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Original Research

SiRNA/DOX lodeded chitosan based nanoparticles: Development, Characterization and in vitro evaluation on A549 lung cancer cell line

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Abstract: High-mobility group AT-hook2 (HMGA2), involved in epithelial mesenchymal transition (EMT) process, has a pivotal role in lung cancer metastasis. Lung cancer therapy with HMGA2 suppressing small interfering RNA (siRNA) has been introduced recently while doxorubicin (DOX) has been used as a frequent cancer chemotherapy agent. Both reagents have been faced with obstacles in clinic which make them ineffective. NanoParticles (NPs) provided a platform for efficient co delivery of the anticancer drugs. The aim of this study was production and in vitro characterization of different pharmacological groups (siRNA, DOX or siRNA-DOX) of carboxymethyl dextran thrimethyl chitosan nanoparticles (CMDTMChiNPs) on cytotoxicity, gene expression, apoptosis and migration of metastatic lung cancer cell line (A-549). CMDTM-ChiNPs were synthesized and encapsulated with siRNA, DOX or siRNA-DOX. Then the effects of HMGA2 siRNA and DOX co delivery was assessed in A549 viability and target genes (HMGA2, Ecadherin, vimentin and MMP9) by MTT and real time PCR, respectively. In addition capability of apoptosis induction and anti-migratory features of formulated NPs were analyzed by flowcytometry and wound healing assays. SiRNA-DOX-CMDTM ChiNPs approximate size were 207±5 with poly dispersity index (PDI) and zeta potential of 0.4 and 16.3±0.3, respectively. NPs loaded with DOX and siRNA were the most efficient drug formulations in A549 cell cytotoxicity, altering of EMT markers, apoptosis induction and migration inhibition. Generally our results showed that co delivery of HMGA2 siRNA and DOX by novel designed CMDTMChiNPs is a new therapeutic approach with great potential efficiency for lung cancer treatment.

Key words: Lung cancer, HMGA2, siRNA, Nanoparticle, Doxorubicin.

Introduction

Lung cancer is the second most common cancer, affecting both genders equally and is the leading cause of cancer related death worldwide. According to statistic documents, around14% of newly diagnosed cancer cases relates to lung cancer with only 15% 5-year survival rate. Both genetic and environmental factors can affect lung cancer development risk, although smoking is the first causative agent (1).

Among the genetic factors influencing cancer incidence, epithelial mesenchymal transition (EMT) process plays important role in cancer metastasis. Several studies have shown that HMGA2 as a transcription factor can impress EMT by regulation of cell adhesion molecules such as E-cadherin and vimentin beside matrix metalloproteases (MMPs) particularly MMP-9 in metastatic cancers including lung cancer (2-6). More over recent reports revealed a novel managing function for HMGA2 as a competing endogenous for promoting lung cancer (7, 8). All of the mentioned oncogenes can become a therapeutic target for prevention of lung cancer metastasis and their suppression may even lead to cure this type of cancer completely.

Current lung cancer treatments are surgery, chemotherapy and radiation therapy, each of which is not effective due to lack of early stage diagnostics, tumor invasion and metastasis. Additionally chemotherapy with well-known anticancer drugs (Paclitaxel, Doxorubicin (DOX)) can lead to tumor drug resistance and worse off-target side effects (9, 10). So finding new therapeutic methods for controlling essential cancer progression molecular pathways seems vital. Small interfering RNAs (siRNAs) have shown the capacity for silencing genes related to cancer progression and metastasis (9). Limitations for siRNAs cancer therapy are their degradation by endonucleases, fast clearance and disability for cell transfection (11). Therefore to achieve safe and efficient delivery, siRNA was encapsulated in nanoparticle platform (12).

Nanoparticles (NPs) are one of the new successful and effective targeted cancer therapy approaches since 1995(13). Different types of NPs have been evaluated for optimum drug delivery. Cationic chitosan (Chi) NPs have gained researchers attention mostly because of chitosan unique characteristics such as no cytotoxicity,

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biocompatibility, biodegradability and controlled drug, protein or peptide releasing (14). Derivations of chitosan (N-trimethyl chitosan (TMC) and mono-N-carboxymethyl chitosan (CMC)) have been shown higher solubility in comparison to chitosan (14). In addition one of the best ways for siRNA efficient delivery is positively charged ChiNPs that facilitate cell endocytosis and prevent lysosmal degradation (15). Combination cancer therapy by anticancer chemotherapy drugs and siRNA can provide a new therapeutic way which needs a smart drug delivery system that can be arranged by NPs.

The aim of the current study is to investigate the potential of novel formulated N-tri methyl-O-carboxy methyl Dextran (TMCMD) ChiNPs for selective delivery of encapsulated DOX/siRNA (knockdown HMGA2 expression) and its anticancer effects on A549 lung cancer cell line.

Materials and Methods

Synthesis of N-tri methyl-O-carboxy methyl dextran(CMDTM) chitosan nanoparticles

Carboxy methyl dextran (CMD) chitosan was synthesized as previously described (16). In brief chitosan (2 gr, 400k Da) was decomposed in 10 ml of 6% v/v acetic acid. Then 10ml of 1mg/ml sodium nitrite ($NaNO_{2)}$ was added and kept stirred at room temperature for 2 hrs to obtain140 kDa chitosan. In order to precipitate depolimerized chitosan, pH was adjusted to 9.0 by sodium hydroxide 4M. Afterwards the sediment was filtered and washed by acetone for three times to remove impurities and immediately dissolved in 40 ml 0.1N acetic acid. For purification, chitosan solution dialyzed against distilled water (DW) by 12k Da cut-off dialysis tube (cut-off 12 kDa, Sigma, Aldrich) followed by freeze drying.

As the second step CMD chitosan attained from first step was resuspended in 60 ml of N-methyl pyrolidone (NMP) and magnetically stirred for 30 minute. 1.5 gr sodium azide, 5 ml NaOH (15% w/v) and 3.8 ml methyliodide were added respectively and the mixture was completely stirred for 3 hrs in 60 ^{o}C. Later 200 ml DW was added and kept under stirring for 2hrs. The final composition (O-carboxy mtheyl N-trimethylchitosan) designated as trimethyl carboxy methyl dextran (TMCMD) chitosan was transferred to 12k Da dialysis tube against DW for 24 hrs and then precipitated by means of acetone. The final compound washed with 15% w/v NaOH and dialayzed against DW for 48 hrs and subsequently lyophilized by freeze dryer. For further application the product kept in 4 ^{o}C.

Preparation of DOX and/or siRNA encapsulated CMDTM ChiNps

Generally, 100 µl of 1mg/ml CMDTM Chitosan was added to different concentrations of DOX (Sigma, USA) (2.5, 5, 10 and 20 µg/ml). For antisense uploading 100 nm HMGA2 specific siRNA (Santacruz, USA) was entered to the prepared solution and vortexed for 15 sec and gradually dissolved in previously prepared CMDTM Chitosan solution (10 mg/ml dissolved in DW, ph=5.5) for siRNA and/or DOX encapsulation. The finalized NPs needed 30 min incubation at room temperature before applying. Each of DOX CMDTM ChiNPs and siRNA CMDTM ChiNPs were generated as described above in absence of siRNA and DOX, respectively.

Determination of particles morphology by scanning electron microscopy (SEM)

Freshly synthesized NPs morphology was evaluated by SEM. In brief small amount of siRNA/DOX CMDTM ChiNPs were spilled on an aluminum disk, as soon as the disk dried in room temperature it was covered by a layer of gold and examined by SEM (ZEISS FE-SEM model SIGMA).

Characterization of NPs (size, poly dispersity index (PDI) and zeta potential)

1mg of NPs was dissolved in 2ml deionized water using bath sonicater. Then different sub groups of CMDTM ChiNPs specific properties such as mean size and PDI and zeta potential were assessed by dynamic light scattering and Laser Doppler Electrophoresis using Zetasizer (Nano-ZS, Malvern, Worcestershire, UK), respectively. All dynamic light scattering measurements were accomplished at a wavelength of 633 nm at 25°C with an angle detection of 90°. To maintain a constant ionic strength, the samples were diluted (1:50 v/v) in NaCl 1 mmol/L (pH 6.5). Three subsequent measurements were determined for each sample.

Fourier transform infrared spectroscopy (FTIR)

Structural properties and precise formation of CMDTM ChiNPs in presence and absence of DOX evaluated by FTIR. By this method particular wavelengths of DOX, CMD, TMC and chitosan assessed by FTIR spectrometer (Magna IR 550; Madison,WI).

Evaluation of siRNA and DOX loading efficiency (LE %)

For evaluating of siRNA loading efficiency the HMGA2 siRNA CMDTMChiNPs were centrifuged (15000 g, 15 min) and the collected supernatant optical density (OD) at 260 nm measured against initial feeding siRNA. The same procedure was done for supernatant of DOX-CMDTM ChiNPs with changes in centrifuging program (12000 g, 20 min) and optimum wavelength(480nm) for spectrophotometery (Scinco, Seoul, Korea). The LE (%) of HMGA2 siRNA and DOX- CMDTM ChiNPs were evaluated as follows.

HMGA2 siRNA loading efficiency % = [(total amount of HMGA2 siRNA – free amount of HMGA2 siRNA)/ total amount of HMGA2 siRNA] × 100

DOX loading efficiency % = [(total amount of DOX – free amount of DOX)/total amount of DOX]

Assessment of serum/heparin stability

Serum stability assays was provided by mixing and soft agitation of 200 µl FBS (Gibco, New York, USA) and 400 µl siRNA loaded NPs (with 5 µg siRNA) in 37^{o}C. Then 20 µl of the incubated sample removed at predicted times (4, 8, 10, 12, 24, 48 and 72 hrs) and maintained in -20^{o}C for gel electrophoresis. Furthermore siRNA CMDTM ChiNPs stability against heparin was investigated by agarose gel electrophoresis. For this purpose 60 µl of CMDTM ChiNPs were exposed with different concentrations of 2 µg/ml heparin (0, 0.6,

Table 1. Primers sequences.

Target gene	primer sequence
HMGA2	F: 5′-TGG GAG GAG CGA AAT CTA AA-3′ R: 5’-TGG TAT TCA GGT CTT TCA TGG-3’
Ecadherin	F:5'-AGTACAACGACGACCCAACCCAAG-3′ R:5'- GCAAGAATTCCTCCAAGAATCC-3′
Vimentin	F:5'- CAGGCAAAGCAGGAGTCCA-3′ R: 5'- AAGTTCTCTTCCATTTCACGCA-3′
MMP9	F: 5'-ATTTCTGCCAGGACCGCTTCTAC-3′ R: 5'-ATCCGGCAAACTGGCTCCTTC-3′
GAPDH	F: 5'-CAACAGCCTCCAAGATCATCAGCAA-3' R:5'-AGTGATGGCATGGACTGTGGTCAT-3'

1.5, 3 µl) and consequently incubated for 1 hr in 37°C, the stability assessed by gel electrophoresis against free siRNA as internal control.

The profiles of NPs in vitro drug-release studies

NPs drug release capability was performed through measuring siRNA and DOX releasing capacity of CMDTM ChiNPs by UV–vis spectrophotometer in 260 and 480 nm respectively. Totally, HMGA2 siRNA or DOX loaded NPs were decomposed in 4 ml fresh phosphate-buffered saline (PBS, PH=5.5, PH=7.4) and transferred to a dialysis membrane sac (cut-off 12kDa). Next the dialyzed membrane sac NPs were floated in beaker containing 50 ml PBS with previously mentioned pH conditions. The beaker was set in an incubator and mixed at 90-100 rpm in 37°C for 144 hrs. In estimated time intervals (0, 1, 3, 6, 12, 24, 48, 72, 96, 120 and 144 hrs) 2 ml of released medium was removed and replaced by the same medium with the exact situation. As the last step the amounts of releasing siRNA and/ or DOX in releasing medium was calculated by a UV–visspectrometry (U.V-1601; Shimadzu, Japan). It is necessary to mention that releasing medium obtained from unloaded NPs was used as blank. The siRNA and/ or DOX releasing profiles were calculated as follows.
Released HMGA2 siRNA (%): [(OD of HMGA2 siRNA in PBS/initial total content of HMGA2 siRNA)] × 100
Released DOX (%): [(OD of DOX in the PBS/initial total content of DOX)] × 100.

A549 cell culture

A549, human lung carcinoma cell line was purchased from Pasteur institute (Tehran, Iran). The cells were cultured in RPMI-1640 (Gibco, New York, USA) with 10% heat inactivated FBS and penicillin–streptomycin (100 U/ml, 1% v/v) (Gibco, New York, USA). Cultured A549 cells were incubated in 37°C with 5% CO_2 and passaged after reaching to the desired confluency.

Cytotoxicity assay

The cytotoxicity effects of prepared CMDTM ChiNPs therapeutic groups were carried out by MTT assay. A549 cells were seeded at a density of 2×10^4 per well in 96 wells cell culture plate (Munc, Denmark) and 24 hrs pre incubated for ensuring cells attachment. The following day cells were exposed to different defined pharmaceutical CMDTM ChiNPs containing DOX (2.5, 5, 10, 20 µg/ml) or siRNA (100 nm) and dual agent NPs, in addition naked siRNA and free DOX for 24 hrs and 48hrs. After 24 hrs or 48 hrs medium of each well was suctioned and 200 µl medium containing MTT solution (5mg/ml) (Sigma,USA) was replaced. The cells were incubated 4 hrs under cell culture condition until the appearance of formazan crystals in living cells. Afterwards for each well 150 µl of medium was aspirated and 100 µl DMSO (Dimethyl sulfoxide) was added as formazan crystals dissolving agent. The plates were shaked for 15 min and the absorbance was determined at 492 nm against 690 nm as reference wavelength by micro titer plate reader (Stat Fax 2100, USA). Viability of the cells was assessed by the following equation:
Viability%= [OD of test/OD of control]×100.

RNA extraction, c-DNA synthesis and real time PCR

A549 cells were seeded in 6 well cell culture plate 24 hrs before treatment. Then the cells were treated with obtained IC50 concentration by DOX-siRNA-CMDTM Chi NPs, DOX- CMDTM ChiNPs and siRNA-CMDTM ChiNPs in comparison with blank NPs and untreated cells as control. Total RNA was extracted by RNXPLUS (SinaClon, Tehran, Iran) and converted to cDNA by reverse transcriptase kit (Thermo Fisher, USA). Then the cDNA subjected to real time PCR for evaluation changes in HMGA2, Ecadherin, vimentin and MMP-9 mRNA expression against GAPDH as internal control using SYBR Green method (Takara, Japan). The PCR reaction condition was 95°C for 5 min for samples denaturation , 95°C for 15 sec, 59°C for 30 sec and 72°C for 1 min. Primers (Bioneer, Korea) sequences were summarized in table 1.

Flowcytometric analysis of apoptosis

The anticancer activities of siRNA-CMDTM ChiNPs, DOX-CMDTM ChiNPs, siRNA-DOX CMDTM ChiNPs, free DOX and naked NPs were examined by annexin V-FITC/PI staining for apoptosis assay (BD Pharmingen, CA, USA). In brief 5×10^5 A549 cells were seeded in 6well cell culture plate 24 hrs before treatment to ascertain attachment. Then the cells were treated with IC50 concentration of above mentioned pharmacologic formulated groups for 24 hrs. Treated cells washed twice by PBS and 5 µl of Annexin V-FITC and propidium iodide were added to each sample immediately which followed by 15 min incubation in darkness and room temperature. Flowcytometry assay was handled by FACS calibur flowcytometer (Becton-Dickenson, Mountain View, CA, USA) and data were analyzed by flowjo software.

Wound healing assay

In order to determine inhibitory effects of different defined NPs on migration of lung cancer cells, 5×10^5

Table 2. Different characteristics of therapeutic formulated NPs.

Np groups	Size	Charge	PDI
Dox-siRNACMDTMC	207±5	16.3±0.3	0.4
DOX-CMDTMC	180±3	14.6±0.2	0.3
SiRNA-CMDTMC	110±3	13.5±0.2	0.2

cells were seeded in a multiwell plate 24 hrs before treatment. As soon as the cells reached to monolayer a wound was created with a pipette tip, afterwards cell treatments were carried out using DOX-siRNA-CMDTM-ChiNPs, , blank nanoparticles and untreated cells as control. The wound healing related events were detected at fixed time points (0, 12, 24, 48 and 72 hrs) by manual imaging.

Statistical Analysis

All experiments were handled in triplicate and reported as mean ± SD. Results were analyzed by Graph pad Prism v6.07 (La Jolla, USA) and non parametric one way- ANOVA. Statistically significant differences were shown by asterisks (*). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Results

CMDTM ChiNPs physiochemical characteristics

Physiochemical characteristics of the formulated Nps including size, zeta potential and PDI which were measured by dynamic light scattering were summarized in table2. Due to our results PDI was not affected after manipulating the NPs whereas the size and zeta potential of siRNA, DOX or siRNA-DOX loaded particles were approximately affected by encapsulation.

Figure 1 represents SEM image of self assembeled DOX-siRNA-CMDTM ChiNPs which revealed an irregular morphology.

Perfect syntax of DOX, CMDTM and chitosan that was measured by FTIR, demonstrated in figure2. Different compounds have specific wavelengths. Carboxyl group in CMD was detected as1741cm^{-1} (figure 2a), amino branches in trimethyl chitosan were shown in 2920 cm^{-1} and 2840 cm^{-1} wave lengths (figure 2b). C-C linkages of DOX were presented in 1217cm^{-1} and 1339 cm^{-1} peak. In figure2d, Wavelengths of ChiNP/DOX/CMD revealed carboxyl group of CMD with 1715 cm^{-1}, amino group of ChNP with1120 cm^{-1} and DOX C–C linkage with 1312 cm^{-1} that arelocated side by side, the overlapping peaks were indicative of successful DOX-CMDTM ChiNPs conjugations.

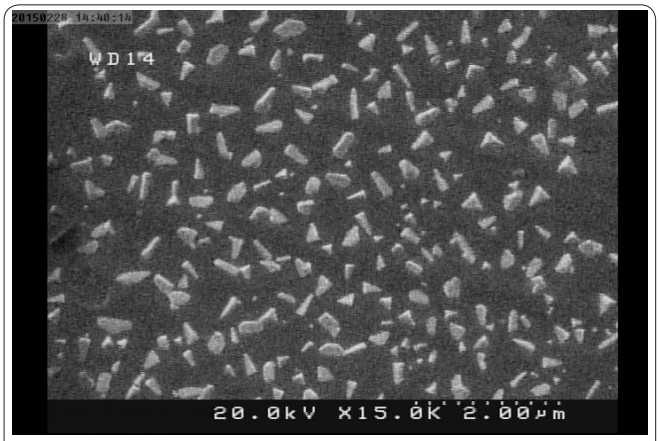

Figure 1. Scanning electron microscopy image of CMDTM-ChiNPs showed polygonal morphology with an irregular shape of siRNA/ DOX loaded ChiNPs.

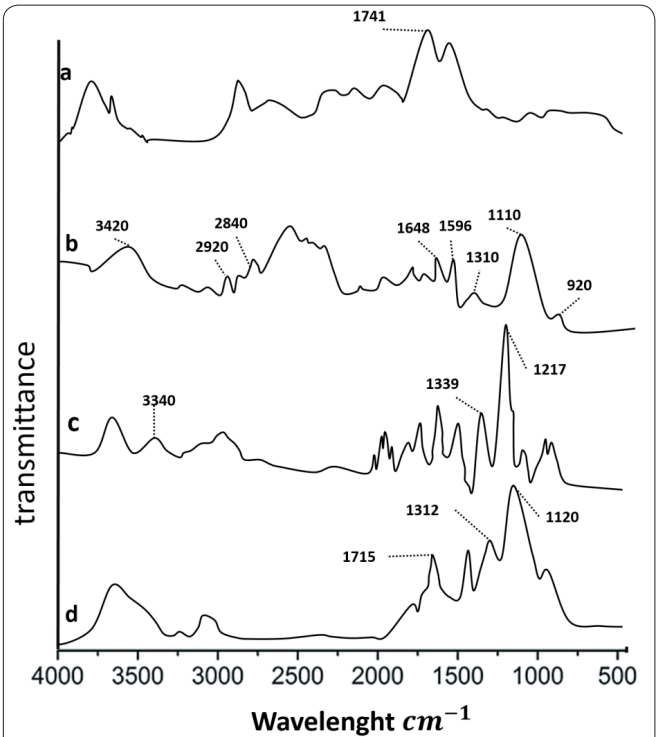

Figure 2. FTIR spectrum of prepared nanoformulations: a: CMD (1731 cm^{-1}), b: TMC (2840, 2920 cm^{-1}), c: Dox C-C linkage (1217,1339 cm^{-1}), d: completely loaded NPs (1715, 1312, 1120 cm^{-1}). As shown overlapped wavelengths were representative of correct self-assembly of CMDTMChiNPs.

SiRNA-CMDTM ChiNPs Serum/heparin stability features

The serum stability of HMGA2 siRNA-CMDTM ChiNPs was performed by time dependently incubation of siRNA loaded NPs in FBS through gel electrophoresis in which siRNA first release was achieved after 12 hrs and the related band sharpened after 72 hrs (figure 3a). Furthermore siRNA loaded NPs were not impressed by various concentrations of heparin (figure 3b).

Profile of DOX and siRNA in vitro release from CMDTM ChiNPs

SiRNA or DOX formulated CMDTMChiNPs in vitro releasing capacity were examined in acidic PBS (pH=5.5) in order to represent tumor microenvironment and compared with neutral physiologic pH (7.4).

The releasing manner of siRNA pointed that releasing rate of siRNA was higher at pH=5.5 rather than pH=7.4 and an initial burst release occurred after 24 hrs which then reached to a plateau phase after 72 hrs (figure 4a).

The pattern of DOX release from the designed NPs in PBS (pH 5.5 and 7.4) is illustrated in figure4b. As figure shown, in contrast with physiological media, DOX have rapid releasing velocity at pH=5.5 which owed it to the better solubility of protonated DOX. The releasing profile of DOX showed different manners in initial burst at acidic media (6 hrs) and physiologic condition (24 hrs) while DOX carried NPs reached to stationary phase at the same hours of incubation (72 hrs) in different mentioned pH (figure 4b).

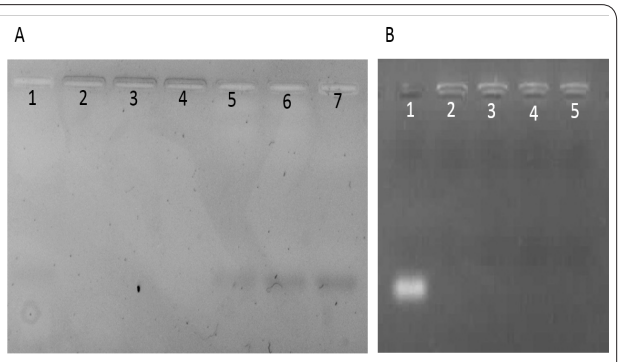

Figure 3. Serum and heparin stability. a) serum stability of siRNA-CMDTMChiNPs after different FBS incubation time intervals(1:4 h, 2:8 h, 3:10, 4:12, 5:24 h, 6:48, 7:72 h). siRNA initial releasing starts at 12hrs and followed by 72 hrs. b) Heparin stability of siRNA encapsulated NPs in various heparin volumes (2 µg/ml) is demonstrative of CMDTMChiNPs high stability against polyanions (1: naked siRNA as internal control, 2: 0, 3: 0.6 µ, 4:1.5 µ and 5:3 µ).

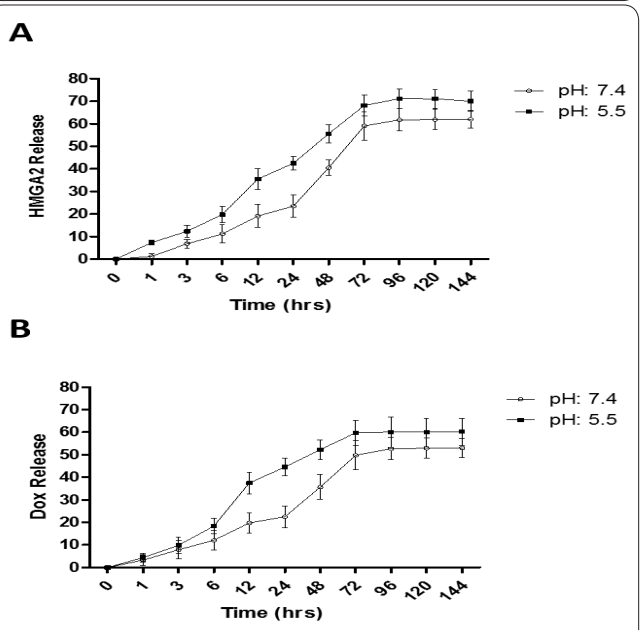

Figure 4. In vitro drug release profile. A) siRNA release in acidic and neutral pH with an initial burst after 24 hrs. B) DOX initial burst release in pH=5.5 was faster (6 hrs) than in pH=7.4 (24 hrs).

Cytotoxicity assay

The in vitro cytotoxicity of different pharmacological formulated NPs including siRNA-CMDTM ChiNPs, DOX-CMDTMChiNPs and siRNA-DOX-CMDTM-ChiNPs with free DOX were assessed in 24 hrs (figure 5a) and 48hrs (figure 5b) against untreated cells and naked CMDTMChiNPs as controls. Then IC50, which defined as the optimum drug concentration for 50% inhibition of cell growth provided by MTT. We found that dual agent NPs (siRNA-DOX-CMDTMChiNPs containing 20µg/ml of DOX) were always more efficient in inducing cytotoxicity in A549 cells (IC50 was 6.38 and 8.88 for 24 hrs and 48hrs, respectively). In addition DOX encapsulated in NPs was more cytotoxic in different concentrations of DOX compared to free DOX at both time intervals.

HMGA2, vimentin and MMP-9 gene expressions were significantly downregulated while E-cadherin markedly reduced particulary in DOX-siRNA-CMDTMChiNPs treated A549 cells

Different pharmacological groups were tested for impressing HMGA2, E-cadherin, vimentin and MMP-9 expression ratio following 24hrs and 48hrs treatment. HMGA2, vimentin and MMP-9 relative expressions were significantly reduced in siRNA-CMDTMChiNPs and DOX-siRNA-CMDTMChiNPs formulated groups after 24 hrs and 48hrs, respectively. After 24hrs, HMGA2 sequential relative expression was 2.41 (0.43±0.11, $p<0.0001$) and 3.18 (0.31±0.048, $p<0.0001$) fold decreased. Following mentioned treatments vimentin expression was 2 (0.5±0.02, $p<0.0001$) and 6.58 (0.15±0.01, $p<0.0001$) fold reduced. MMP9 expression dropped down to1.93 (0.52±0.01, $p<0.0001$) and 3.46 fold (0.29±0.04 $p<0.0001$). In parallel, after 48hrs expression fold values of the mentioned genes were as followed: 1.59(0.63±0.06, $p<0.0001$) and 11.77 (0.08±0.01, $p<0.0001$) for HMGA2, 6.49 (0.49±0.01, $p<0.0001$) and 7.15 (0.14±0.009, $p<0.0001$) for vimentin and finally 2.87 (0.37 ±0.11, $p<0.0001$) and 6.26 (0.16 ±0.008, $p<0.0001$) for MMP9, respectively. In general our data revealed time dependent behavior of formulated NPs whereas free DOX and encapsulated NPs did not affect gene expression compared to control cells even in 48hrs treatments. On the other hand expression of E-cadherin markedly upregulated to 1.59(24hrs) and 1.6 (48hrs) fold by siRNA-CMDTMChiNPs treatments (1.59±0.02, $p<0.0001$ in 24hrs and 1.6±0.009, $p<0.0001$ in 48hrs). Also DOX-siRNA-CMDTMChiNPs treatments led to 1.74 (1.74±0.02, $p<0.0001$) and 2 fold changes (2±0.11, $p<0.0001$ for 24 and 48hrs, respectively) in a time dependent manner (figure 6).

SiRNA-DOX-CMDTMChiNPs powerfully induced apoptosis among other pharmacological NP formulations

The cells treated with different nanoformulated drugs showed various percentages of apoptosis. Compared with other groups, siRNA-DOX-CMDTMChiNPs

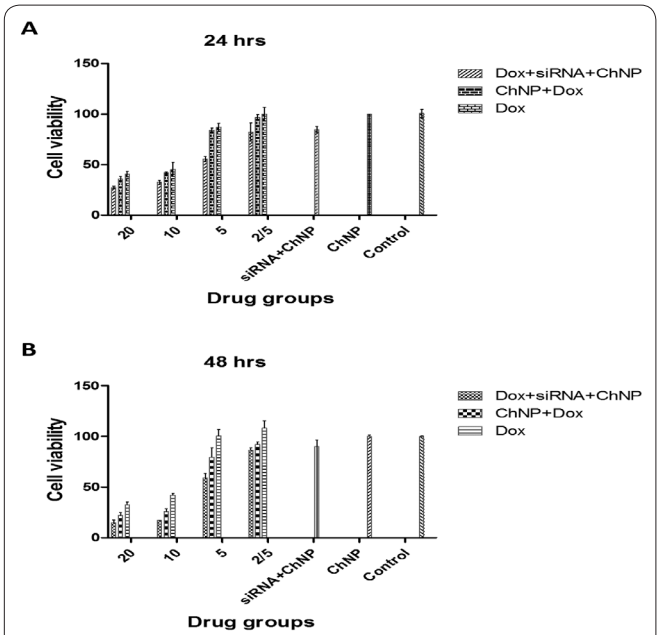

Figure 5. MTT assay of A549 treated cells with naked NPs, free DOX, siRNA-CMDTMChiNPs, DOX-CMDTMChiNPs and siRNA-DOX-CMDTMChiNPs in 24 hrs (A) and 48 hrs (B). DOX was tested in 2.5, 5, 10 and 20 µg/ml concentrations in different formulations. (IC50 =6.38 and 8.88 µg/ml for 48 and 24 hrs respectively).

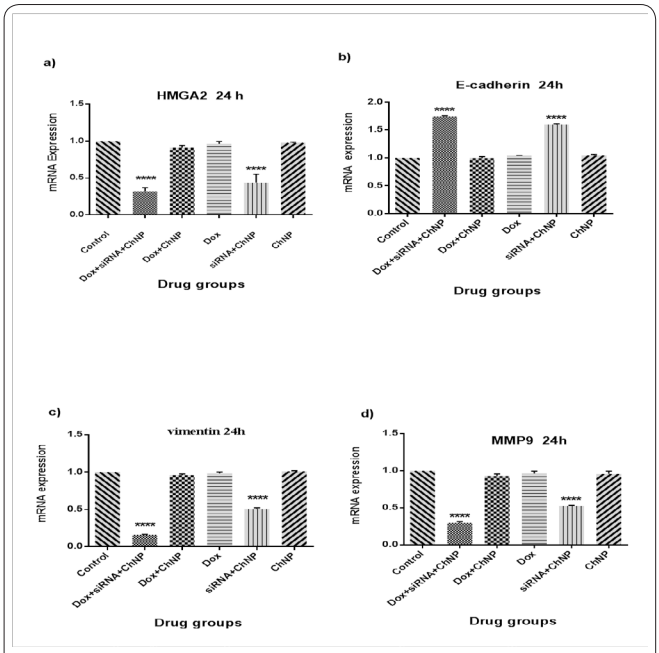

Figure 6. Relative mRNA expression of target genes after 24 hrs treatment with nakedNPs, free DOX, siRNA-CMDTMChiNPs, DOX-CMDTMChiNPs and siRNA-DOX-CMDTMChiNPs. HMGA2, vimentin and MMP9 expression values showed significant down regulation in siRNA-DOX-CMDTMChiNPs and Ecadherin in opposite upregulated in the same drug groups a) HMGA2 b) E-cadherin c) Vimentin d) MMP9.

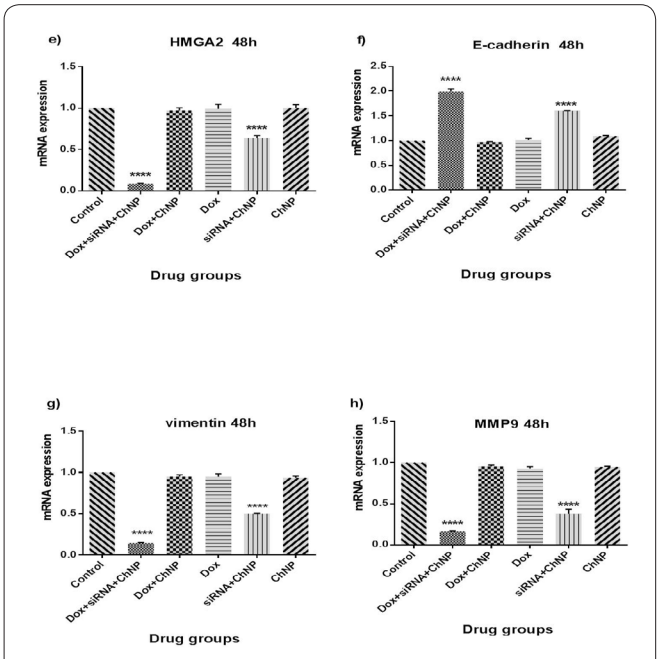

Figure 7. mRNA fold changes of target genes after 48 hrs of treatment with different defined pharmaceutical agents. The strongest group is siRNA/DOX CMDTMChiNPs which showed significant down regulation of EMT related gene expression. e) HMGA2 f) E-cadherin g) Vimentin h) MMP9.

showed great potential to inducing apoptosis in cancer cells (47.06±5% p<0.0001, figure 7B).

DOX, either encapsulation in NPs or in solution was effective in inducing apoptosis in lung cancer cells (22.86±5, p<0.0001 and 11.93±4.7-, respectively, figure7A-e, B). Furthermore DOX loaded NPs were more effective than siRNA-CMDTMChiNPs (11.37±4.6, p<0.0001, figure 7A-d). It is important to add that naked NPs approximately had no apoptotic effects compared to control group.

Migration inhibitory effects of siRNA-DOX-CMDTMChiNPs on A549 cells were outstanding.

The migration inhibitory effects of siRNA-DOX-CMDTMChiNPs were observed after 72hrs and compared with no treatment cells. Figur 8A, B showed that siRNA and DOX encapsulated NPs can efficiently suppress A549 cells migration assay in the artificial wound compared to untreated cells (figure 8C, D). The percentage of migration inhibition of dual agent NPs

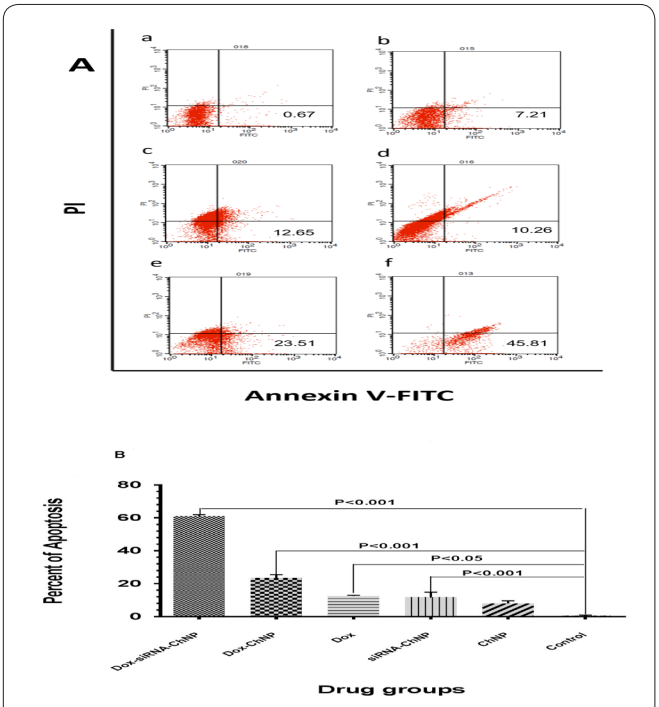

Figure 8. Apoptosis profiles of A549 cells after a 24 hrs based on Annexin V-FITC/PI staining. A) Representative dot plots demonstrating early and late apoptosis and necrosis: A-a) Control, A-b) Naked NPs, A-c) Free DOX, A-d) siRNA-CMDTMChiNPs, A-e) DOX-CMDTMChiNPs and A-f) siRNA-DOX-CMDTMChiNPs. B) Apoptosis percentage in different pharmacological groups. Results indicated significant increase in apoptosis percentage of siRNA-DOX-CMDTMChiNPs treated cells.

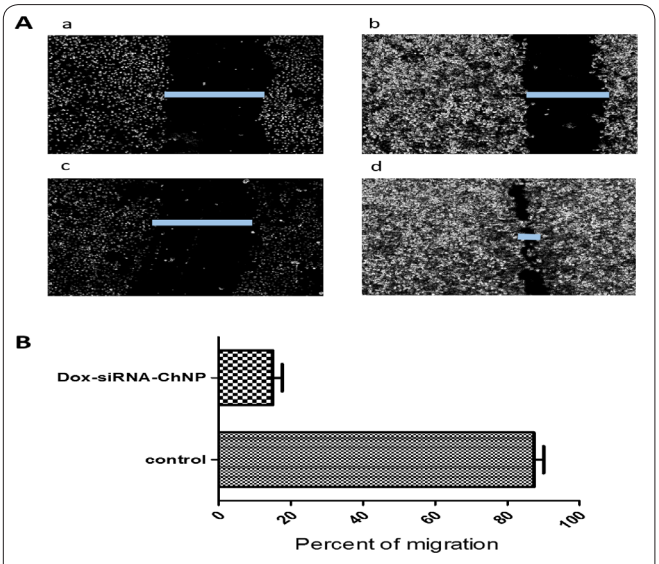

Figure 9. Migration inhibition effects of siRNA-DOX-CMDTMChiNPs on A549 cells after 72 hrs of treatment. A-a) siRNA-DOX-CMDTMChiNPs treated cells in time 0 A-b) siRNA-DOX-CMDTMChiNPs treated cells after 72 hrs A-c) Untreated cells in time 0 A-d) Untreated cells after 72hrs B) migration percentage in siRNA-DOX-CMDTMChiNPs compared to control group.

was 5 folds (87.33±2.72) greater than control group (15.00±2.64) (figure 9).

Discussion

Lung cancer is one of the most invasive and globally extended cancer types with poor therapeutic reactions to current cancer therapies. Although several mechanism might contribute to poor therapeutic reactions of lung cancer cells, however off-target side effects plays major role in failure of chemotherapy (17). In the present study, it was proved that CMDTM ChiNPs were the efficient carriers for dual delivery of HMGA2 antisense and DOX time/dose dependently for manipulation of EMT related gene expressions including HMGA2, Ecadherin,vimentin and MMP9. Besides these formulated NPs significantly inhibited migration and induced apoptosis in A549 cells.

As our first achievement, generated CMDTM ChiNPs have been completely suitable for drug delivery according to their physiochemical features. ChiNPs size and PDI were changed by loading of CMDTMC, HMGA2 siRNA and DOX in which the size and PDI of siRNA-DOX-CMDTM was the highest (207nm, 0.4, respectively). FTIR results confirmed quite assembly of CMD (1731cm^{-1}), DOX (1217,1339 cm^{-1}) and ChiNPs(1715,1312,1120cm^{-1}). Secondly the loaded NPs were completely stable against serum and heparin. Moreover DOX and siRNA invitro release profiles showed better results in acidic pH versus physiologic pH which was indicating that this formulation was adapted for tumor microenvironment. MTT assay by different defined pharmacological groups has been shown that DOX approximately did not have any cytotoxic effect on A549 cells addition of HMGA2 siRNA to DOX-CMDTMC ChiNPs were mostly cytotoxic as expected and the cytotoxic effects increased by 20µg/ml concentration of DOX. Most importantly naked NPs were not cytotoxic compared to control group.

According to our gene expression results, siRNA-CMDTMChiNPs could significantly influence mesenchymal markers (HMGA2/ vimentin/ MMP9) as well as E-cadherin as an epithelial marker involved in EMT process and tumor invasion relative gene expressions in A549 cells and this effect remarkably increased by presence of DOX in a time/dose dependent manner. In case of siRNA-CMDTMChiNPs after 48 hrs HMGA2 and MMP9 were mostly affected whereas vimentin down regulation mainly occurred in 24 hrs treatment. On the other hand vimentin down regulation followed by HMGA2 silencing showed no differences in 24 hrs and 48hrs therapies revealing that vimentin gene expression was not impressed by time intervals. Interestingly HMGA-2 silencing led to E-cadherin over expression by the same pharmacological groups. Recent studies have been evidenced that HMGA2 was over expressed at mRNA and protein levels in many malignancies. A group of studies suggested that HMGA2 expression in tumor cells is closely associated with carcinogenesis, progression and prognosis. Consistently, knock down of HMGA2 in colon cancer contributes to in vitro and in vivo inhibition of tumor proliferation, motility and invasion (18). Sun et al have been shown that in breast cancer, silencing of HMGA2 could reduce growth and metastasis of tumor cells (19). These results are comparable and in agreement with our results showing that delivery of HMGA-2 siRNA in combination with DOX or alone resulted in down regulation of MMP-9 and vimentin and up regulation of E-cadherin which ultimately led to inhibition of tumor metastasis and tumor growth. We also confirmed that addition of HMGA-2 siRNA had improved apoptotic effects of DOX-CMDTMChiNPs on A549 cells. Moreover DOX-CMDTMChiNPs compared to free DOX, DOX were more efficient in apoptosis induction demonstrating that DOX encapsulation in NPs significantly improved the apoptotic effect of DOX. Currently some findings admitted HMGA2 leading role in important molecular changes of EMT related signaling pathways. Zha etal (20) reported that HMGA2 activated EMT by effecting Wnt/β catenin. In other related studies by Thuault etal (21),Morishito etal (22) and Xia etal (23), HMGA2 involved in TGFβ/Smad pathway that also induced EMT. All of the mentioned pathways were under strong control of HMGA2 and dysregulation of these multipathway signaling networks may results in blocking EMT and cancer metastasis. By siRNA-DOX-CMDTM ChiNPs, HMGA2 ideally silenced and partially many EMT signaling pathways were inhibited.

HMGA2 could indirectly over expressed MMP9 which was a main leader of migration therefore the formulated NPs could perfectly reduce tumor cell migration (24). The antimigratory effects of siRNA-DOX-CMDTMChiNPs seem to be optimized after72hrs due to the time dependent nature of siRNA-DOX-CMDTM ChiNPs. The wound healing assay confirmed the changes in mRNA expression of target genes. HMGA2 and MMP9 counted to be the late modifiers of tumor migration which were mainly suppressed by extending time interval. In line with our findings Alinejad, V et al revealed that dual agent Np based treatment resulted in significant silencing of NF-κB and Bcl-2 relative gene expression, induction of apoptosis and inhibition of migration in MDA-MB361 cells (25).

According to our knowledge this was the first study that evaluated the anti cancer effects of novel designed CMDTMChiNPs and in relation with Souto,G.D. et al study formulation pharmaceutical ingredients in nanoparticles results in a delayed release of the drug to the cell, leading to a delayed cellular response (26). We recommended western blotting analysis for evaluation of protein levels of target genes. Of note, Tan et al reported that in MDA-MB-231 breast cancer cells HMGA2 knockdown could effectively up regulate mRNA of Ecadherin where as it failed to re express Ecadherin proteins (27). Cytokine profile of A549 cells would be better to assess after siRNA-DOX-CMDTM Chi NPs therapy due to its possible effects on signaling pathways. Investigation of siRNA-DOX-CMDTM-ChiNPs effects on other lung cancer cell lines, preclinical and clincal samples would be helpful in future for lung cancer therapy by CMDTMChiNPs. Due to immunologic properties of chitosan such as ability for macrophage and CTL activation(28) along with immunological adjuvant features of chitosan which has been confirmed for induction of a wide range of antigens from bacteria, viruses and tumors(29-31), it seems that naked CMDTMChiNPs may have immunostimulatory effects needs to be checked on immune cells. Taking

our findings into account siRNA as a representative of new anticancer agents and DOX as an indicator of prevalent chemotherapeutic drugs combat with different features of cancer immune resistance mechanisms and CMDTMChiNPs provided a suitable platform for co delivery of these reagents in order to reduce their side effects and enhanced their synergistic effects.

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