

# TARGETED DELIVERY OF ANTI-CANCER DRUGS TO BREAST CANCER CELLS USING BIO-FUNCTIONAL CARBONATE APATITE NANO-PARTICLES

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## INTRODUCTION

Breast cancer is one of the leading causes of mortality worldwide. Chemotherapy is the only option for treating breast cancer in the malignant condition for increasing the life time of the patient. Cisplatin and doxorubicin are two traditional cancer drugs commonly used in chemotherapy for killing breast cancer cells [1-3]. However, these drugs can also kill normal cells causing severe side effects. One promising approach for overcoming the side effects is using nanoparticles for carrying the drugs specifically to the breast cancer cells [3-6]. In active targeting, drug carrying nanoparticles are functionalized with a ligand for delivery of the drugs to specific cells which possess the receptor for the ligand. In passive targeting, drug-loaded nanoparticles of defined sizes (~100 nM) are allowed to penetrate across the blood vessels in the cancer cells, which are more permeable than those in normal cells.

Liposomal and polymeric particles have been used for delivery of cancer drugs. However, the existing systems are not so efficient for delivery purposes. While liposomes are unstable in the physiological conditions and easily release drugs before reaching the target site (cancer cells), the polymeric carriers are very stable and after cellular internalization, drugs still remain bound to the polymers. Free drugs at a sufficient concentration are needed in cytoplasm to kill a cancer cells. Recently, we have developed an efficient macromolecular drug (DNA) delivery and expression system based on pH-sensitive inorganic nanocrystals of carbonate apatite with capability of effective intracellular delivery and release of associated drug molecules leading to very high level of transgene expression in cancer and primary cells. [6, 7]. However, these nanoparticles are also highly potential for delivery of small cancer drugs in targeted fashion.

The objective of the project is to deliver anti-cancer drugs, such as cisplatin and doxorubicin specifically to breast cancer cells by using pH-sensitive carbonate apatite nanoparticles being coated with erythropoietin (EPO) protein or HER2-specific antibody that can recognize EPO or HER2 receptor on the breast cancer cells for the targeted delivery. Here we will show fabrication and characterization of doxorubicin-loaded nanoparticles and their effects on cancer cell killing in vitro.

## METHODOLOGY

## **Cell culture**

sw480 and NIH:OVCAR-3 cell lines were cultured in 75-cm<sup>2</sup> flasks in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) and RPMI medium (Gibco BRL) respectively supplemented with 10% fetal bovine serum (FBS), 50 µg penicillin/ml, 50µg streptomycin/ml, 100 µg neomycin/ml at 37 °C in a humidified 5% CO<sub>2</sub>-containing atmosphere.

## **Complex (Apatite-DOX) formation and delivery to the cells *in vitro***

sw480 and NIH:OVCAR-3 cells from the exponentially growth phase were seeded at 40,000 cells per well into 24-well plates the day before drug delivery. 1 to 5 µl of 1 M CaCl<sub>2</sub> was mixed with 20 to 80µM of DOX and added to bicarbonate (44 mM)-buffered DMEM medium (pH 7.5), followed by the incubation at 37°C for 30 min, for complete generation of apatite-DOX particles. Then the particle suspensions were centrifuged at 15000 rpm for 3 min and the pellets were washed with the same medium. This repeated centrifugation and washing were done thrice to remove all the free unbound DOX. Apatite-DOX crystal suspension (DOX load 10-1000nM) in 1 ml DMEM or RPMI containing 10% FBS was added, respectively, onto sw480 and NIH:OVCAR-3 cells being cultured in 24-well tissue culture plate. After an additional 72 hr culture, cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

## **Evaluation of the DOX amount loaded onto Apatite**

Following generation of carbonate apatite-doxorubicin particles as described above, using 3 mM Ca<sup>2+</sup> and 20-80 µM of DOX, Centrifuged at 15000 rpm for 3 mins and discard the supernatant and washed 3 time with the same medium. The resulting pellet was then dissolved with 10 mM EDTA-PBS (100 µl). 100ul of dissolved particle was taken to assay plate and quantify the florescence by spectrophotometer( $\lambda_{ex}$  470nm,  $\lambda_{em}$  585nm). Free DOX (1, 10, 100 nM & 1, 10, 50, 100µM) in PBS was quantified and plotted to make the calibration curve. DOX loading was quantified by comparing from the calibration curve.

## **Size determination by dynamic light scattering (DLS) method**

Dynamic light scattering (DLS) measurement for particle suspension was carried out with a Super-dynamic Light Scattering Spectrophotometer, 'Photal' (Otsuka Electronics) at 75 mW Arlaser. Apatite-DOX particles were formed by mixing 1-5mM Ca<sup>2+</sup> in DMEM along with 20-80 µM, followed by the incubation at 37°C for 30 mins and added 10% FBS.

## **MTT Cell viability assay**

Drugs were delivered into sw480 and NIH:OVCAR-3 cells and cultured for 72 hours as described above. 30 µl of MTT solution (5mg/ml) was added to each well and incubated for 4 hours at 37°C. 0.5 ml of DMSO was added after removal of media. After resolving

crystals and incubating for 5 min at 37°C, absorbance was measured in a micro plate reader at 570 nm with a reference wavelength of 630 nm.

## **RESULTS AND DISCUSSION**

### **Generation and molecular characterization of apatite-DOX particles**

Turbidity analysis of the particle suspension at 320 nm demonstrated that the growth of carbonate apatite and binding affinity of DOX to apatite particle at a fixed concentration of  $\text{Ca}^{2+}$  (3mM) and  $\text{HCO}_3^-$  (44 mM), increased almost proportionately with an increase of DOX concentrations (0 to 80 $\mu\text{M}$ ) in  $\text{HCO}_3^-$ -buffered DMEM (pH 7.5) prior to incubation at 37 °C for 30 min for particle formation (Fig. 1). DOX showed the binding affinity for carbonate apatite as the fluorescence analysis of the dissolved apatite-DOX particles proved the incorporation of doxorubicin into apatite particles. DOX incorporation is increased with higher DOX concentration maintained during the formation of apatite-DOX particle (Fig. 2). Maximum 2.785 mol% DOX was incorporated in the carbonate apatite particle. We assume that this apatite-DOX formation is based on electrostatic interaction where the positively charged doxorubicin binds to negatively charged carbonate or phosphate-rich domains in the apatite particle. To clarify our assumption, we measured the surface charge of conjugates particles and found that  $\zeta$ -potential of the apatite-DOX particle is more electropositive than that of only apatite particle. Moreover,  $\zeta$ -potential of the apatite-DOX particle (higher doxorubicin loading) is more electropositive than that of lower loading (data not shown here). The positively charged doxorubicin can electrostatically bind with the negative ions in apatite like carbonate and phosphate during conjugate formation, making the ultimate charge of the conjugate more electropositive than the apatite only.

### **Size measurement of apatite-DOX particles**

The sub-micron size of nanoparticles offers a number of distinct advantages over microparticles. Nanoparticles have, in general, relatively higher intracellular uptake compared to microparticles. Although one of the famous targeting strategies like the EPR effect (34) do not specify the size of drug carriers, it is generally known that nano-sized carriers are a prerequisite for efficient drug targeting. When carrier systems possessing a diameter larger than that of a capillary are injected in the bloodstream, carriers physically clog the blood vessels. However, carrier systems of 300 nm diameter or smaller are used for drug targeting, whereas the smallest diameter of capillary blood vessels is approximately 5mm. We carried out the size measurement of generated apatite-DOX particles by dynamic light scattering method which revealed that the average size of the apatite-DOX is within 200~300 nm and incorporation of DOX in the apatite particles increased the size significantly (Fig. 4a, 4b).

### **Apatite-DOX mediated cytotoxicity on sw480 and NIH:OVCAR-3 cell line**

The efficacy of an anti-cancer drug delivery system is measured in terms of apoptotic cells. That is why we performed the cytotoxicity assay which is called MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. We found the profound

cell killing ability of apatite-DOX conjugated particles in colon carcinoma cell like sw480 and ovarian carcinoma cells like NIH:OVCAR-3 cells in comparison with free DOX. The IC<sub>50</sub> of free DOX was 500 nM and 750 nM whereas IC<sub>50</sub> of apatite-DOX conjugated particle was 75 nM and 80 nM after 72 hours in sw480 (Data not shown here) and NIH:OVCAR-3 cell lines (Fig 7) respectively, thus exhibiting 7 times and 10 times more cytotoxic than free DOX in those cell lines respectively possessing negligible level of cytotoxicity caused by biodegradable carbonate apatite carrier only. This enhanced cytotoxic effect may be due to the high uptake level and quick release of drugs from the apatite at low pH, which can avoid extra and intracellular drug inactivation and drug resistance due to the entry through diffusion. Moreover, the enhanced entry of conjugated anticancer drugs facilitated by the endocytosis mediated entry may be a good reason for enhanced cytotoxicity.

## CONCLUSION

Thus we have developed an advanced technology for drug delivery of anticancer drugs like doxorubicin to different type of cancer cells based on the generation of nano-sized as well as highly acid soluble carbonate apatite-DOX conjugate nanoparticles having wide and potential applications from laboratories to clinical medicine. This apatite-DOX conjugate may be a good candidate for individually and combined therapy with specific siRNA, oncogenic DNA and so on. Moreover, the new findings give us insights to create a new era for inorganic crystal-based drug delivery for cancer treatment.

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