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Protamine-carboxymethyl cellulose magnetic nanocapsules for enhanced delivery of anticancer drugs against drug resistant cancers

Rajasegaran Elumalai[a], Shilpa Patil[b], Naseer Maliyakkal[b], Annapoorni Rangarajan[b], Paturu Kondaiah[b], Ashok M. Raichur[a,*]

[a]*Dept. of Materials Engineering, Indian Institute of Science, Bangalore*
[b]*Dept. of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore*

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Abstract

Multidrug resistance is a major therapeutic challenge faced in the conventional chemotherapy. Nanocarriers are beneficial in the transport of chemotherapeutics by their ability to bypass the P-gp efflux in cancers. Most of the P-gp inhibitors under Phase II clinical trial are facing failures and hence there is a need to develop a suitable carrier to address P-gp efflux in cancer therapy. Herein, we prepared novel protamine and carboxymethyl cellulose polyelectrolyte multi-layered nanocapsules modified with Fe_3O_4 nanoparticles for the delivery of doxorubicin against highly drug resistant HeLa cells. The experimental results revealed that improved cellular uptake and enhanced drug intensity profile with greater percentage of apoptotic cells were attained when doxorubicin loaded magnetic nanocapsules were used in the presence of external magnetic field. Hence, we conclude that this magnetic field assisted nanocapsule system can be used for delivery of chemotherapeutics for potential therapeutic efficacy at minimal dose in multidrug resistant cancers.

Key words: Magnetic nanocapsules; Drug resistant cells; Doxorubicin delivery; Apoptosis; Targeted delivery

Background

Cancer is one of the malignant diseases responsible for more than 20% of all deaths.[1] It is defined as uncontrolled proliferation of cells and rapidly spread to healthy tissues. Worldwide, more than 10 million new cases of cancer have been reported, 85% of which are solid tumors.[2] The conventional method of solid tumor management is surgery followed by radiation and chemotherapy.[3] Majority of the conventional chemotherapy fails in cancer therapy due to multidrug resistance (MDR). Cells attain MDR effect by two important mechanisms such as over expression of drug efflux proteins on the cellular membrane and by enhancing the anti-apoptotic pathways.[4] Cancers constitute heterogeneous populations of drug sensitive and drug resistant malignant cells. The drug sensitive (DS) cells are killed upon treatment with therapeutic agents but a higher proportion of drug resistant (DR) cells remain intact hampering the effective treatment of cancer.[3] Molecular pumps actively expel chemotherapeutic drugs and are overexpressed in the cellular and nuclear membranes of tumor cells.[3] These molecular pumps comprise of P-glycoprotein (P-gp) and multidrug resistant proteins (MRP). The P-gp binds with neutral or positively charged molecules whereas the MRP bind with negatively charged molecules. This results in efflux of molecules across intracellular or extracellular membranes depleting the drug molecules inside the cytoplasm and nucleus. This phenomenon is called efflux pump related cell resistance.[3,5-8]

Thirty different types of inhibitors were used for more than 150 clinical trials, but P-gp inhibitors are yet to be approved by the food and drug administration. P-gp inhibitors are classified into three generations like first, second and third. Each type of

Abbreviations: PRO, protamine; CMC, carboxymethyl cellulose; Dox, doxorubicin; MN, magnetic or ferrite nanoparticles; MF, magnetic field; NCp, polyelectrolyte multilayered nanocapsules; MNC, magnetic nanocapsules; Dox-NCp, Dox loaded nanocapsules; Dox-MNC, Dox loaded magnetic nanocapsules; DS-HeLa, drug sensitive HeLa cells; DR-HeLa, drug resistant HeLa cells; (+ Field), presence of external magnetic field; (− Field), absence of magnetic field; (SD1 and SD2), schematic diagram 1 and 2.

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*Corresponding author at: Department of Materials Engineering, Indian Institute of Science, Bangalore, India.
E-mail addresses: amr@materials.iisc.ernet.in, ashok.raichur@gmail.com (A.M. Raichur).

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generation has its own limitations and drawbacks at *in vitro* and *in vivo* level. For example, first generation inhibitors are more active only at *in vitro* studies and the required dose is causing high toxicity *in vivo*. Such inhibitors are cyclosporine A and verapamil. Dexverapamil and Valspodar are the second generation inhibitors which are more effective against P-gp pumps but they modify the pharmacokinetic parameters. Most of the third generation inhibitors are under Phase II clinical investigation and are attaining failures in clinical trial.[9,10]

To overcome these problems raised by P-gp inhibitors in cancer therapy, researchers are designing alternative delivery systems that can deliver the drugs against DR cancers to minimize the drug efflux. Nanomedicine is an emerging and promising strategy to overcome this drug efflux. It involves the interaction of nanomaterials with tissues or cells to achieve maximum clinical therapeutic efficacy at the target site.[11,12] A large number of nanoparticulate systems such as dendrimers, micelles and liposomes have been utilized for encapsulation of therapeutic molecules including conventional drugs, nucleotides and recombinant proteins for delivery at the site of disease. The unique property of external carriers in drug delivery is that they ignore the reorganization of encapsulated drug molecules by transmembrane proteins in DR cancers. Also, the subcellular size carriers relatively enhance the cellular permeability,[13-16] half-life in physiological fluid, modify the kinetics, protect the sensitive biomolecules and release encapsulated molecules at specific rates at the desired site. However, they possess few drawbacks in clinical studies in terms of poor bioavailability and physical stability.[17-19] In the current scenario, a lot of research is focused on the introduction of novel biodegradable and biocompatible materials for fabrication of cargo to deliver encapsulated molecules into tissues and cells.[20]

In this study, we have used two oppositely charged polyelectrolytes protamine (PRO) and carboxymethyl cellulose (CMC) to prepare **multi-layered** nanocapsules by **layer-by-layer** (LbL) deposition on sacrificial silica template. These polyelectrolytes are already used in clinical applications such as in cardiac surgeries where PRO helps to neutralize the heparin while CMC is used to treat renal failure, facilitate **wound** healing and as a support for implant materials.[21] Cancer chemotherapy uses Doxorubicin (Dox) as one of the effective drugs.[22] We have utilized these **multi-layered** nanocapsules (NCp) for delivering Dox against Dox resistant HeLa cells (DR-HeLa). It is a topoisomerase inhibitor and induces apoptosis upon interaction with the cell nucleus. The mechanisms of drug resistance and inhibition of proliferation of the cancer cells by Dox are still unclear.[23-27]

Among the various nanoparticles, **super-paramagnetic** iron oxide (Fe_3O_4) nanoparticles, also called the ferrite nanoparticles (MN), have attracted wide spread attention in the fields of **bio-imaging**, biosensors, targeted delivery and magnetically induced hyperthermia applications.[23,28,29] Hence in the current study, MN modified PRO-CMC **multi-layered** nanocapsules called magnetic nanocapsules (MNC) were prepared by LbL technique to study the effect of magnetic field enhanced drug delivery. The novel MNC were utilized to study the cellular uptake, intracellular fluorescent intensity, cytotoxicity and programmed cell death in DR-HeLa cells at different treatment time points in the presence and absence of an external magnetic field (MF). Also, the MF assisted drug delivery enabled the control of **bio-distribution** in an *in vivo* scenario.

Methods

Materials

See supplementary information.

Preparation of multi-layered nanocapsules and magnetic nanocapsules

The PRO and CMC polyelectrolyte solutions were prepared by 1 mg/ml concentration in 1 M NaCl and adjusted to pH 5 using 0.1 M NaOH. The polyelectrolytes were deposited alternatively on charged silica nanoparticles by LbL technique **up to three bilayers and finished with PRO as the outer layer.** The silica template was removed by acid etching to produce hollow nanocapsules (NCp). The percentage of silica dissolution was analyzed by EDS (supplementary materials). The charge at the surface of each layer after deposition and the particle size distribution were measured by Zetasizer. The ferrite nanoparticles were synthesized and characterized by electron microscopy. The MNC were prepared by attaching MN on the NCp surface by incubating nanocapsule suspension with MN overnight. The unattached MN was separated by dialysis. The brief protocol for preparation of NCp and NMC is given in supplementary materials. The **schematic diagram (SD)** of LbL deposition and preparation of MNC is given in supplementary materials SD1.

Electron microscopy characterization of nanocapsules

The characteristics of the nanocapsules such as size, shape and morphology were studied by field emission scanning electron microscopy (FE-SEM) and field emission transmission electron microscopy (FE-TEM). Nanocapsule suspension was sonicated and dropped on cleaned silicon wafer for drying overnight in a desiccator. Electron conductivity was created externally to the sample by sputtering with gold nanoparticles using a gold sputter (JEOL JFC 1100E Ion sputtering device) and later analyzed by FE-SEM (FEI-SIRION, Eindhoven, Netherlands) at 5 kV, 6WD using TLD. The percentage of silica dissolution was quantified by EDX software at 15 kV and 6 spot size. Similarly for FE-TEM, the sample suspension was placed on copper gird (300 mesh, carbon coated, Toshniwal Bros SR Pvt Ltd, India) and dried overnight under vacuum condition. Images were obtained using FE-TEM (Tecnai F30 FEI-Eindhoven, Netherlands) at 120 kV.

Dox loading and release studies

A brief protocol for Dox loading and determining encapsulation efficiency is given in supplementary materials. Dox release profile was studied in DMEM supplemented with 10% FBS in the presence of stimuli such as pH and enzyme. For pH responsive release, Dox-NCp suspension was adjusted to pH 6 and 7.4. Similarly, for enzymatic release, Dox-NCp was treated with Trypsin (10 μg/ml concentration in sterile DPBS) at pH 6 and 7.4. Dox-NCp suspension containing 100 μg Dox was incubated at 37 °C, 170 rpm (Orbitek, Scigenics Biotech, India). After every 1 h interval, samples were centrifuged at 2000 rpm,