Intracellularly triggered biopolymer based polyelectrolyte nanocapsules for targeted delivery of anticancer drugs

Pulakkat S.[1], Balaji S.[2], Rangarajan A.[2] and Raichur A.M.[1*]
Materials Eng, Indian Institute of Science, Bangalore, India (amr@materials.iisc.ernet.in)

INTRODUCTION AND OBJECTIVE

Targeted delivery of anticancer therapeutics can minimize most of the drug-originated systemic toxic effects. Herein, we report a polyelectrolyte nanocapsule system capable of targeting CD44 receptors on breast cancer cells and releasing its cargo at intracellular lysosomal pH. Hollow nanocapsules were fabricated by Layer by Layer (LbL) technique using hyaluronic acid (HA) and protamine (PR). HA, besides being the structural component of the capsule, also acts as a targeting ligand.

The size, morphology and surface characteristics of the nanocapsules were characterized followed by loading of an anticancer drug, doxorubicin (dox). The cellular uptake of the nanocapsules was evaluated by fluorescence-activated cell sorter (FACS) and confocal microscopy. MTT assay revealed that the drug loaded nanocapsules exhibited superior antitumor effect over the free drug.

MATERIALS AND METHODS

Materials

Hyaluronic acid, protamine, Dulbecco's modified eagle medium and doxorubicin hydrochloride were purchased from Sigma Aldrich.

Preparation of nanocapsules

LbL technique involves sequential deposition of oppositely charged polyelectrolytes onto a sacrificial silica template. Briefly, silica nanoparticles were incubated alternatively with PR and HA solutions (0.5 mg/ml, pH 5.5) and after deposition of 6 layers, the template was removed to obtain hollow capsules.

Characterization of nanocapsules

The surface charge of the coated particles was measured after every adsorption step. Morphological characterization of the nanocapsules was done by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

Drug loading and release studies

The hollow nanocapsules were incubated overnight with 300µM dox in acetate buffer solution (pH 3). The sample pH was then raised to 5.5 followed by several washings. The sample was then centrifuged and the amount of drug in the supernatant was estimated by measuring the absorbance at 490 nm using a spectrophotometer. The dox loaded nanocapsules (HA-PR-dox) were later suspended in pH 4 acetate buffer to monitor the drug release, and the cumulative release percentage was estimated.

Cell culture

Breast cancer cell lines MDAMB 435S (CD44+) and BT 474 (CD44-) were used for *in vitro* studies. About $1x10^5$ cells were grown with DMEM supplemented with 10% FBS at 37°C incubator and 5% CO_2 supply.

Cellular uptake studies

The binding efficiency of nanocapsules to CD44 receptor was evaluated by incubating 1µM of free dox and HA-PR-dox with MDAMB 435S and BT 474 cells for 1 hour followed by PBS wash and trypsinization. To study the effect of HA pretreatment, the cells were incubated with 1mg/ml HA solution for 2 hours before adding free dox and HA-PR-dox nanocapsules. Both samples were then subjected to FACS analysis. Confocal images were obtained after incubating $5x10^4$ MDAMB 435s cells with 1 µM of free dox and HA-PR-dox for 12 hours.

Cytotoxicity assay

The *in vitro* toxicity of the HA-PR-dox nanocapsules was assessed using MTT assay. $8x10^3$ cells of MDAMB 435s were seeded in 96 well plates for 12 hours followed by addition of different concentrations (0.25 to 1.5 µM) of free dox and HA-PR-dox. After 48 hours, 20µl of 5mg/ml of MTT was added and again incubated for 4 hours. Later, the media was removed completely and 100 µl of DMSO was added before taking reading at 550nm. The IC50 values for free dox and HA-PR-dox were also determined.

RESULTS AND DISCUSSION

Preparation and characterization of nanocapsules

The LbL assembly was carried out at an assembly pH of 5.5 considering the pKa and pI values of HA and PR respectively. At this pH, both polyelectrolytes are highly charged resulting in strong electrostatic attraction between the multilayers (Szarpak 2010). The zeta potential measurements yielded a saw tooth graph indicating the charge reversal due to alternate deposition of positively and negatively charged PR & HA respectively (Fig 1A). The coated particles were then treated with a buffer (0.2 M HF, 0.8 M NH_4F) to yield hollow capsules. The SEM and TEM analysis showed nanocapsules in the size range of 150-200nm (Fig 1B, 1C). The removal of the template was confirmed using EDX analysis (data not shown here).

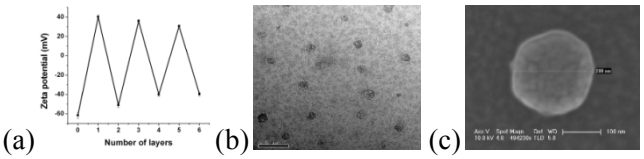

Fig 1: (a) Zeta potential (b) TEM and (c) SEM analysis of hollow nanocapsules

Drug loading and release studies

Dox was loaded into the nanocapsules at an acidic pH and 64% loading was achieved. At pH 3, which is close to the pka value of HA, the electrostatic attraction between the multilayers decreases leading to increased permeability and hence diffusion of drug into the capsules. Drug release studies at pH 4 showed a sustained release of dox over a period of 48 hours, the cumulative release being 42% (data not shown here).

Cellular uptake studies

The cellular uptake of nanocapsules were studied in MDAMB 435S (CD44+) and BT474 (CD44-) cell lines. FACS studies showed that HA-PR-dox nanocapsules, with HA as the final layer, are internalised more rapidly in MDAMB 435S cells. It was also observed that more amount of encapsulated dox was found inside the cells than free dox (Fig 3A, 3B). When the cells were pre-treated with 1 mg/ml HA solution, the uptake was found to be less (Fig 3C). This indicates that the CD44 receptors were saturated with free HA molecules, thereby confirming that nanocapsules are internalised by receptor mediated endocytosis. CLSM images also revealed an increased delivery of drug inside the cells using HA-PR-dox capsules (Fig 2).

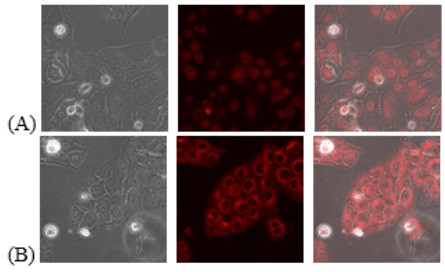

Fig 2: Confocal images of MDAMB 435S cells treated with (A) free dox and (B) HA-PR-dox

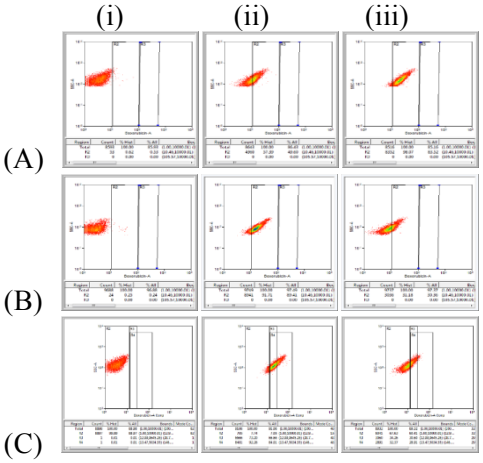

Fig 3: FACS analysis (i) unstained cells, (ii) free dox and (iii) HA-PR-dox (A) MDAMB 435S cells, (B) BT 474 cells, (C) Cells treated with HA

Cytotoxicity assay

MTT assay in MDAMB 435S revealed that HA-PR-dox was more cytotoxic than free dox (Fig 4A). The IC50 values obtained for free dox (0.6814 µM) and encapsulated dox (0.3825 µM) also confirmed that HA-PR-dox was more efficient in killing cancer cells (Fig 4B). The acidic pH and enzymes in the lysosomes trigger the sustained release of dox, thereby enhancing the antitumor effect of HA-PR-dox.

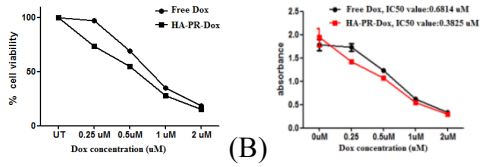

Fig 4: Comparison of (A) cytotoxicity and (B) IC50 value of free dox and HA-PR-dox nanocapsules

CONCLUSION

In summary, 6 layered HA/PR nanocapsules were prepared and characterized. The pH permeability of capsules enabled efficient loading and release of dox. The final layer HA, being a ligand for CD44 receptors, facilitated targeted delivery of HA-PR-dox in CD44 over expressing breast cancer cells. The enhanced uptake of nanocapsules and the sustained release of the encapsulated dox ensured superior anticancer effect than free drug.

REFERENCE

- Anna Szarpak et al. (2010) *Designing Hyaluronic Acid Based Layer-by-Layer Capsules as a Carrier for Intracellular Drug Delivery*, Biomacromolecules,11, 713-720

[2]Dept. of Molecular Reproduction, Development and Genetics, IISc, Bangalore (anu@mrdg.iisc.ernet.in)