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Bacterial exopolysaccharide based nanoparticles for sustained drug delivery, cancer chemotherapy and bioimaging

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ABSTRACT

Introduction of a novel biocompatible, stable, biomaterial for drug delivery application remains always challenging. In the present study, we report the synthesis of an extremophilic bacterial sulfated polysaccharide based nanoparticle as a stable biocompatible material for drug delivery, evaluation of anticancer efficacy and bioimaging. Mauran (MR), the sulfated exopolysaccharide extracted from a moderately halophilic bacterium, *Halomonas maura* was used for the synthesis of nanoparticles along with chitosan (CH). MR/CH nanoparticles were synthesized by simple polyelectrolyte complexation of anionic MR and cationic CH. The MR/CH hybrid nanoparticles formed were ranging between 30 and 200 nm in diameter with an overall positive zeta potential of 27.5 ± 5 mV and was found to be stable under storage in solution for at least 8 weeks. *In vitro* drug release studies showed a sustained and prolonged delivery of 5-fluorouracil (5FU) for 10–12 days from MR/CH nanoparticles under three different pHs of 4.5, 6.9 and 7.4 respectively. Cytotoxicity assay revealed that MR/CH nanoparticles were non-cytotoxic towards normal cells and toxic to cancer cells. Also, 5FU loaded MR/CH nanoparticles were found more effective than free 5FU in its sustained and controlled manner of killing breast adenocarcinoma cells. Fluorescein isothiocyanate (FITC) labeled MR/CH nanoparticles were used for cell binding and uptake studies; thereby demonstrating the application of dye tagged MR/CH nanoparticles for safe and nontoxic mode of live cellular imaging. We report the introduction of an extremophilic bacterial polysaccharide, MR, for the first time as a novel biocompatible and stable biomaterial to the world of nanotechnology, pharmaceutics and biomedical technology.

1. Introduction

Sulfated polysaccharides (SPSs) are gaining attention since last few decades because of their exceptionally best physico-chemical properties and bioactivities. Owing to their unique properties like stable structure, composition, fluid dynamics, extreme stability, biodegradability and biocompatibility, they are widely exploited in modern biotechnology and material science (Arad & Levy-Ontman, 2010; Xu, Zhang, Nichols, Shi, & Wen, 2007). Naturally occurring plant (algal) and animal sulfated polysaccharides are of great therapeutic importance apart from their food and industrial applications. They are proved to be effective in retroviral inhibition and cancer therapy. Most of the mammalian cell receptors that help in the adhesion of various growth factors during angiogenic processes (Cheng, Huang, Lur, Kuo, & Lu, 2009) and interacting with viral glycoproteins are SPS (Luscher-Mattil, 2000). Despite of their varying structure and composition, most of them are widely studied for their antiviral, anti-inflammatory, antitumor, anti-parasitic and anti-angiogenic activities as well as activation of immune system and effect of smooth muscle proliferation (Toshihiko, Amornrut, & Robert, 2003). These polymeric carbohydrate structures form important constituents of plants, animals and micro-organisms either as structural component or as storage molecules. Among various SPS, algal and animal polysaccharides were extensively studied; whereas bacterial polysaccharides are thus far been overlooked. Most of the SPS are polyanionic in nature and can bind to positively charged molecules *via* polyelectrolyte complexation or ionotrophic gelation. Hence they are widely used for nanotechnology applications including the synthesis of nanoparticles and microspheres for drug delivery purposes (Argandona et al., 2005; Bouchotroch, Quesada, Moral, Llamas, & Bejar, 2001; Calvo, Martinez-checa, Mota, Bejar, & Quesada, 1998; Quesada et al., 1990).

Extreme environments are proved to be interesting as a valuable source for industrially important bacteria that produces enumerable active biomolecules like polysaccharides, proteins and small peptides. Focus on active microbial sulfated exopolysaccharides (EPS) especially from extremophilic bacteria is of prime concern in current research for an ideal formulation of nanodrug carrier to encapsulate an anticancer test drug and demonstrate its sustained release pattern as well as anticancer efficacy. Halophiles are such extremophilic bacterial species that are seen under high

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salt conditions of about 5–25% of NaCl. *Halomonas maura* is a moderately halophilic bacterium, which is capable of producing highly sulfated EPS residues into the external milieu. As reported by Arias et al. (2003), *Halomonas* polysaccharides are rich in sulfate residues and hence posses various biological properties. Polysaccharides of extremophilic bacterial origin have remarkably excellent properties that show their better future in the field of nanodrug drug delivery. Biologically active sulfated polysaccharide produced by *H. maura* is called MR and it has exceptionally high sulfate content and uronic acid content.

MR is a high molecular weight acidic polysaccharide with repeating units of mannose, galactose, glucose and glucuronic acid. It is highly anionic in nature due to the presence of sulfate and uronic acid moieties. Viscoelasticity, pseudoplasticity and thixotropic behavior of MR make it an ideal molecule for material science applications. Similarly rheological properties of MR are not easily affected by the presence of any salts, sugars, surfactants, lactic acid, and changes in pH and freeze thawing (Llamas et al., 2006). Another important striking property of MR is the ability to with stand various harsh conditions like temperature, freeze thawing, extreme pH values and salt conditions. High temperature over 55 °C has detrimental effect on viscosity, although it can regain its 70% of its property on cooling to 25 °C (Arias et al., 2003). MR can form stable gels on binding to various metal ions that helps in efficient removal of toxic ions from the polluted environments and water. The unusually high sulfate content of MR contributes to immunomodulating and antiproliferative effects on human cancer cells (Llamas et al., 2006).

Present study involves the extraction of MR from *H. maura* for the synthesis of nanoparticles along with CH, to encapsulate anticancer drug 5FU and labeling of free nanoparticles with a fluorescent moiety facilitating cell binding and uptake studies. CH is a widely used polysaccharide, produced by the deacetylation of chitin, a long chain polymer seen in the exoskeleton of crustaceans and cell walls of fungi. Basically CH is a linear polysaccharide with β(1–4) linked D-glucosamine and N-acetyl D-glucosamine residues with variable deacetylation percentage. It is rich in positively charged amino functional group and can easily undergo coacervation or ionic gelation with anionic polymers, macromolecules and polyanions upon contact in an aqueous medium (Grenha et al., 2010; Lin et al., 2009; Liu, Jiao, Liu, & Zhang, 2007). Drug loaded MR/CH nanoparticles are formed during polyelectrolyte complexation of 5FU-entrapped MR with CH under constant stirring. 5FU is a broad-spectrum antitumor drug that interferes with the DNA synthesis and inhibits the action of thymidylate synthase in solid tumors. However, serious side effects limit its wide application in the medical field. With an aim of reducing side effects and enhancing therapeutic index of 5FU many combinations of polymers are being tried so far (McCarron, Woolfson, & Keating, 2000; Sivabalan et al., 2011). Encapsulation of a hydrophilic drug within a biomaterial based nanoparticle will be less toxic and safer for biomedical applications. Also, sustained release of the drug for a prolonged period of time can enhance the efficacy of therapy and reduce patient compliance. Hereby, we report the synthesis of stable biocompatible nanoparticles using bacterial EPS, especially from an extremophilic origin, with multiple applications of drug delivery, cancer chemotherapy and bioimaging for the first time to best of our knowledge.

2. Materials and methods

2.1. Materials

CH and FITC was purchased from Tokyo Chemical Industry (TCI, Kasei), Japan. 5FU was procured from Nacalai Tesque Inc., Japan. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from Sigma-Aldrich, USA and Gibco respectively. Alamar blue from Invitrogen, USA and all other chemicals used were of reagent grade.

2.2. Bacterial strain and mammalian cell lines

Moderate halophilic bacteria *H. maura* (ATCC 700995) was purchased and propagated as per the procedure mentioned in the product information sheet. On solid medium, the colonies were creamy, raised, glistening, circular and entire (Bouchotroch et al., 2001). Mouse connective tissue (L929) fibroblast cells and breast adenocarcinoma cells (MCF7) were procured from RIKEN Bioresource Centre, Japan.

2.3. Bacterial culture and MR production

The strain was grown in MY medium as mentioned elsewhere (Xu et al., 2007). Briefly, the growth medium composition: NaCl, 51.3 g; $MgCl_2{\cdot}6H_2O$, 9 g; $MgSO_4{\cdot}7H_2O$, 13 g; $CaCl_2{\cdot}2H_2O$, 0.2 g; KCl, 1.3 g; $NaHCO_3$, 0.05 g; NaBr, 0.15 g; $FeCl_3{\cdot}6H_2O$, traces; glucose, 10 g; yeast extract, 3 g; malt extract, 3 g; proteose peptone, 5 g; trace salt solution, 0.00325 g. Bacto agar (2 g/L) was added for the preparation of solid medium. Liquid medium was prepared, sterilized and inoculated with 1 ml of 48 h culture grown in the same medium (OD_{520} = 2.5) and incubated at 32 °C in a rotary shaker at 110 rpm for 15 days. Bacterial growth and EPS production were monitored in batch cultures of 500 ml Erlenmeyer flasks with 100 ml of medium in each. The experiment was performed in triplicates. At the end of incubation culture was centrifuged using an ultracentrifuge, Himac, CF12RX at 12,000 rpm for 1 h at 4 °C. Supernatant was precipitated out with cold ethanol and again centrifuged. Pellet was dissolved in ultra pure distilled water and purified using dialysis against distilled water (3–4 exchanges) for 48 h using Snakeskin pleated dialysis tubing from Thermo scientific of 10,000 MWCO. Purified MR was freeze dried and subjected to characterization. Total carbohydrates (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and proteins (Smith et al., 1985) were estimated as described elsewhere.

2.4. Electron microscopy of the strain

Cells were taken from mid exponential phase culture of *H. maura* for electron micrographic study of the EPS layering the bacterial cell. Negative staining and ultra thin sectioning was performed as mentioned elsewhere with slight modifications in the protocol and viewed through transmission electron microscope (TEM) (JEOL, JEM-2200FS). *H. maura* bound to poly-L-lysine coated glass slides were chemically fixed as mentioned elsewhere (Bouchotroch et al., 2001) and sputter coated with Pt. Images were recorded using scanning electron microscope (SEM) (JEOL, JSM-7400F).

2.5. MR characterization

Partially purified MR was subjected to X-ray photoelectron spectroscopy (XPS) (KRATOS). Analysis was carried out under a basic pressure of 1.7×10^{-8} Torr and the X-ray source used was anode mono-Al with pass energy of 40 (survey scan). XPS spectra for MR with peaks of C, N, O, S, and P were obtained. Fourier transform infrared (FTIR) spectroscopy was performed to characterize the structure of sulfated polysaccharide, MR. 2–3 mg of MR powder was mixed with 200 mg of dry KBr pellets, ground thoroughly and mixture was pressed into a 16-mm-diameter mold to prepare pellets for FTIR analysis. Infrared spectra were recorded (Perkin Elmer US) with a resolution of 4 cm^{-1} in the region of 4000–400 cm^{-1}