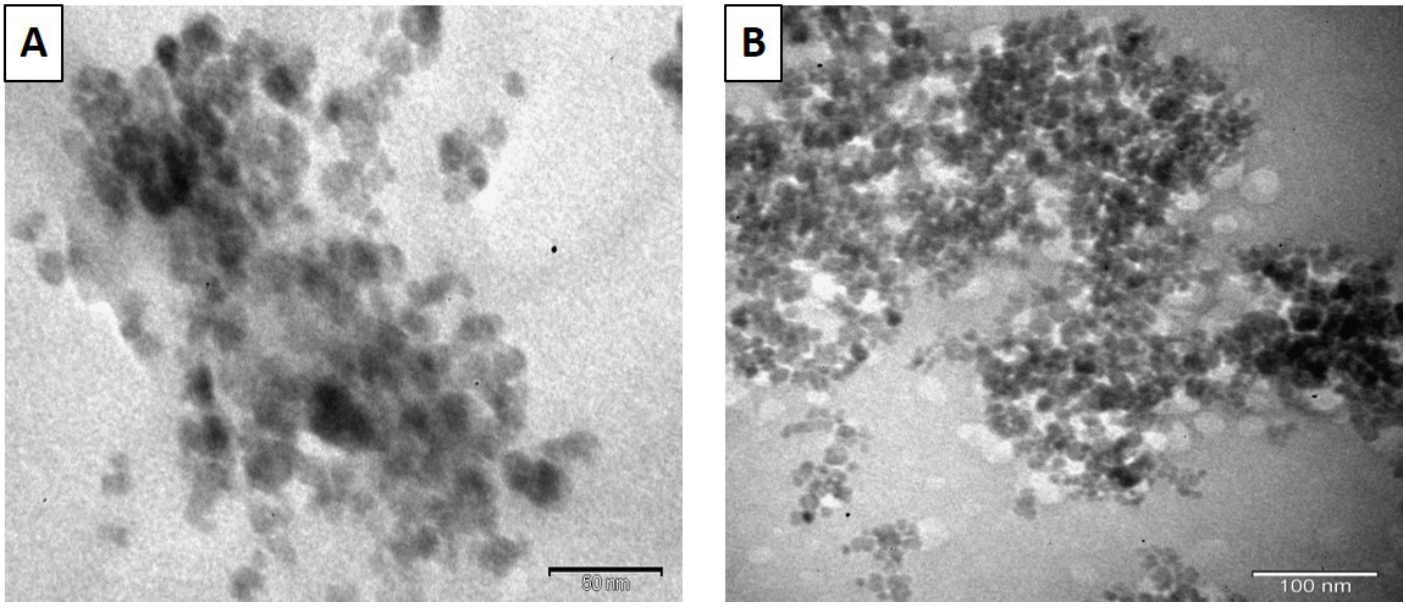

Figure 4. a, b. TEM images of; GMTMC (a), GMC (b).

that gemcitabine adsorption yield for MTMC was higher than that of MC. 82.05 µg drug was loaded on per mg MTMC and this value for MC is 75.75 µg. It was understood that MTMC had higher drug loading capacity due to the trimethylated structure. Figure 3b shows FTIR spectra of gemcitabine, GMTMC and GMC. The signal at 1020 cm^{-1} belongs to fluoride group of the drug which was found at the structure of GMTMC. Moreover N-O stretching peak at 1535 cm^{-1} was seen in the spectrum of GMC. This peak is specific for gemcitabine. Based on those data, it was indicated that gemcitabine was bound on MTMC and MC successfully, even higher drug amount was analyzed in GMTMC.

Hydrodynamic sizes of GMTMC and GMC were found as 462.1±201.5 nm and 345.0±96.2 nm (Figure 3c and d) with PDI values of 0.247 and 0.302, respectively. In addition, TEM images (Figure 4) demonstrated spherical shapes of both GMTMC and GMC. Those data were convenient with the previous works (22, 23, 25, 27).

In vitro Drug Release

In vitro drug release studies were performed by dialysis method. As can be seen in Figure 5a and b, maximum release percentage from GMTMC and GMC at pH 6 in 28 h was 88% and 9%, respectively, while those values were calculated as 53% and 5% at pH 7.4. Drug release from free formulation was reached to 95% after three and a half hours at both buffer media (data not shown). Drug release from nanoparticles was proceeded in a controlled manner throughout the study period and also slower than free gemcitabine. In a study, methotrexate release from chitosan nanoparticles was found as 36% within 5.5 hours, but almost all of the free drug was released in 1.5 hours (15). Drug release profiles from nanoparticles depend on the nature of the carrier system and the structure of active agent. The first rapid release of gemcitabine may be thought to be due to gemcitabine adsorbed on the surface of nanoparticles (12, 28). In our study, drug release from GMC was found slower to be toxic against cancer cells in comparison with GMTMC both at pH 6 and 7.4 buffers. On the other hand, higher drug release was determined in acidic medium than physiological medium meaning higher drug release could be occurred at cancerous tissue than circulation and healthy tissues.

Cytotoxicity Tests

CRL5807 and A549-luc-C8 (non-small cell lung cancer cells) cell lines were chosen for cytotoxicity studies. Empty chitosan and TMC nanoparticles were tested as blank (data not shown) and not lead to cell death at those concentrations. Figure 6 shows viabilities (%) of cells treated with drug groups for 72 h. All the data demonstrated that cell survival treated with GMTMC and GMC decreased with increasing gemcitabine doses in nanoparticles suggesting cytotoxicity caused by only active agent. IC_{50} values of gemcitabine, GMTMC and GMC at 72 h for cell lines can be seen from Table 3. There were statistically significant differences in IC_{50} values of all drug groups for both cell lines ($p<0.05$).

Table 3. IC_{50} values (µg/mL) of gemcitabine and gemcitabine loaded nano-particulate systems against A549-luc-C8 and CRL5807 cell lines at 72 h

Cell Line	IC_{50} value (µg/mL)		
	Gemcitabine	GMTMC	GMC
A549-luc-C8	1.57±0.54	10.65±0.20	18.67±3.08
CRL5807	0.58±0.16	0.95±0.03	3.31±1.07

GMTMC: gemcitabine loaded magnetic trimethyl chitosan nanoparticles; **GMC:** gemcitabine loaded magnetic chitosan nanoparticles.

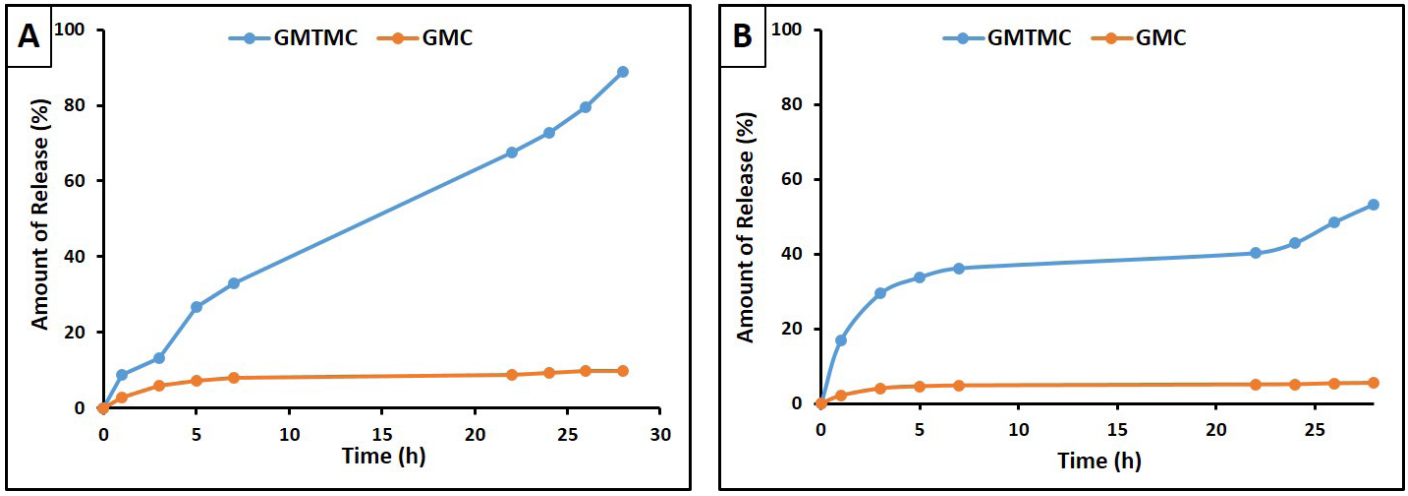

Figure 5. a, b. Gemcitabine release profile (%) at; pH 6 **(a)** and pH 7.4 **(b)** buffers from GMTMC and GMC.

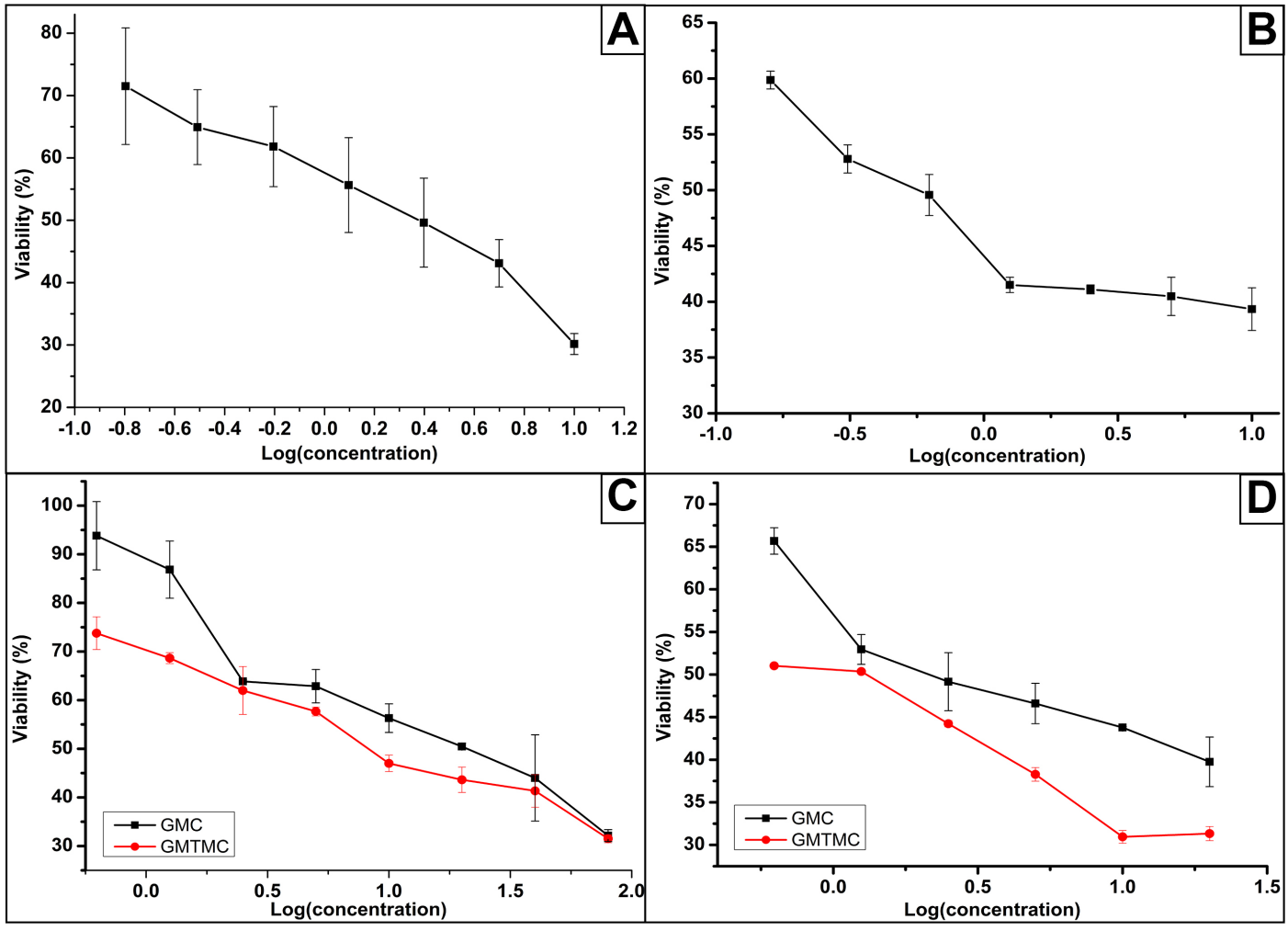

Figure 6. a–d. Dose-dependent cytotoxic effects of drug groups at 72 h: Gemcitabine against A549-luc-C8 cells **(a)**, Gemcitabine against CRL5807 cells **(b)**, GMTMC and GMC against A549-luc-C8 cells **(c)** and GMTMC and GMC against CRL5807 cells **(d)**.

IC_{50} values of gemcitabine against A549-luc-C8 cell line was consistent with a previous work (29). Polymers could increase the internalization of gemcitabine by influencing its passive diffusion through the bio-membranes. The IC_{50} values of nanoparticles were higher than ($p<0.05$) free drug owing to slower release from nanostructures, especially GMC demonstrated in the previous section. For A549-luc-C8 cell line, IC_{50} value of GMTMC was 1.75 fold lower and for CRL5807 cell line 3.48 fold lower in comparison with GMC. Cell survival difference between cells might be explained by drug sensitivity of cells as well as uptake of drug carrier into cell. For both cell lines GMTMC was found as more effective than GMC likely for drug release test.

CONCLUSION

Chitosan is a biocompatible and biodegradable polymer found abundantly in nature. Gemcitabine is an anticancer agent but its usage is limited despite its anticancer activity against many cancer types. GMC and GMTMC systems were developed in order to deliver of gemcitabine and accumulate it in the tumour area. MC and MTMC were first synthesized and gemcitabine was loaded on nanoparticles. Characterization studies, FTIR, TEM and particle size analyzes were performed. Drug release from both drug delivery systems was slower and controlled and nano-particulates had cytotoxicity effects against lung cancer cell lines. In addition, GMTMC and GMC have magnetic properties with drug targeting ability. GMTMC was found as more effective and cytotoxic against lung cancer cells and it can be said that GMTMC is superior to GMC. Both nanoparticles, especially GMTMC have anticancer and magnetic drug targeting potential for future studies.

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