

Synthesis and Evaluation of the Cytotoxic and Anti-Proliferative Properties of Dox-ZnO Quantum Dots Loaded Chitosan Nanoparticles against MCF-7 and SKBR-3 Human Breast Cancer Cells

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Abstract— Doxorubicin (Dox) is a potent chemotherapeutic agent used in the treatment of cancer. In the present study, pH responsive chitosan polymer coated Dox nanoparticle (Composite) was developed to investigate targeted drug delivery against breast cancer. The anticancer drug DOX-ZnO QDs was loaded to the chitosan nanoparticles. The synthesized free and drug loaded nanoparticle were analyzed using Fourier transmission electron microscopy (FTIR) and UV-Visible spectroscopy (UV-Vis). The particle size was measured using Transmission Electron Microscopy (TEM). Further, the composite was evaluated for its anticancer effects. Drug release analysis showed significantly larger amount of drug released in acidic pH of 5.0 compared to pH 7.4. The composite was significantly more cytotoxic to the breast cancer cells MCF-7 and SKBR-3. The composite was however, less toxic to HEK-293 human embryonic kidney cells confirming minimum side effects on normal cells and cytotoxic to tumor cells. DAPI staining showed nuclear degradation in composite treated breast cancer cells. The cellular uptake of the composite was analyzed by confocal microscopy. The composite induced a G0/G1 phase arrest in breast cancer cells and the number of colonies formed by the composite treated breast cancer cells formed less number of colonies compared to free NP. Our results showed that our composite could serve as a promising therapeutic approach to improve clinical outcomes against various malignancies.

Keywords— Dox-ZnO QDs, iron-oxide, chitosan, Nanoparticle, Apoptosis, Breast cancer.

I. INTRODUCTION

Chemotherapy is the most preferred treatment for various types of cancers; however, the low therapeutic efficacy and high toxicity are major drawbacks [1]. Drug resistance exhibited by the tumor cells is also major challenge for effective treatment of cancer [2]. Nowadays, there are many anticancer drugs applied for cancer treatment such as Doxorubicin, a well-known anticancer drug for treatment of several types of cancers including breast cancer. Doxorubicin, a potent anticancer drug interacts with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of topoisomerase II, an enzyme which relaxes supercoils in DNA for transcription [3]. Doxorubicin is reported to induce apoptosis of the cancer cells. It controls the cancer cell proliferation [4]. Dox is reported to induce cell cycle arrest at G0/G1 phase [5,6]. Despite such significant anticancer activity, the major drawbacks are the side effects, cellular uptake and drug resistance of cancer cells [7,8].

Nanotechnology based approach is growing extensively in recent years in every research areas such chemistry, physics, medicine, mechanical, optical, chemical, and electronics respectively [9–11]. In clinical approach, extensive research is carried out on nano biotechnology based applications predominantly diagnostic and therapeutic applications [12,13]. In cancer research, nanocarriers offers huge opportunity for anticancer drug delivery. Nanocarriers are developed through various organic and inorganic materials especially those that are bio degradable and nontoxic to

normal cells [14,15]. Iron oxide, an organic nanoparticle offers multiple applications in various fields including biomedical applications [16]. For clinical aspect, iron oxide nanoparticle is applied for different approaches such as imaging, targeted drug delivery and for driving the drug to target tissues [17]. This is due to high magnetic property and particle size distribution that favors iron oxide nanoparticle for targeted cancer drug delivery and hyperthermia application [18]. Also, iron oxide nanoparticles are biocompatible and its 1000 times smaller than human cells so it can easily penetrate into the cells, enzymes, receptor and proteins [19]. Current approaches focus on applying iron oxide nanoparticle for magnetically driven drug delivery particularly for cancer treatment [20]. However, the low stability and aggregating nature of magnetic iron oxide nanoparticles owes to the practical difficulties to use this as a carrier. To surmount this, surface modification by biocompatible materials are widely accepted method [21]. Chitosan is a natural, bio-degradable, non-toxic polymer derived from deacetylation of chitin. Chitosan has several reports for its clinical applications such as burns, wounds, nanoparticle based treatments and tissue regeneration [22].

ZnO quantum dots also finds significance for its applications in many areas for its attractive fluorescence properties [23]. ZnO quantum dots have superior fluorescence properties than the other semiconductors quantum dots. Basically semiconductor ZnO nanoparticle is soluble in water phase so in targeted drug delivery cancer treatment it is applicable for imaging and diagnostic properties [24]. In conventional drug delivery system have many issues such as the drug leakage, instability of the carrier etc. [25]. Hence, the modern day cancer therapy uses stimuli responsive drug delivery system such as pH, thermal, magnetic, optical, redox, etc. Among these, pH responsive delivery system is highly suitable for targeted drug delivery. Due to the acidic pH exhibited by cancer cells, change in pH benefits drug to be released at these sites [26].

In this study, pH responsive polymer encapsulated Doxorubicin-ZnO QDs was synthesized. The composite characterization was performed. The composite was used for analyzing its cytotoxicity in MCF-7 and SKBR-3 breast cancer cells and also HEK-3 human embryonic kidney cells. The effects of the composite on cancer cell proliferation was also evaluated.

II. MATERIALS

Chemicals and reagents

Chitosan, Doxorubicin hydrochloride and $\text{ZnAc}_2 \cdot 2\text{H}_2\text{O}$ were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Iron (2) chloride tetrahydride, iron (3) chloride hexahydrate and ammonium hydroxide were purchased from Merck & Co., Inc. (West Point, PA, USA). DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride), Fluoromount™ Aqueous Mounting Medium, Crystal violet solution, Propidium iodide, DMSO, MTT (Thiazolyl Blue Tetrazolium Blue) were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lines

HEK-293 human embryonic kidney cell line, human breast cancer cell lines MCF-7 and SKBR-3 were purchased from Korea Cell Line bank (South Korea). All the cells were grown in RPMI-1640 media supplemented with 10 % Fetal bovine serum (FBS) and 1% Penicillin/streptomycin. Cells were maintained at 37° C with 5% CO₂ in a humidified incubator. The cells were washed with 1x Dulbecco's PBS and trypsinized with Trypsin-EDTA solution (1x). All the reagents used for cell culture were purchased from WELGENE (South Korea).

III. METHODS

Preparation of chitosan encapsulated iron oxide nanoparticle:

Chitosan encapsulated Iron oxide nanoparticles were synthesized through co-precipitation method as per previous reports [19,27]. Accordingly, Iron (II) chloride tetra hydride Fe(II) 1.99 g and iron (III) chloride hexahydrate Fe (III) 5.41 g precursors were dissolved in 100ml of water. This mix was transferred to five necked glass balloons provided with heating mantle. The solutions were mechanically stirred continuously. Additionally, nitrogen gas was continuously supplied to the five necked balloon to prevent oxidation. To the solution, 30ml Tripolyphosphate was added and NH₄OH was added dropwise in order to achieve uniform and small sized nanoparticles. Finally, the chitosan coated colloidal solution was washed with deionized water.

Preparation of ZnO Quantum dot

ZnO quantum dots was prepared from $\text{ZnAc}_2 \cdot 2\text{H}_2\text{O}$. 10mmol of $\text{ZnAc}_2 \cdot 2\text{H}_2\text{O}$ was mixed to anhydrous ethanol and the solution was refluxed at 70°C for about 4h. On the other hand, 3mmol LiOH.2H₂O precursor solution was dissolved in 10ml anhydrous ethanol. This precursor

solution was actively added into the $\text{ZnZc2.2H}_2\text{O}$ precursor solution to achieve the ZnO quantum dots. To this, 20ml N-hexane was added to ZnO quantum dots solution and centrifuged for 10-15min. The precipitated particle was dissolved using anhydrous ethanol and clearing top solution was separated and kept for drying to obtain ZnO quantum dots powder. This ZnO quantum dots was dispersed in N-methylpyrrolidone and sonicated to get uniform suspension[28].

Preparation of DOX-ZnO QDs loaded on chitosan

NMP dispersed ZnO QDs was prepared by adding 20 μl of 3-Aminopropyltriethoxysilane and the mixture were stirred at 100°C for 20 min. The amine modified ZnO QDs was collected and washed with NMP. 1 mg of folic acid was dissolved in dimethyl sulfoxide (DMSO), to this 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride was added and stirred for 30 min, after that the amine functionalized ZnO QDs were added and stirred. DOX was mixed into the amine functionalized ZnO QDS and the reaction was continued for some time by continues stirring. Finally, ZnO-DOX mixture was transferred in to the chitosan coated colloid solution and continuously stirred for 12 hours(Fig.1).

Characterization of the composite

The morphology, physical and chemical characteristics of the prepared composite was analyzed through different characterization methods such as Transmission electron microscopy(TEM), Fourier transmission infrared microscopy(FTIR), Ultraviolet -visible spectroscopy (UV-Vis) and Photoluminescence spectroscopy (PL).

Time based drug -release study:

The Dox drug release from the carrier nanoparticle was investigated in time dependent manner in different buffers at pH 7.4 and pH 5.0 for 12 hours at 37° C. Accordingly, 1 ml of drug loaded carrier solution was transferred to the dialysis membrane tube (3.5 KDa). The released drug in the different pH buffers was analyzed at different time intervals by using the UV-visible spectrometer at 480 nm.

In vitro cytotoxicity assay

Cytotoxicity was evaluated on HEK-293 human embryonic cells, MCF-7 and SKBR-3 breast cancer cells. Cells were seeded on a 96-well plate in RPMI-1640 medium with 10% FBS and 1% P/S at cell density of 1×10^4 cells per well and incubated overnight. Cells were treated with Doxorubicin-

loaded chitosan nanoparticles containing Dox concentrations at 1 μM , 2 μM , 3 μM were mixed with RPMI-1640 media. After 48h incubation, cells morphology was taken images using Phase contrast microscope. Following this, 20 μl of MTT reagent was added to each well. After 4 h of incubation in 37° C, the medium was removed and 100 μl of DMSO was added to each well. The OD was measured at 570nm using Microplate reader.

DAPI staining

MCF-7 and SKBR-3 cells were seeded on a coverslip in a 35mm dish and allowed to adhere for 24h. Cells were then treated with empty and doxorubicin-loaded chitosan nanoparticles for 48h at 37° C. The cells were fixed with 100% methanol for 20min. Cells were washed three times with 1x DPBS and stained with DAPI for 20sec. The cells were then washed with 1x DPBS and mounted. Nuclear morphology was taken images using confocal laser scanning microscope.

Cellular uptake

Cellular uptake of doxorubicin loaded chitosan nanoparticles was assessed. Briefly, MCF-7 and SKBR-3 cells were seeded on a coverslip in a 35mm dish and allowed to adhere for 24h. Cells were treated with Empty and doxorubicin-loaded chitosan nanoparticles for 48h at 37° C. The cells were fixed with 100% methanol for 20min. Following this, cells were washed three times with 1xDPBS and stained with DAPI for 20sec. The cells were further washed thrice with 1xDPBS and mounted. The cellular uptake of Doxorubicin was observed using confocal laser scanning microscope.

Colony formation assay

Breast cancer cells MCF-7 and SKBR-3 were harvested from log phase of growth and plated at densities of 500 cells in a 60mm dish. Next, 24h after plating, the medium was replaced with fresh medium with empty or doxorubicin-loaded iron oxide nanoparticle. After two weeks, the cells were washed in 1x DPBS and fixed with 100% methanol for 15min. The fixed cells were further washed twice with 1x DPBS and stained with 0.5% crystal violet for 15min at room temperature. The cells were washed until the stain was removed completely. The plated showing the number of colonies in the empty nanoparticle and doxorubicin-loaded iron oxide nanoparticle treated cells were photographed with a digital camera.

Cell cycle analysis

MCF-7 and SKBR-3 cells were seeded into 6-well plates and treated with empty and doxorubicin-loaded iron oxide nanoparticles. The cells were incubated for 48h at 37° C. Cells were trypsinized, centrifuged at 3000rpm for 3min. Cells were washed twice with ice cold 1x DPBS and fixed with 70% cold ethanol for 1h. Following this, cells were washed twice with ice cold 1x DPBS and stained with 10 μ l Propidium iodide (1mg/mL) for 30 min at 37° C in dark. The amount of cells in each cell cycle phase was analyzed using flow cytometry BD LSRFortessa Instrument.

Statistical analysis

All the experiments were performed in replicates of three. Results are presented as means \pm standard deviations (SDs). Statistical significance was evaluated using the Student's t-test and P values of < 0.05 were considered significant.

IV. RESULTS AND DISCUSSION**Morphology analysis**

The size and shape of iron oxide nanoparticle was analyzed using TEM. Results showed the total size of the particle approximately 30 nm and the shape of the particle expressed irregularly arranged iron oxide nanoparticle (Fig. 2a). Approximately 90% of the iron oxide nanoparticle revealed needle like nano rods similar to the previous reports [29]. The morphology and size of the ZnO QDs appeared sphere like quantum dots structure and size of the QDs is approximately 8-10nm (Fig. 2b).

Analysis of chemical composition of composite using FTIR

FTIR analysis was done for chitosan, iron oxide + chitosan, free Dox and Dox loaded with Iron oxide and Zinc oxide nanoparticles. Simple Iron oxide and ZnO showed band at 560 cm⁻¹ and 463 cm⁻¹ indicating the Fe-O and Zn-O stretching. Bands at 3369 cm⁻¹ and 1630 cm⁻¹ and the absence of band at 560 cm⁻¹ in INP-Chitosan composite confirms the encapsulation of iron oxide by chitosan. The observed FTIR band for free DOX assigned as follow, 3454 & 2920 cm⁻¹ and band at 1410 cm⁻¹ is due to the presence of C-C, and at 1046 cm⁻¹ is due to C-O. In Dox loaded composite nanoparticles, FTIR spectrum peak is shifted to 3386 cm⁻¹ due to overlap of N-H and O-H peaks and showed broader peaks at 2901 cm⁻¹ (C-H), 1408 cm⁻¹ (C-C) and 1020 cm⁻¹ (C-O). These peaks confirmed successful loading of Dox in the composite. Iron showed absorption peak at

560 cm⁻¹ and ZnO quantum dots at 461 cm⁻¹ in FTIR spectrum (Fig. 3).

UV-Visible spectrometry analysis of the composite

The UV-Visible analysis of the free Dox and Dox loaded composite nanoparticle was analyzed by the UV-Visible spectrometer. Free Dox exhibited the two major peaks at 234nm and 487 nm. However, the composite showed peak shift at 256 nm and 570nm. Also the composite peak exhibited a slight hump peak at 358nm exhibiting the presence of ZnO (Fig. 4). These results confirmed that Dox was successfully loaded in the composite. These results further confirmed the FTIR results.

Photoluminescence

Photoluminescence spectrum of the ZnO quantum dot and composite nanoparticle showed multiple emission peaks. ZnO quantum emission attribute at 376nm, 408 and 558nm. The peak 376nm denoted ZnO QDs. The peak at 558nm denoted the oxygen vacancy level. Composite nanoparticle also showed three major peaks at 366, 422 and 580nm respectively. The peak at 366nm exhibit ZnO QDs peak shift from 376nm to 366nm in the composite. Chitosan peak was seen at 422nm and finally DOX emission peak was seen at 580nm (Fig. 5).

Drug release from carrier nanoparticle

The DOX drug released from the carrier nanoparticle in different pH levels at 37°C in different pH buffered solution at pH 5.0 and pH 7.4 is shown in (Fig. 6). Maintaining the temperature at 37°C mimics the normal physiological temperature. Poor drug release was observed at pH 7.4 at 12 hours but at pH 5.0 the drug released was nearly 80%. The release study suggested the carrier was completely acidic pH responsive thus releasing the drug at acidic pH.

The cytotoxicity of Doxorubicin- loaded iron oxide nanoparticles

MTT assay was performed to determine the cytotoxicity of Doxorubicin-loaded iron oxide nanoparticles. Dox concentrations ranging from 1 μ M to 3 μ M was treated to HEK-239 embryonic kidney cells and MCF-7 and SKBR-3 breast cancer cells. The percentage of viable cells after 48h of treatment was calculated (Fig. 7 a, b). Doxorubicin when introduced as nanoparticle composites to the cells, is shown to confer less toxicity to the normal cells. There are previous reports that Doxorubicin-loaded colloidal nanoassemblies confer less cytotoxicity compared to direct

treatment with Doxorubicin in NIH3T3 mouse fibroblast cells [30]. Corresponding to this, our results from MTT assay showed Doxorubicin-loaded iron oxide nanoparticles induced cytotoxicity to the MCF-7 and SKBR-3 breast cancer cells at specific concentrations. However, HEK-293 human embryonic kidney cells were not affected at the same concentrations. Thus the composite can be effective in inducing cytotoxicity to the cancer cells while leaving the normal cells unharmed. The results confirmed a significant cytotoxicity of the composite containing Dox concentration at 3 μ M in MCF-7 and SKBR-3 breast cancer cells. On the other hand, this concentration did not show any cytotoxicity to HEK-293 cells.

Cellular uptake analysis using CSLM

The cellular uptake of Dox was assessed by CSLM. The nucleus is stained with DAPI and cellular uptake of Dox was seen as red fluorescence inside cells. The intensity of the red fluorescence was also seen to be increased in the cells whose nuclear morphology is changed indicating apoptosis in these cells induced by Dox (Fig. 8). The most important criteria for developing different drug-loaded nanoparticle composites is increased cellular uptake by the cancer cells [31]. The mechanism through which chitosan encapsulated nanoparticles enter the cancer cells are discussed extensively. The most common being endocytosis. [32] The particles are endocytosed into human cancer cells, drug is then released and increases cytotoxicity than free drug. Images from the CSLM of the composite treated MCF-7 and SKBR-3 breast cancer cells showed immense cellular uptake of the composite 48h after treatment.

Doxorubicin-loaded iron oxide nanoparticles induce nuclear breakage

Condensed and fragmented nuclei are characteristics of apoptosis. MCF-7 and SKBR-3 breast cancer cells treated with Doxorubicin-ZnO QDs loaded chitosan nanoparticles for 48h were subjected to DAPI staining. The nuclear morphology of the empty nanoparticle treated and Doxorubicin NP treated cells was evaluated using Confocal microscope. The morphology of the cells treated with empty nanoparticle remained intact and regular. On the other hand, nuclear condensation and abnormal nucleus are seen in Doxorubicin-loaded chitosan nanoparticles indicating cell apoptosis (Fig. 9). Similar to these results, changes in nuclear morphology of cancer cells upon Dox treatment is previously reported. MCF-7 and SKBR-3 breast cancer cells treated with the composite showed similar nuclear

degradation as observed in confocal images upon DAPI staining. There are several reports on different molecular mechanisms underlying this [33].

Doxorubicin-loaded chitosan nanoparticles control the cell proliferation of breast cancer cells

One important feature of Doxorubicin is the regulation of cancer cell proliferation. To confirm the regulation of breast cancer cell proliferation by the composite, colony formation assay was performed. MCF-7 and SKBR-3 breast cancer cells were treated with empty and composite for 48 h and the number of colonies formed was evaluated after two weeks. MCF-7 and SKBR-3 cells treated with empty NP formed many colonies. On the other hand, MCF-7 and SKBR-3 cells treated with composite formed significantly less number of colonies than empty NP treated cells. These results indicate that composite controls the breast cancer cell proliferation (Fig. 10 a). Dox treatment is reported to control the cell proliferation of many types of cancer cells such as colon, lung, liver, breast, etc.[34][4]. Here, we have shown that our composite could appreciably reduce the cell proliferation.

Doxorubicin-loaded chitosan nanoparticles induce G1 phase arrest in breast cancer cells

Besides apoptosis, our composite also induced cell cycle arrest in breast cancer cells. MCF-7 and SKBR-3 breast cancer cells were treated with empty NP and composite. 48h after treatment, cells were subjected to Propidium iodide (PI) staining and flow cytometry analysis was used to determine the cell cycle distribution. MCF-7 and SKBR-3 breast cancer cells treated with composite showed a significant reduction of cells in G0/G1 phase compared to the empty NP treated cells (Fig.10 b).

V. CONCLUSION

In summary, DOX-ZnO QDs loaded with the chitosan nanoparticles were successfully developed and evaluated for their anticancer effects. The composite was nanosized at around 30nm and with uniform needle like nano rods morphology. The drug was loaded to the shell effectively. Drug release analysis showed significantly larger amount of drug released in acidic pH of 5.0 compared to pH 7.4. The composite induced significant cytotoxicity only to MCF-7 and SKBR-3 but very less toxic to HEK-293 cells at particular concentrations emphasizing cytotoxic effect against breast cancer cells specifically. The cellular uptake of the composite in the breast cancer cells were quite

significant. The composite also induced nuclear damage upon treatment. The proliferation of the breast cancer cells drastically decreased compared to the control. Further, the composite induced cell cycle arrest at the G1 phase in these cells. Based on our results, we believe that our composite can effectively kill the breast cancer cells *in vitro* and needs for more *in vivo* studies in future to translate this approach into the clinic (Fig.11). Iron oxide in the composite facilitates driving the composite to cancer cells specifically using magnetic field and ZnO quantum dots can act as a dye for imaging purpose in *in vivo* models. Additionally, there are several reports on the anticancer properties of ZnO therefore this Dox-ZnO QDs formulation will have higher anticancer effect compared to other Dox formulations. Hence, as a next step, we intend to continue the effective targeted therapy of the composite against breast cancer in xenograft models.

CONFLICTS OF INTEREST

The authors report no conflicts of interest about this work.

ACKNOWLEDGMENT

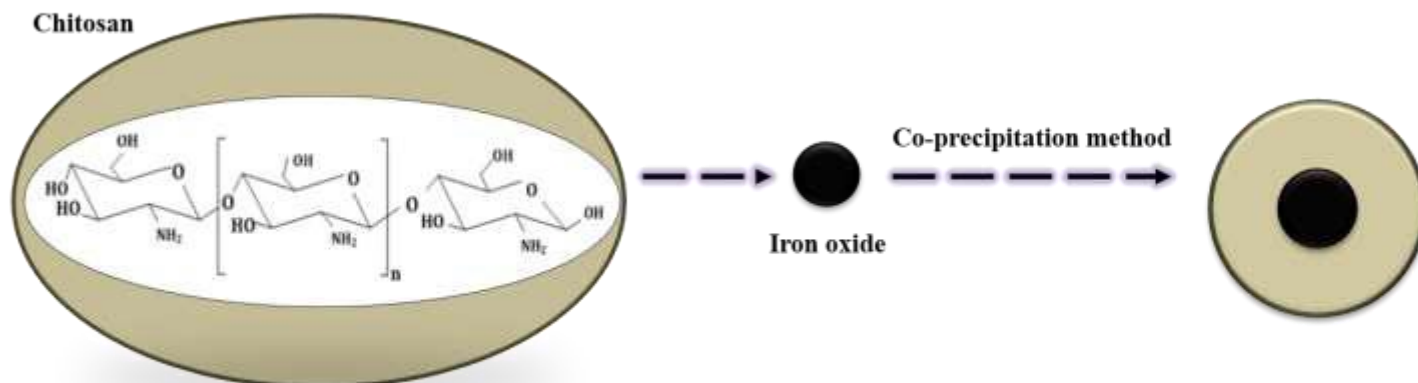
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Step1: Chitosan coated iron oxide



Step2: Functionalized DOX-ZnO

DOXORUBICIN + ZnO

Continuous stirred



Step3: DOX-ZnO QDs loading on chitosan

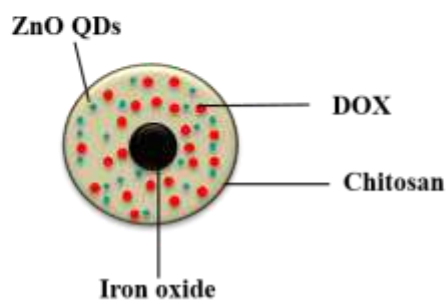


Fig.1: Schematic diagram showing preparation of Dox-ZnO QDs and subsequent loading of Dox-ZnO QDs to Chitosan nanoparticles.

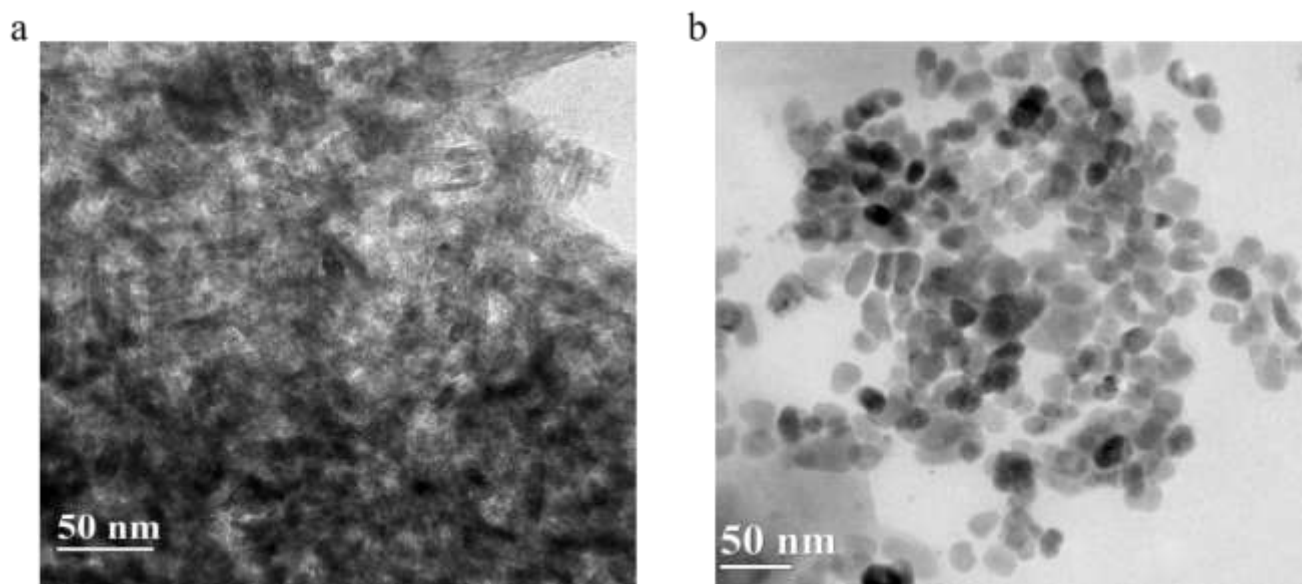


Fig.2: TEM images. (a) iron oxide nanoparticle, (b) ZnO QDs.

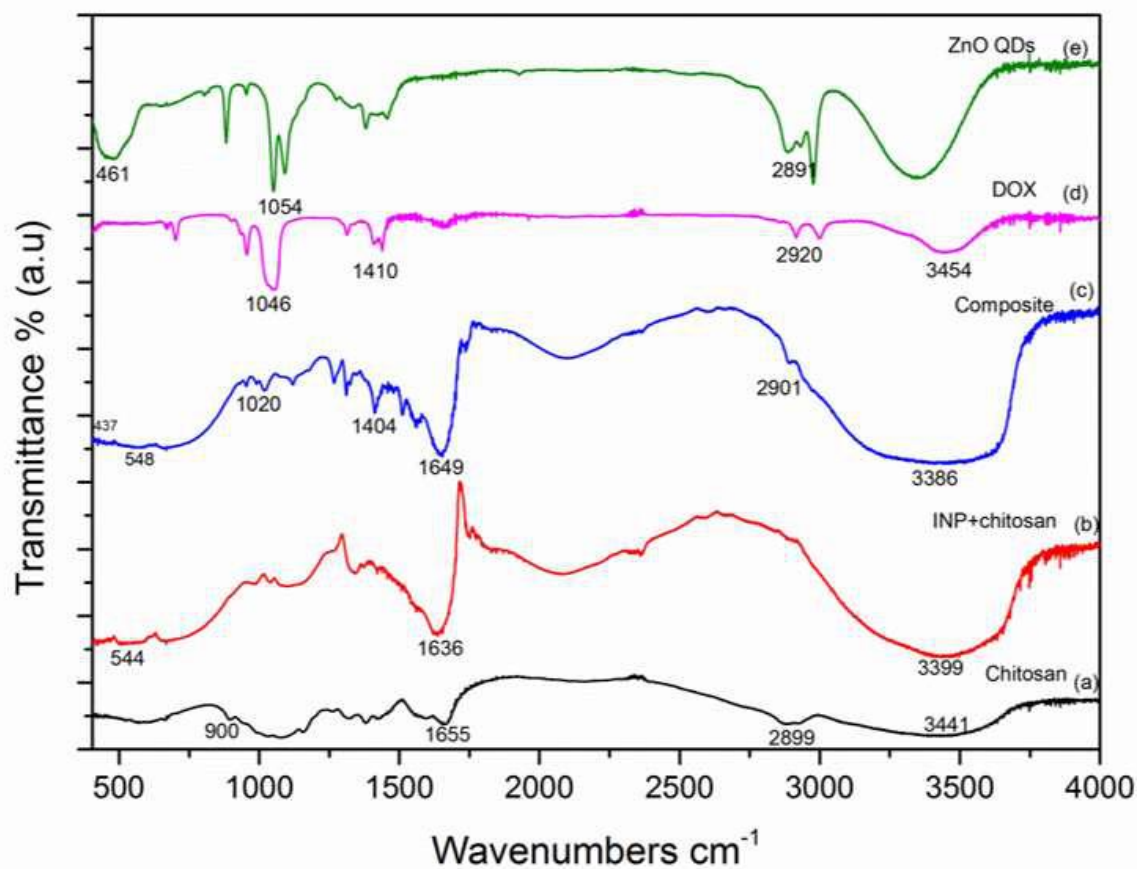


Fig.3: FTIR spectrum of the empty nanoparticle, composite and free DOX. (a) chitosan alone, (b) Iron oxide+ chitosan, (c) composite, (d) free DOX, (e) ZnO QDs.

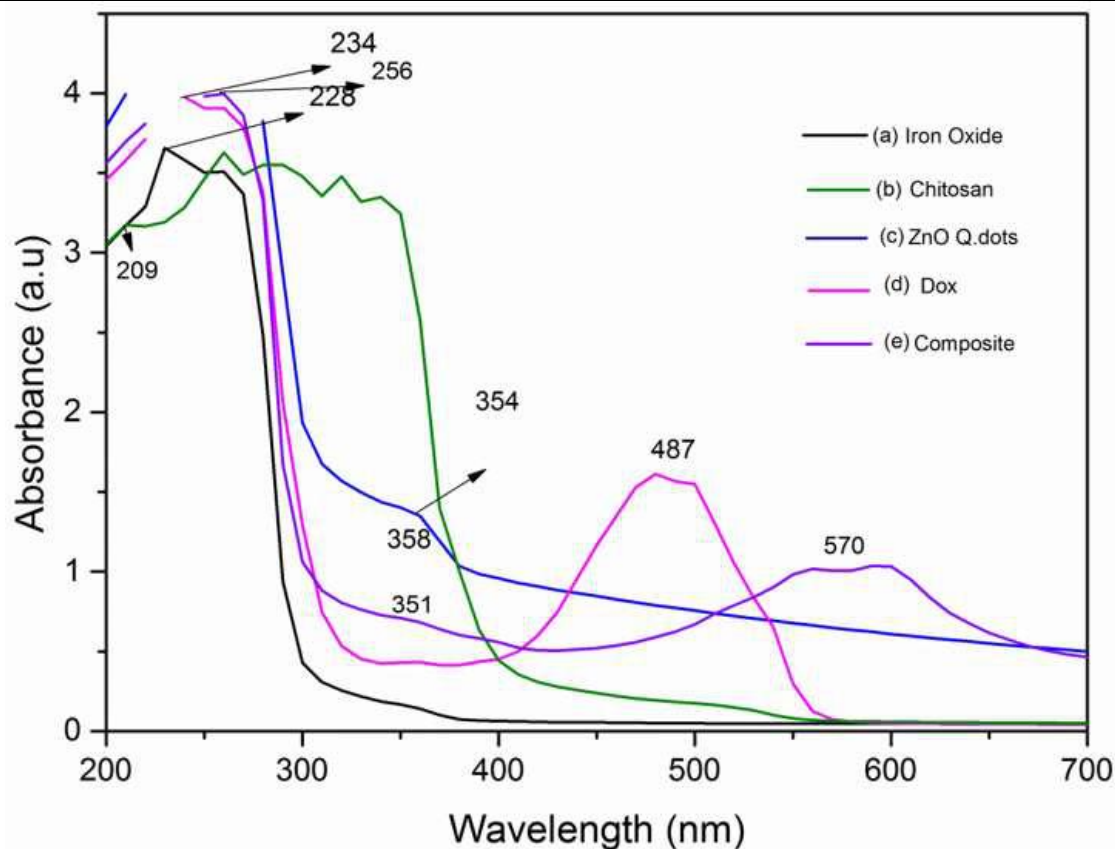


Fig.4: Absorption spectra of the DOX and DOX loaded nanoparticle. (a) Iron oxide, (b) Chitosan, (c) ZnO QDs, (d) free DOX, (e) Composite.

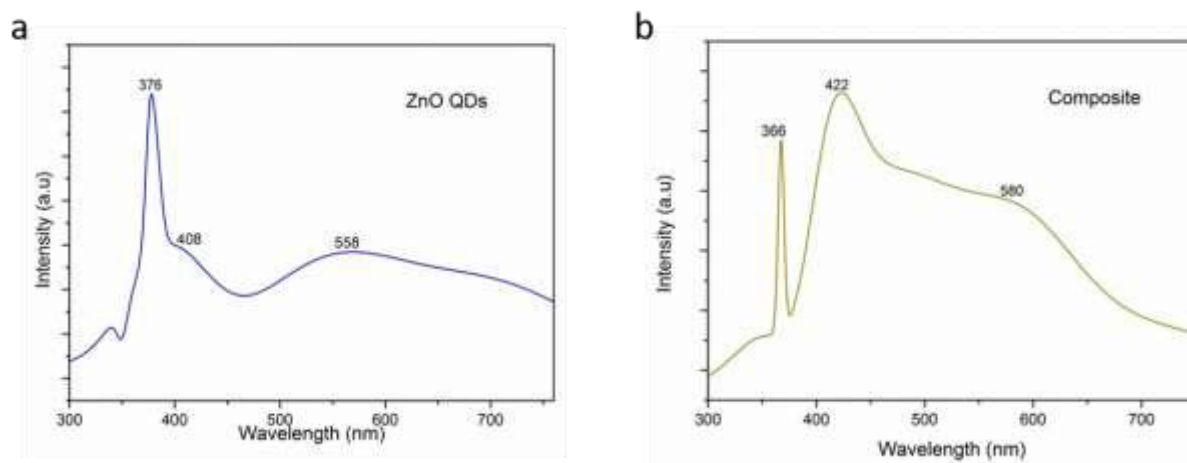


Fig.5: Fluorescence spectra. (a) ZnO QDs, (b) DOX loaded composite nanoparticle.

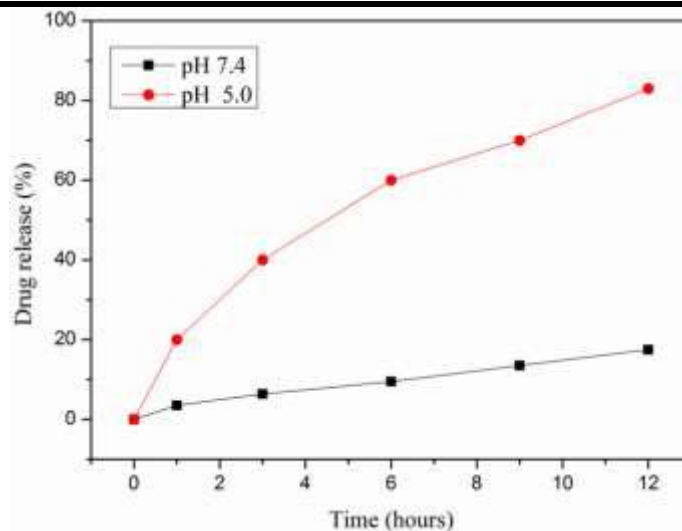


Fig.6.: pH dependent release of DOX from DOX-ZnO QDs loaded chitosan nanoparticles. DOX release studies were carried out by calculating the amount of DOX in acetate (pH 5.0) and phosphate (pH 7.4) buffer dialysis filtrate at different time intervals.

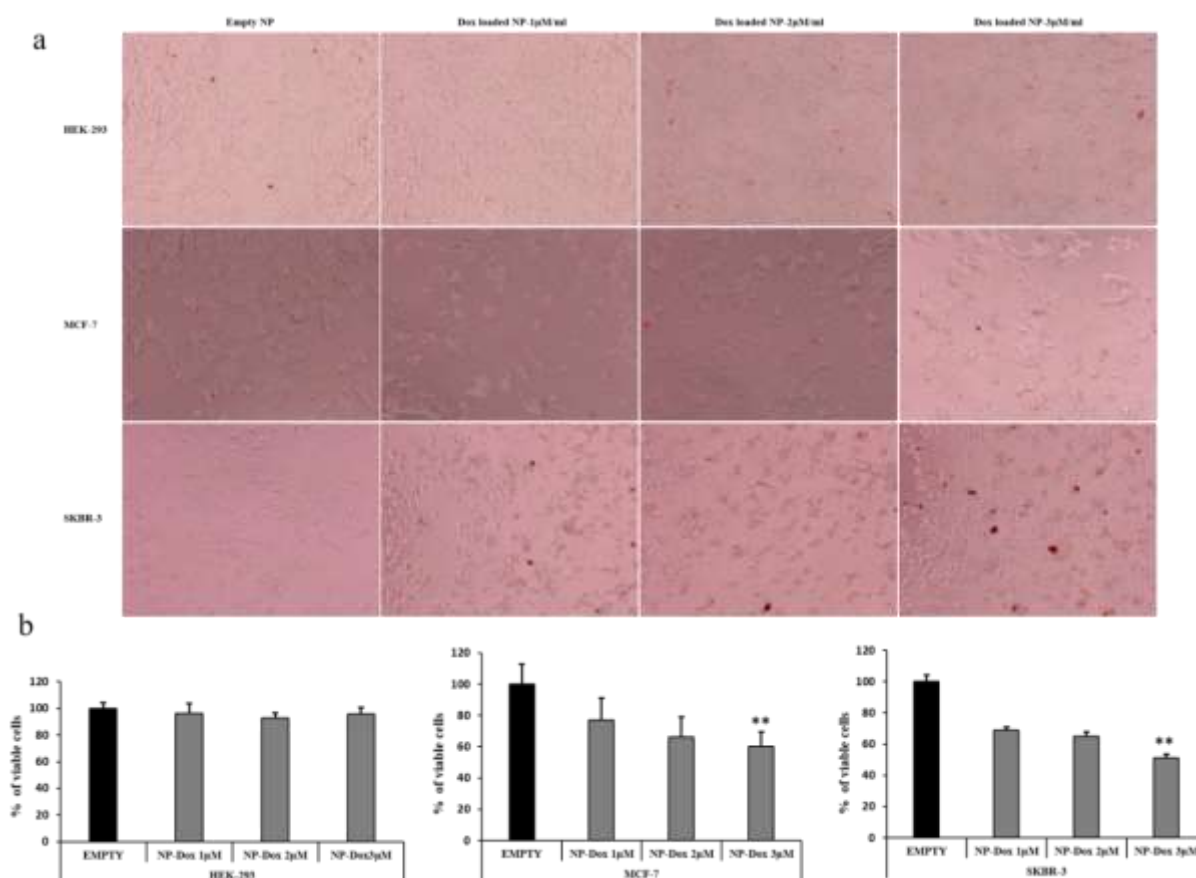


Fig.7: In vitro antitumor cytotoxic effect of various concentrations of Dox in the composite in HEK-293 human embryonic kidney cells, MCF-7 and SKBR-3 breast cancer cells after 48h incubation. (a) Cell death observed using phase contrast microscope. (b) percentage of viable cells using MTT assay. Each value represents the mean \pm SE (n=5).

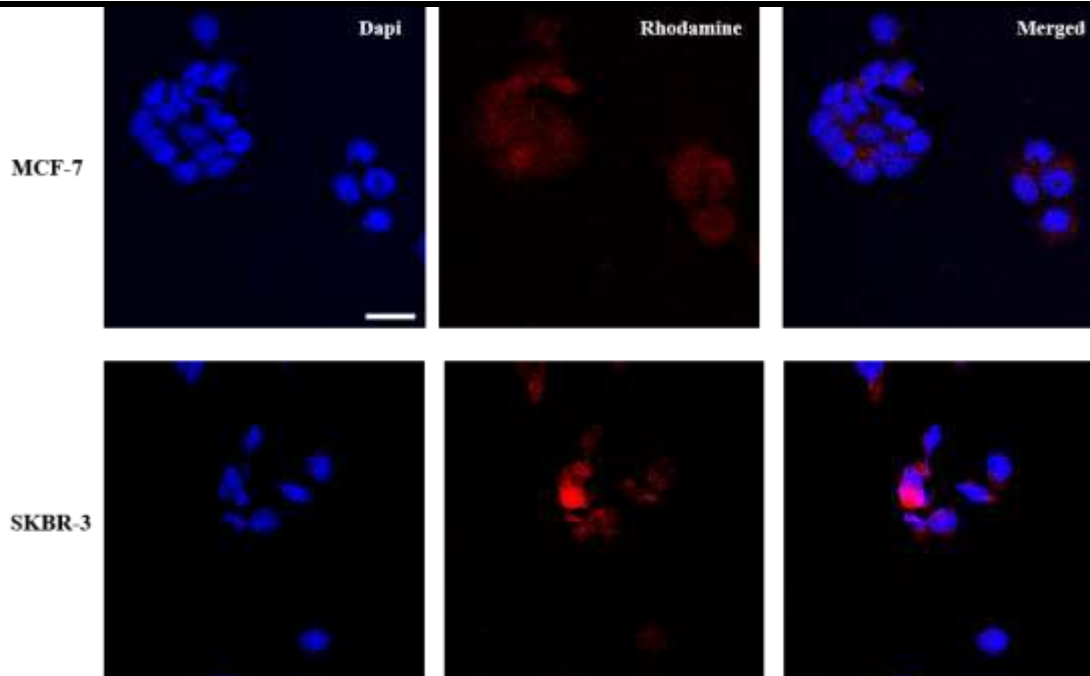


Fig.8: In vitro cellular uptake of the composite by MCF-7 and SKBR-3 breast cancer cells after incubation for 48h. The cells were observed using confocal laser scanning microscopy (DAPI: blue, Dox: red).

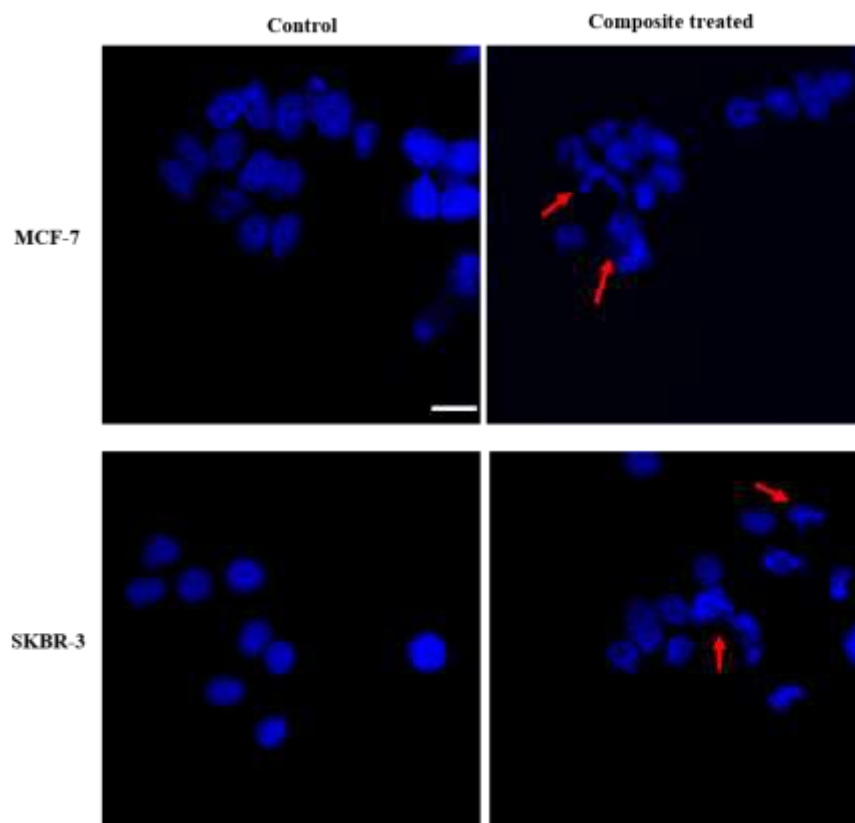


Fig.9: Nuclear damage of MCF-7 and SKBR-3 breast cancer cells. Cells were treated with indicated concentrations of composite for 48h. The cells were fixed, nucleus was stained and observed using confocal laser scanning microscopy (DAPI: blue).

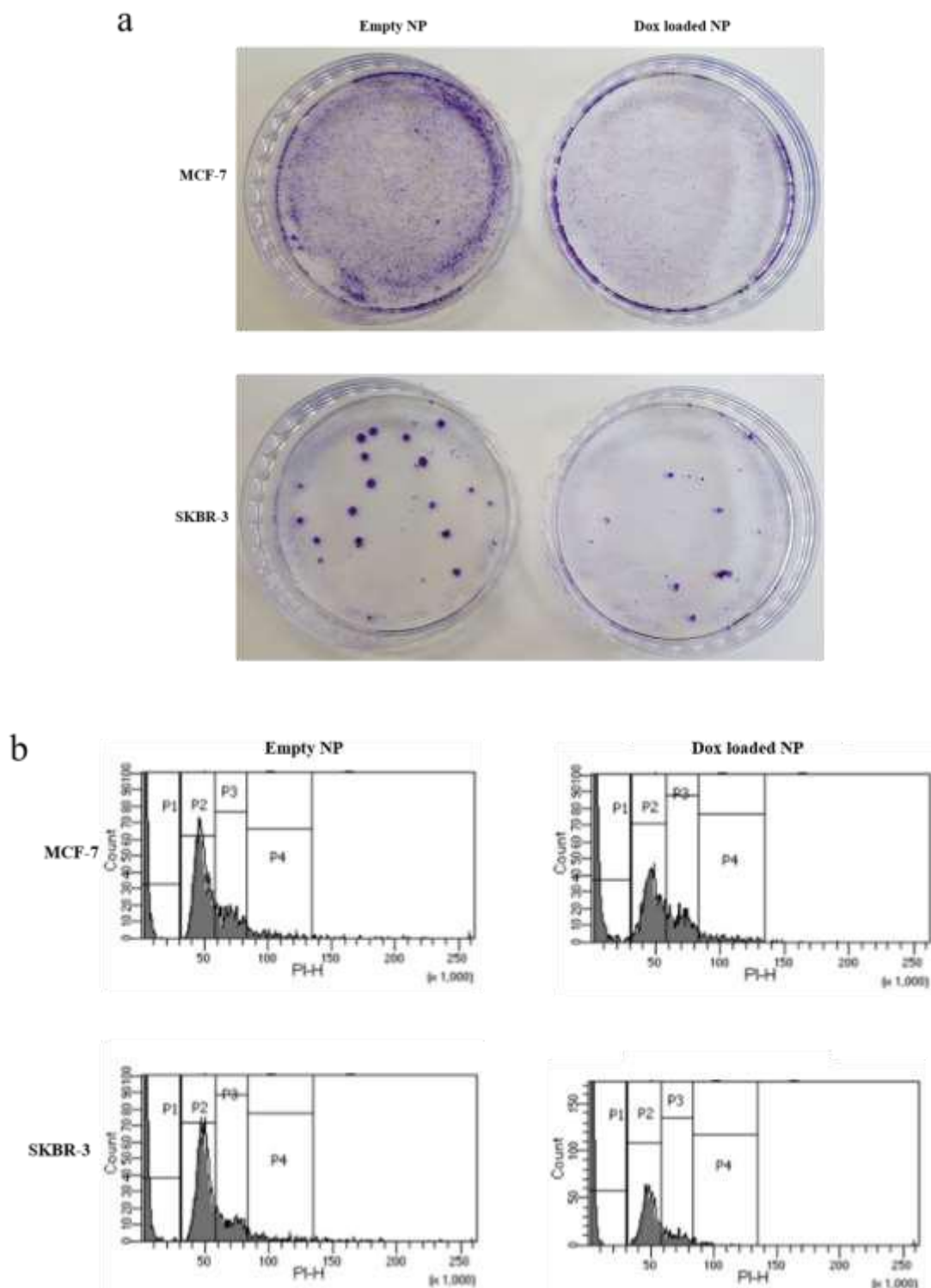


Fig.10: (a) Composite inhibited cell proliferation of breast cancer cells. (a) MCF-7 and SKBR-3 cells were treated with indicated concentrations of composite for 48h. Clonogenic assay was performed to validate colony formation ability of treated cells. (b) Cell cycle analysis was performed after treating MCF-7 and SKBR-3 cells with indicated concentrations of composite for 48h using flow cytometry.

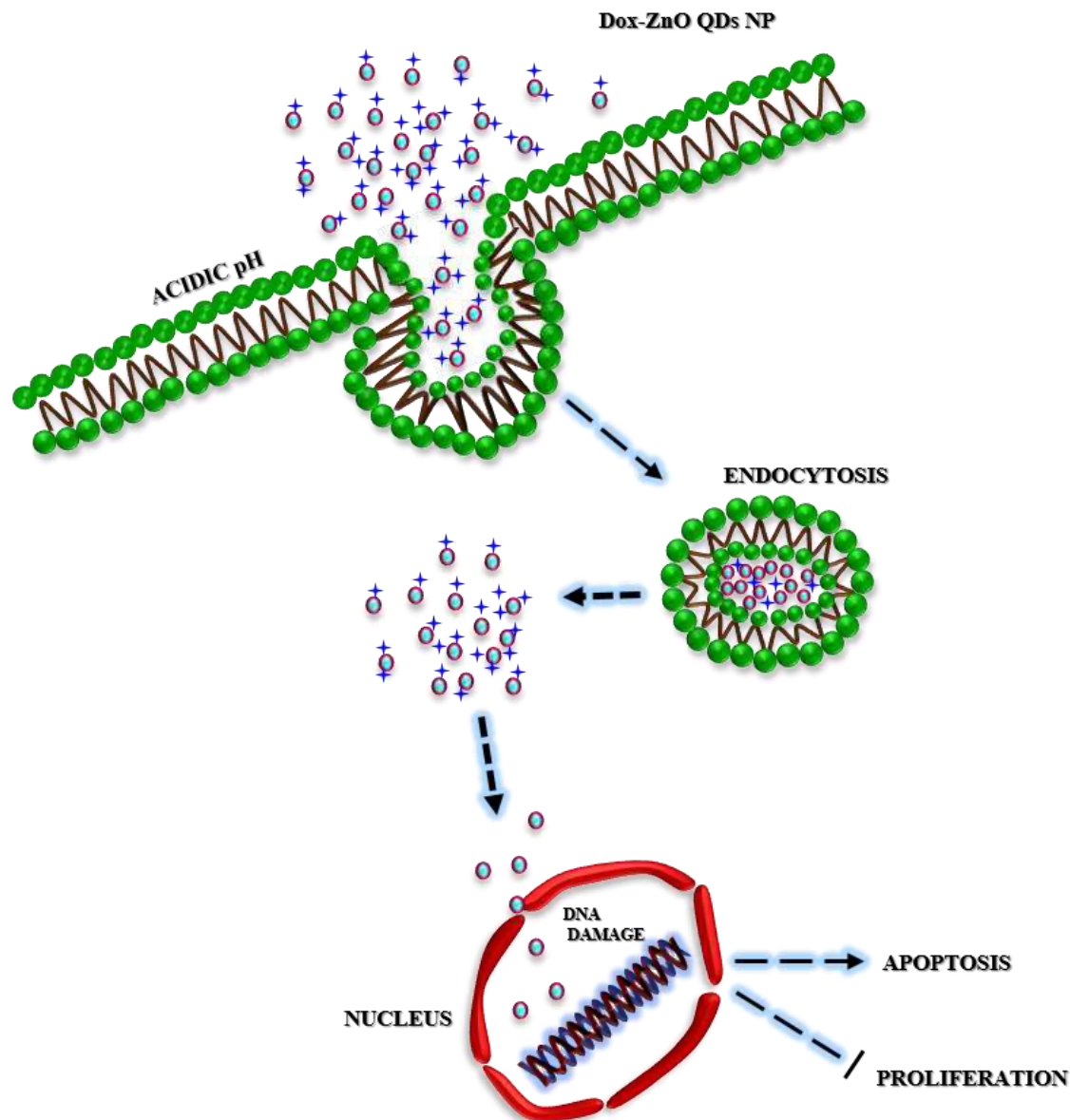


Fig.11: Schematic illustration of mechanism for the interaction of ZnO QDs-breast cancer cells and their cytological death.