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Synthesis and Characterization of Nanodiamond-Doxorubicin (Dox) Conjugate for Effective Delivery against MCF-7 Cell Lines

Sweta Garg[1], Ashish Garg[1], Nitendra K. Sahu[1*], Awesh K Yadav[2]

[1]RKDF College of Pharmacy, SRK University, Bhopal, 462026 M.P. India

[2]Drug Delivery and Nanotechnology Laboratory, Bhagyodaya Tirth Pharmacy College, Sagar, 470002 M. P. India

ABSTRACT

In this work, we have introduced a carbon nanomaterial (nanodiamond), to bind with anticancer drug doxorubicin (DOX) with via amide bond conjugation for cancer drug delivery and therapy. Nanodiamond (ND) was initially carboxylated by the surface modification along the treatment with strong alkaline solution (H_2SO_4:HNO_3) and then activated the carboxyl moiety of ND with the addition of EDC. Anticancer drugs were bound to the ND through a succession of chemical modifications by adipic acid dihydrazide (ADH). The ND-Drug conjugate was analyzed by Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy, Fourier Transform Infrared (FTIR) Spectroscopy and Mass Spectroscopy (MS), Atomic Force Microscopy (AFM), Particle size, Zeta potential, Drug release, SRB assay against MCF-7 cells, and DNA fragmentation. Spectroscopic analysis confirms the conjugation of nanodiamond with different anticancer drug. AFM photomicrograph represents the surface morphological features of ND-DOX conjugates. *In- vitro* investigation showed that ND-DOX conjugates have slow and sustained drug release characteristics. *In-vitro* cytotoxicity studies, an enormous cytotoxic potential of ND-Drug conjugates were showed against cancer cell line. Above all findings were suggested that the ND-DOX conjugates may be a potential inhibitor of MCF-7 cancer cells to act as a drug candidate. According to all these data it can be confirm that the ND-DOX conjugates could be an effective agent for drug delivery and could be promising in future for tumor targeting strategy.

Keywords: Nanodiamond, Sustained Release, Drug Delivery, Cytotoxicity, Conjugates

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***Address for Correspondence:** Nitendra K. Sahu, RKDF College of Pharmacy, SRK University, Bhopal, 462026 M.P.

INTRODUCTION

As a major cause of mortality, cancer remains a global public health concern. To date, the most common treatment of cancer has been chemotherapy, the therapeutic effect of which is far from optimal due largely to the nonspecific toxicity of chemotherapeutics. That is why the idea of cancer nanotechnology is put forward, which provides a unique approach against cancer by applying nanotechnology in cancer management[1, 2]. Chemotherapy is a process for treating cancerous diseases; but due to improper selective recognition of cancerous and normal cells, there are various side effects are seen in patients during the chemotherapy. With the progress in nanotechnology, in the treatment of cancerous disease, drugs conjugated or adsorbed into nanocarriers presented various advantages related with free drugs, like controlled drug release, altered drug biodistribution, prolonged drug circulation in the blood, and anti-multidrug resistance; are now became keen topic for research [3, 4].

Nanodiamond or diamond nanoparticles are diamonds having particle size range less than one micrometre. Nano scale-size diamond fragments are propitious components for research due to their comparably narrow and small size allocation, flexible surface complex and chemical dormancy, altogether which constitute them as affirmative elements for various biological and electronic applications[5-8]. In current research work, we investigate the effectively of ND-DOX conjugates against breast cancer cell line, and also explore the drug retention in cancer cell line. The anticancer drug substance bind effectively with the surface modified nanodiamond via amide bond. The resultant complex, ND-DOX, was further characterized by different assay. Importantly, we show that ND-DOX conjugates enhance sensitivity of resistant of drug in breast cancer cells, possibly mediated via increased drug retention in the tumor cells. Additionally, it's also posses the prolonged and sustained drug release mechanism from ND-DOX conjugates. This work suggests the use of nanodiamond as a promising and effective drug delivery platform for anticancer drug against tumor cells.

MATERIALS

Nanodiamond powder (ND), Adipic acid dihydrazide (ADH) was procured from Sigma Aldrich (USA). Doxorubicin (DOX) was obtained as generous gift sample from Dabur India Limited, Ghaziabad, India. N-hydroxysuccinimide (NHS), dialysis membrane, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl), N, N' Dicyclohexyl Carbodiimide (DCC), Pluronic F-68 were purchased from Himedia laboratories, Mumbai, India. Acetone, ethanol, concentrated HNO_3, sulphuric acid, hydrochloric acid, isopropyl alcohol and acetonitrile purchased from Merck Limited, Mumbai, India. Other chemicals which are consumed are of investigative chemical grade and used as brought.

METHODOLOGY

Synthesis of carboxylated ND (ND-COOH)

The commercially available synthesized nanodiamond powder having particle size of <10 nm, were carboxylated by following the standard procedure[9]. Primarily the obtained nanodiamond powder was treated with the mixture of H_2SO_4 and HNO_3 (3:1 v/v) at room temperature for 48 h and then diluted with the addition of deionized water and centrifuged at 900 rpm for 30 min to separate out the ND particles. The obtained particles were rinsed by using deionized H_2O. Then obtained solution was again centrifuged at 900 rpm to separate out the ND particle, which was further heated at 90ºC for 2 h in 0.1 M NaOH solution. Then ND sample was heated again with 0.1 M HCl for 2 h at 90ºC, and washed by using deionized water to solution became weakly acidic. The obtained carboxylated-NDs were separated and dried under vacuum for further procedure [9].

Synthesis of activated nanodiamond

Firstly, 50 mg of ND powder was dispersed in 10 ml of distilled water (5 mg/ml) and then 250 mg of EDC-HCl was added with continuous stirring (Remi, Mumbai, India) for 12 h and maintain the pH 5.8 with the addition of 0.1N HCl. The EDC-HCl provides the effective activation of the carboxyl moiety of the NDs end group which enhances the attachment of NDs with another group of next chemical compound ($-NH_2$ moiety of the adipic acid dihydrazide). Subsequently addition of 125 mg of NHS into nanodiamond dispersion with constant rate of stirring and maintain the pH 5.8 with the addition of 0.1N HCl.

Syntheses of ND-DOX conjugate

To synthesize the different ND-DOX conjugate, the anticancer drugs (DOX) was conjugated with activated carboxylated NDs (ND-COOH). Primarily, the anticancer drug (DOX) was dissolved in aqueous system (distilled water) according to their solubility parameters. DOX (50 mg) was dispersed in 10 ml of aqueous medium, and then the prepared drug solution was activated with the addition of DCC and NHS. Surfactant (Pluronic F-68) medium of different concentration (0.25%, 0.5% and 1%) was formed by dissolving Pluronic-F-68 in acetone. 10 ml of drug dispersion (DOX) and ND-COOD (5 mg/ml) was added in drop wise manner in alternating sequence into separate Pluronic-F-68 (surfactant) medium with continuous stirring for 12 h to formulate drug conjugated nanodiamond i.e. ND-DOX conjugate. All the reaction mixtures were dialyzed to remove unreacted ND, DOX from all the formulated ND-DOX conjugate The obtained different ND-DOX was separated by membrane filter (0.45µm) and centrifuged at 15,000 rpm for 30 min (Remi, Mumbai, India). After centrifugation discarded the supernatant and lyophilized and preferred for subsequent analysis.

Characterization parameters of ND-DOX conjugate

The ND-DOX conjugate was prepared and authenticated by different spectroscopic instrumental methods i.e. nuclear magnetic resonance (^{1}H-NMR) (Bruker AvII-400), and Fourier transform infrared (FTIR) (8400S, Shimadzu) spectroscopic technique. FTIR is most common technique used to determine the different functional group present in the prepared nanodiamond conjugate.

Particle morphology, particle size characteristic and surface charge (zeta potential)

The size of ND-DOX conjugate and the surface charge characteristics of synthesized ND-DOX conjugate were done by HORIBA SZ-100 series, (Horiba Scientific, Kyoto, Japan). The surface characteristics of prepared ND-DOX conjugate were examined by Variable Pressure Field Emission Scanning Electron Microscope (Supra 55, VP FE-SEM, Carl Zeiss). The application was done at various pressure 2-133Pa with accelerating voltage 0.1 to 30kV. The surface morphology of formulated ND-DOX conjugate was also be analyzed with Atomic Force Microscopic method (AFM) (Alpha300RA AFM, WITec, Germany). For taking AFM photomicrograph of the ND-DOX dispersion was spread on glass substrate on AC mode.

Drug loading efficiency of ND-DOX conjugate

The loading proficiency of different synthesized ND-DOX conjugate by HPLC system. The anticancer bioactive (DOX) conjugated nanodiamond were separately dispersed in 10 ml of PBS (pH 7.4) medium then the conjugate dispersed medium was filled in centrifuge tube and then centrifuged at 15000 rpm in cooling centrifuge (C-24BL, Remi, Mumbai, India) for 5 min. Then after centrifugation the supernatant were taken out and analyzed by using high performance liquid chromatography (HPLC) (Shimadzu, Japan). The HPLC system consist reverse phase Cosmosil C_{18} column (4.6 mm × 250 mm, 5 µm, China) with UV detector was used to analyze the entrapment efficiency of prepared ND-DOX conjugate. The temperature of column should be maintained at 30 °C, for detection of UA content, methanol: phosphate buffer (pH 7.4) at (70:30 v/v) was taken as mobile phase with the flow rate 0.8 ml/min and detection of DOX was done at 485 nm. The amount of drug loaded in ND was calculated from unbound drug in the supernatant, the loading efficiency of ND-DOX conjugate was analysed.

In-vitro release profile

The *in-vitro* drug release characteristics of the prepared ND-DOX conjugate were performed by modified dissolution technique. The prepared conjugate were placed separately in dialysis bag (Himedia Lab Limited, Mumbai, India) and dipped into the separate container containing 10 ml of phosphate buffer saline (PBS) having different pH range (pH 7.4, 6.5 and 5.5) and the medium was stirred with the rate of 100 rpm at 37±0.5°C. At a definite time interval an adequate quantity of the medium was taken out and replaced with the same quantity of the fresh PBS medium to maintain the sink condition. The collected medium was centrifuged at 5000 rpm for 15 min, subsequently the supernatant was taken out from the centrifuge tube and analyzed by HPLC system as previously described to carry out the amount of percentage cumulative drug released from ND-DOX conjugate.

Hemolytic toxicity

For hemolytic activity, whole human blood was amassed and collected in a collection vial from authorized pathology centre as denoted in Bhadra et al., 2005[10]. Firstly human blood was centrifuged at 10,000 rpm for 15 min for complete separation of RBC and plasma. The plasma was discarded and RBC was taken for further procedure. The red blood corpuscle (RBCs) (1ml) was incubated separately with 10 ml of phosphate buffer solution pH 7.4 (taken as 100% hemolytic standard). In this hematocrit solution the ND-DOX conjugate and unmodified anticancer drugs (DOX), were added separately on hematocrit solution. The collection tube was allowed to stand for 1-2 h at 37°C, after that the ND-DOX conjugate in hematocrit mixture was centrifuged at 5000 rpm for 10 min, and then the absorbance was taken of supernatant at 540 nm to optimize the effect of ND-DOX conjugate and plain (unmodified) drugs against RBCs, which was useful to predict the percentage hemolysis.

SRB (sulforhodamine B) assay

Initially the cell line were grown in the RPMI 1640 medium containing fetal bovine serum (FBS 10%) and L-glutamine (2mM) further cell were inoculated on 96 well microtiter well plate containing 100µl plating densities. After it microtiter plates were incubated at temperature 37°C, 5% CO_2, 100% relative humidity and 95% air for 24 h before addition of formulations (i.e. ND-DOX conjugate, ND, plain anticancer drugs). Firstly conjugate were dissolved in dimethyl sulfoxide at 100 mg/ml further diluted to 1mg/ml using distilled and kept frozen before use. Aliquots for frozen concentrate diluted to 100µg/ml, 200µg/ml, 400µg/ml and 800µg/ml during the addition of different conjugate with total medium consisting test articles. Final concentration of 10µg/ml, 20µg/ml, 40µg/ml and 80µg/ml was prepared by adding 10µl of different dilutions in microtiter wells consisting 90µl of medium. Plates were incubated after compound was added at standard conditions for 48 h and assay was performed with cold trichloroacetic acid (TCA) and incubated at 4°C for 60 minutes further the supernatant was discarded and plates were cleaned. After than all the wells were treated with sulforhodamine B (SRB) solution (50µl) at 0.4 % (w/v) in 1 % acetic acid and incubated at room temperature for 30 minutes. The plates were dried and the absorbance was noted at 540 nm with 690 nm reference wavelength. Percent growth of cell was determined against control wells by plate-by-plate basis and further calculated as: [Ti/C] x 100 %. Finally the morphology of MCF-7 cell lines was determined by the light microscope[11].

DNA fragmentation analysis

Genomic DNA with low-molecular-weight was extracted as detailed previously (Yawata, 1998; http://www.abcam.com/protocols/apoptosis-dna-fragmentation-analysis-protocol) [12]. A breast cancer cell (MCF-7) was firstly treated with ND-DOX conjugate then cell was lysed in a buffer solution consisting 5mM EDTA (ethylene diamine tetra acetic acid), 5mM of pH 7.5 Tris HCl and 0.5% Triton X-100 intended for an about 30 min on ice. The prepared lysate media were vortexed by vortex shaker and then centrifuged at 30,000 for about 20mins. The fragmented DNA present in the supernatant was reacted with RNAse, after then treated with proteinase K digestion, and added on the mixture of phenol, chloroform and isoamyl alcohol in different volume concentration (25:24:1) extraction and isopropanol precipitation. The separation of DNA was carried out through agarose gel (1.5%), and then stained by using ethidium bromide solution (0.1µg/ml) and interpretation of fragmentation can be visualized under UV source.

Autophagy

MCF7 cells expressing LC3 EGFP were utilized for examining the autophagy inducing capability. The stable cells were grown on glass bottom plates and upon reaching 70% confluency, the cells were subjected to the decided concentration (20, 40 and 80 µg/ml) of ND-DOX conjugate for 48 hrs. The cells were imaged for LC3 punctae formation by using 40x objective of a fluorescent microscope Nikon Tie. The images were captured using EMCCD camera, iXON 889 using NIS element software. Ultimately, the green punctae signifies evidence for autophagy[13-15].

Cell cycle assay

MCF7 cells were developed on the 24-well plates with indicated ND-DOX conjugate for 48 hrs. After 48 hrs, adherent and non-adherent cells were gathered and settled with 70% cold ethanol at 4°C overnight. Later the cells were trypsinized with one microgram/ml of Hoechst for 20 minutes. The cells were rinsed and investigated by employing FACS. Then the cells were stimulated with 405m laser line and emission at 430/30 was collected for the DNA cell cycle study.

Statistical analysis

Final research outcome were showed as mean ± SD. All processes were performed thrice.

RESULTS AND DISCUSSION

The main objective behind the current study was to synthesize different ND-DOX conjugate for improved the targeting toward tumor cell as well as enhancing the potency of anticancer drug to provide maximum therapeutic effect. The surface of nanodiamond can be modified by many functional groups for providing higher stability of conjugate.

Spectroscopic analysis (^{1}H-NMR and FTIR)

The ^{1}H-NMR spectra of synthesized ND-DOX conjugate was illustrated in Figure 1. In ^{1}H-NMR spectrum of ND-DOX conjugate different distinctive peak was obtained. the proton assignment of ND was obtained at 1.02 ppm (2H, S) and 1.04 ppm (2H, S), shows presence of alkyl group, the peak at 2.17 (1H, S) shows hydroxyl moiety (R-OH). The proton assignment of DOX was obtained at ppm 1.2 ppm (3H, S) and 3.5 ppm (3H, S), 5.9 (1H, S) and 7.8 (1H, S). Beside of these proton assignment there are additional major peak was observed at 8.4 ppm (1H, S) and 8.9 ppm (1H, S), justify the presence of amide, which formed due to carbodiimide conjugation of carboxyl moiety of ND and amine group of DOX. All the proton assignment justifies the presence ND and DOX in the conjugate.

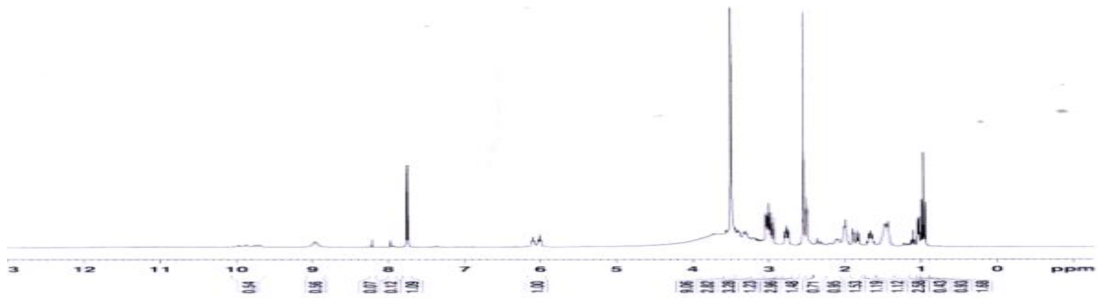

Fig. 1 ^{1}H-NMR spectra of ND-DOX conjugate

The FTIR spectra of ND-DOX conjugate were shown in Figure 2. The conjugate ND-DOX, displayed distinctive peak at 727 cm^{-1}, 857 cm^{-1}, 927 cm^{-1}, 2919 cm^{-1} shows C-H stretch, at 1285 cm^{-1} attribute to presence of C-O-C stretching, at 1588 cm^{-1} has a predominantly mixed C=C stretch and C=O stretch, at 1650 cm^{-1} displayed the presence of C=O stretch, a broad spectrum at 3250 cm^{-1} shows the presence of N-H stretch.

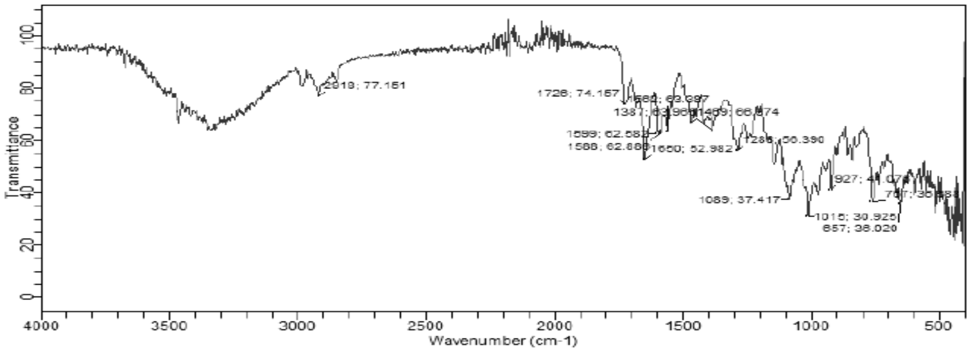

Fig. 2 FTIR spectra of ND-DOX conjugate

Surface characteristics

The surface characteristics like shape, size and texture of ND-DOX conjugate was examined by using atomic force microscopic technique (Alpha300RA AFM, WITec, Germany).

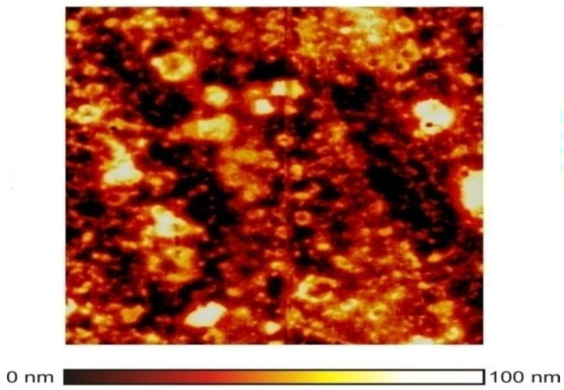

Fig. 3 AFM image of ND-DOX conjugate

The prepared ND-DOX conjugate having nanometric size range as monitored by AFM image Figure 3. The photomicrograph shows the uniform arrangement as well as similar height of the different ND-DOX conjugate. Summarizing, we have shown that the prepared ND-DOX conjugate displayed flake-like structure with preferred crystal orientation as well as its also clearly depicted that the particles are certainly not all the same. The photomicrograph from AFM, it was displayed that some particles show clearer sharp edges than others, according to these finding we assume, however, that sharp edges on that length scale would be quite reactive.

Zeta potential determination, particle size analysis and drug entrapment proficiency

According to the result depicted in Table 1, the particle size evaluation of ND-DOX conjugate was carried out using particle size analyzer and the particle size of ND-DOX conjugate were observed 59.2 nm. All the data obtained from particle size analysis, it was confirming that the ND-DOX conjugate having nanometric size range. Zeta potential of ND-DOX conjugate was obtained to be -14.7 mV. The negative value of the zeta potential analysis of ND conjugate may be due to the carboxyl moiety of the nanodiamond. In the charge particle having increased zeta potential cause formation of more stable particles due to higher repulsive interaction. It was observed that the lower negative zeta potential may increase the stability of the system[16]. The loading efficiency of unconjugated (unbound) anticancer drug measured by HPLC and found to 94.3 ± 1.25%.

Table 1 Ingredients and concentration using in the formulation of ND-DOX conjugate

Formulation	Nanodiamond Concentration (mg)	Distilled water (ml)	DOX	Acetone/ Ethanol (ml)	Particle size (nm)	Zeta potential	% Drug loading Efficiency
ND-DOX Conjugate	50	10	50	10	59.2nm	-14.7mV	94.3±1.25

In-vitro drug release pattern

The sustained release nature of drug from ND-DOX conjugate system illustrated in Figure 4. It is recognized that well-organized release of drug from a drug delivery system is essential for therapeutic action of most of the anticancer drug conjugated formulations. Assimilation of acidic environment between the carrier and drug capable the liberation of an anticancer drug from the carrier (ND) into the cancer cell (having slightly acidic environment (pH 6.5)) then after endocytosis phase in the endosomes consisting pH range 5-6 and lysosomes (pH 4-5) of cancer cells. For this circumstances, the percentage release of drug from ND-DOX conjugate was performed at 37°C under simulated physiological conditions (phosphate-buffered saline, pH 7.4) and an acidic environment (phosphate-buffered saline, pH 5.5, an acidic endosome environment and pH 6.5, a simulated tumor environment) to analyse the feasibility of ND-DOX conjugate as an drug delivery system for anticancer drug. As depicted in Figure 4 (a), the amount and rate of drug released from ND-DOX conjugate were dependent on the pH of the medium. ND-DOX conjugate displayed a rapid release pattern of anticancer drugs at pH 5.5 then pH 6.5 and then at pH 7.4. The release rate of drug from different ND-DOX conjugate after 96 hrs was found to be 35.5% at pH 5.5, whereas 24.6% was found at pH 6.5 and 16.2% of drug release was obtained at pH 7.4 correspondingly. Drug conjugated with ND via amide bond which is relatively more stable than physical absorption, thus slowing down the release of anticancer drug from ND conjugate [17, 18]. This is due to because of surface modification of ND via carbodiimide conjugation between of the carboxyl group of nanodiamond with the amine group of adipic acid dihydrazide and formation of crosslinked core-shell micelle, which having less solubility, may promote sustained release[19]. The data also has been suggested by various researchers, that acidic pH condition triggered the cleavage of amide bond[20-23]. The slow rate of anticancer drug (DOX) release from ND-DOX conjugate was calculated at pH 7.4, which mimics the physiological environment of the bloodstream and establishes that reduced amount of anticancer drugs, is liberated from conjugate in the blood circulation. It was assume that ND-DOX conjugate would adhere preferentially in the site of tumor cell via enhanced permeability and retention (EPR) effect. Figure 4 (b), also represents the release of unmodified anticancer drug (DOX), and the plateaus by approximately 30mins at pH 7.4, 6.5 and 5.5 respectively, with a highest release of 98.5% at pH 7.4, the release 96.7% occurs at pH 6.5, and 94.8% at pH 5.5 correspondingly. In contrast with ND-DOX conjugate, the free or unmodified anticancer drugs cause damage cells in a normal physiological environment, causing serious side effects.

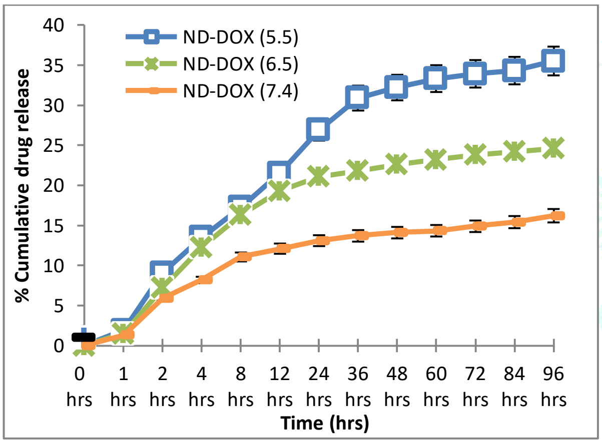

Fig. 4 (a) Drug release profile of ND-DOX conjugate

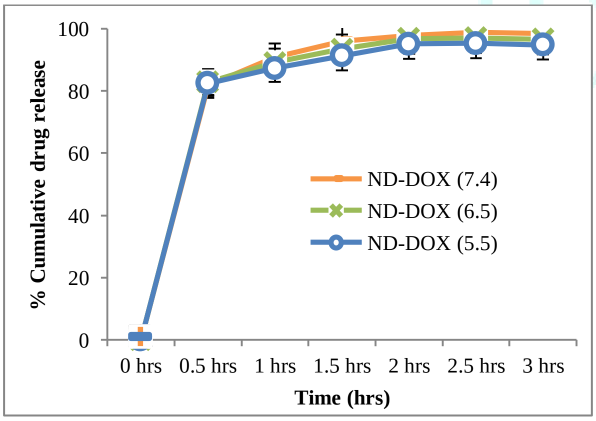

Fig. 4 (b) Drug release profile of Unmodified (DOX) at different pH conditions

Hemolytic toxicity study

The hemotoxic effect of the prepared ND-DOX conjugate was estimated by hemolytic toxicity study. The ND-DOX conjugate exhibited hemolytic toxicity upto, 5.18±0.25%. Whereas the plain drug (DOX) represents hemolytic toxicity 55.12±0.5%. There was decline in hemolytic toxicity by different ND-DOX conjugate in compare to plain drug, caused due to delayed release of encapsulated drug molecules in the nanodiamond conjugate. The repression of hemotoxicity of drug can be linked among other similar studies described previously [24].

SRB (sulforhodamine B) assay

The *in-vitro* cytotoxicity screening of ND-DOX conjugate in human breast cancer cell line (MCF-7) was established by SRB assay. The result obtained by the assay affirm dose dependent assessment of cytotoxicity in which the cellular bioavailability decreased with increasing the concentration of sample (ND-DOX conjugate and anticancer drugs DOX). The result of percentage growth inhibition of cell illustrated in Figure 5. Which revealed that higher concentration of sample ND-DOX conjugate inhibit the cell growth. Furthermore, the cell viability also gets declined with increase in the concentration of sample. ND-DOX conjugate formulations were experiential to be cytotoxic to a greater amount with the concentration between 10-80μg/ml. The Figure also states the cytotoxic effect of ND and unmodified anticancer drugs (DOX). The finding concluded that cytotoxic effect of optimized ND-DOX conjugate discovered to have greater inhibitory effect.

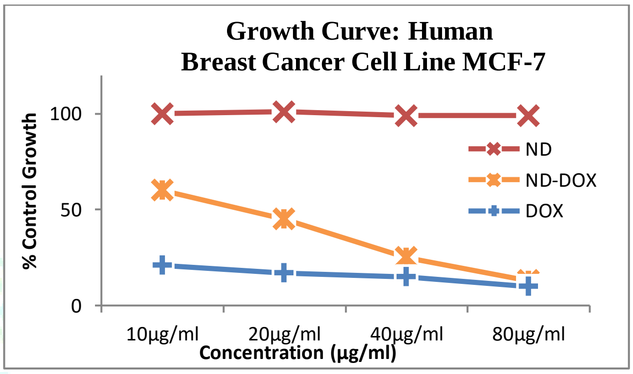

Fig. 5 Cell-line study of ND-DOX against MCF-7 cells

DNA fragmentation analysis

To assess the apoptosis mechanism of tumor cell by ND-DOX conjugate, the DNA fragmentation analysis was performed. The cell was treated with ND-DOX conjugate for about 48 hrs and then isolates the DNA and analyzed by agarose gel electrophoresis. After 48 h incubation, the cell treated with drug showed high number of DNA ladder formation. In the result obtained from agarose gel electrophoresis of MCF-7 cells, internucleosomal fragmentation was observed in MCF-7 cells after 48 h of with ND-DOX conjugate treatment Figure 6. These finding suggested that ND-DOX conjugate is an effective inducing agent of apoptosis against MCF-7 cell lines.

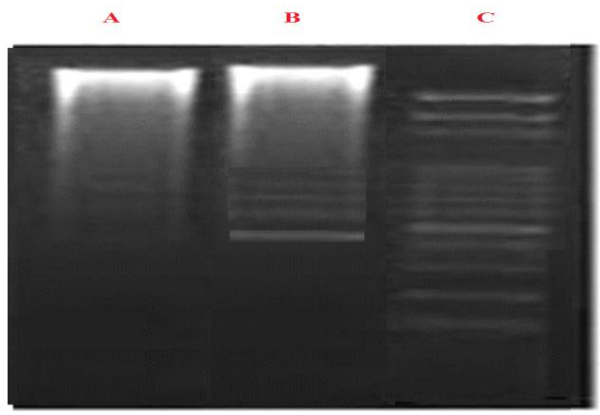

Fig. 6 Agarose Gel Electrophoresis of ND-DOX conjugate nanocomplex (DNA Fragmentation study). A: Marker, B: Control, C: ND-DOX (100μM)

Autophagy analysis

To investigate the dynamic behavior of autophagosomes noted the dynamic behaviors of LC3-positive vesicles present in the cell bodies and processes after transfection of EGFP-LC3, which is a standard procedure for autophagosome recognition. ND-DOX conjugate strongly induced autophagy in the cells as observed by the increase in a green dot like punctae. All the concentration displayed indication for autophagy Figure 7.

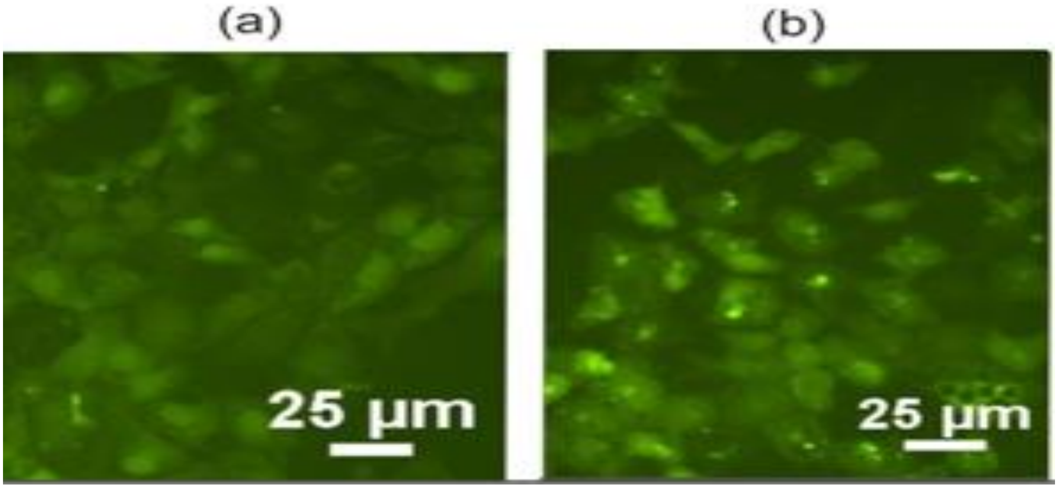

Fig. 7 Autophagy Assay (a) Control; (b) cell treated with ND-DOX

Cell cycle assay

The antitumor mechanisms of prepared ND-DOX conjugates have consorted with mitosis, apoptosis and cell cycle arrest in the G2/M phase[25]. Consequently, the enhancement in G2/M phase arrest, signify the improved beneficial and therapeutic effect of conjugate. The effect of the arrest of G2/M phase (cell cycle) was investigated by the various range of concentration of conjugates against MCF-7 cells (i.e. 20μg/ml, 40μg/ml and 80μg/ml). These conclusions suggested that ND-DOX conjugate at different concentration could encourage cell cycle arrest in MCF-7 cells.

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