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# Extremophilic polysaccharide nanoparticles for cancer nanotherapy and evaluation of antioxidant properties

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ABSTRACT

Polysaccharides that show finest bioactivities and physicochemical properties are always promising for bionanoscience applications. Mauran is such a macromolecule extracted from halophilic bacterium, *Halomonas maura* for biotechnology and nanoscience applications. Antioxidant properties of MR/CH nanoparticles were studied using biochemical assays to prove the versatility of these test nanoparticles for biomedical applications. Here, we demonstrate the prospects of extremophilic polysaccharide, mauran based nanoparticles for scavenging reactive oxygen species in both *in vitro* and *ex vivo* conditions. 5-fluorouracil loaded MR/CH nanoparticles were tested for anticancer proliferation and compared their therapeutic efficiency using breast adenocarcinoma and glioma cells. Fluorescently labeled nanoparticles were employed to show the cellular uptake of these nanocarriers using confocal microscopic imaging and flow cytometry.



## 1. Introduction

Natural polysaccharides are interesting macromolecules that have been abundantly used for the synthesis, stabilization and functionalization of various micro and nano sized particles. Their role crucially decides the fate of several sugar-modified nanoparticles (Nps) and structures in terms of biocompatibility [1]. As an alternative to poly (ethelene glycol), polysaccharides are widely used for passivating various Nps and employed for drug delivery applications [2–4]. Among polysaccharides, sulfated oligosaccharides are more precisely applied for biomedical applications owing to their therapeutic and bioactive properties [1]. Sulfated polysaccharides (SPS) are widely studied for their antiviral [5–10], anticancerous [5,7,11,12], anticoagulant [13,14], antiproliferating [12,13], and immunomodulating [5,7] properties, which makes them attractive for bionanoscience applications. Chitosan (CH) [15,16], heparin [17–19], chondroitin-6-sulfate [20–22], carrageenans [23–26], and fucoidans [27,28] are typical polysaccharides that are extensively used for formulating carrier matrix and stabilization of various Nps. It is evident that these materials are less cytotoxic and offers versatile material properties to polysaccharide-modified Nps [19]. Similarly, SPS modified Nps have a better cellular interactions due to their charge distribution and functional specificity. Most of the SPS are the integral components of extra cellular matrix; they include proteoglycans and sulfated GAG receptors in the cell surface, which play a vital role in attachment of various binding agents [29]. Thus, applications of SPS based Nps can mimic cell proteoglycans and compete with them in binding various pathogenic agents.

Natural and synthetic SPS are proved to be effective in biomedical applications, irrespective of their source. Degree of sulfation and molecular weight are the two important factors essential for its enumerable biological activities. Mauran (MR) is an anionic SPS extracted from a moderately halophilic bacterium, *Halomonas maura*. It is a stable polysaccharide with high uronic acid content and posses' properties like pseudoplasticity, viscoelasticity and thixotrophy. Also, it maintains its viscosity under a wide pH range of 3–11. Ability to bind various cationic molecules makes it an ideal molecule for nanotechnological applications [30–35]. MR on polyelectrolyte complexation with CH can form MR/CH Nps, which can be used for drug delivery applications and cell imaging studies.

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In our earlier research paper, we reported the novel synthesis of MR/CH Nps and encapsulation of 5-Fluorouracil (5FU) as test drug for conducting drug release kinetics. It was found that the 5FU encapsulated MR/CH Nps were capable of sustained drug delivery over a period of time with anticancer effect. Also, it was shown that they could be fluorescently modified and applied for cell uptake studies [31]. 5FU, pyrimidine analog with a broad-spectrum anticancer activity was used as the test drug for studying the anticancer activity against tumors [36]. With an aim of reducing the associated side effects and improving therapeutic index of 5FU, we used 5FU-MR/CH Nps as test nanodrug formulation for *in vitro* anticancer studies. Cellular uptake of Nps and confocal imaging of cells were performed using fluorescent MR/CH Nps. MR and CH was separately conjugated with fluorescent dyes, sypro ruby (SR) biofilm matrix stain and fluorescein isothiocyanate (FITC) respectively. Apart from the anticancer property, MR was found to be a potential antioxidant molecule [32]. In the present work, we are comparing the anticancer mechanism of drug loaded MR/CH Nps using 2 different cancer cell lines: glioma and breast adenocarcinoma and finding the possibility of its application as a novel incipient polysaccharide nanoparticle for defending oxidative stress.

## 2. Experimental

### 2.1. Materials

MR (Average molecular weight, $4.7 \times 10^6$ Da) [30] was extracted from *Halomonas maura*, ATCC strain 700995, and purified before lyophilization and collected in a powdered form. CH (Average molecular weight, $2.6 \times 10^6$ Da; viscosity, 484 mPa/S and 80% of deacetylation after drying) and FITC was purchased from Tokyo Chemical Industry (TCI, Kasei), Kita-Ku, Tokyo, Japan. 5-Fluorouracil was procured from Nacalai Tesque Inc., and Fetal bovine serum (FBS) from Gibco. Thiobarbituric acid (TBA), Sodium dodecyl sulfate (SDS), glutathione reduced (GSH), glutathione oxidized (GSSG), dithio-bis-2-nitrobenzoic acid (DTNB), hydrogen peroxide ($H_2O_2$), disodium hydrogen phosphate ($Na_2HPO_4$), sodium dihydrogen phosphate ($NaH_2PO_4$), and ethylene diamine tetra acetic acid (EDTA), and Dulbecco's modified Eagle's medium (DMEM) were procured from Sigma Aldrich, USA and BCA reagent kit (Qiagen, Germany). Alamar blue and Sypro-Ruby were purchased from Invitrogen, USA. All other chemicals and reagents used were of analytical grade.

### 2.2. Cell lines

Mouse fibroblast like cell line (L929) (RIKEN-RCB2619), Glioma (GI-1) cells separated from gliosarcoma (RIKEN-RCB0763) and breast adenocarcinoma (MCF7) (RIKEN- RCB1904) cells were procured from Riken bio resource center, Japan.

### 2.3. Electron tomography of H. maura

Electron tomography (ET) has been performed to obtain a detailed 3D structure of MR adhesion to the bacterial cell surface and the quantity of its release at different stages of growth using a high resolution transmission electron microscopy (HRTEM). *H. maura* (ATCC 700995) was propagated in MY medium for 15 days at 32 °C under an uniform shaking speed of 110 rpm. 5 ml of culture was taken after second and fourth day of incubation separately and ultrathin sectioning was performed [37] using TEM (JEOL, JEM-2200FS). Initially 2D images were taken by passing an electron beam at incremental degrees of rotation around the center of the bacterial sample. Finally all the images were collectively assembled for the generation of the 3D image of the bacteria with supra- molecular structure MR adhered to it.

### 2.4. Preparation of MR/CH Nps

MR was extracted from *Halomonas maura*, grown in MY medium as described elsewhere [30]. MR/CH Nps were synthesized by polyelectrolyte complexation of CH and MR solutions under strong magnetic stirring [31]. Polyanionic molecules can interact with polycationic molecules *via* ionotrophic gelation for the formation of Nps [38]. Here, 3 mg of polyanionic MR was dissolved in 1 ml of ultra pure distilled water with pH 4.3, maintained using 0.1 N HCl. 3 mg of CH was dissolved in 1% acetic acid (w/v). Later, CH solution was added drop wise to MR solution under strong magnetic stirring for nanoparticle formation. After 30 min constant stirring, the solution was centrifuged and the pellets were collected. Pellets were washed twice and resuspended in ultra pure water.

### 2.5. Encapsulation of 5FU

2.5 mg of 5FU was dissolved in 1 ml of methanol and mixed with MR solution before polyelectrolyte complexation. 5FU containing MR solution was stirred thoroughly for 30 min and later followed MR/CH synthesis as mentioned previously [31]. Drug loaded Nps were washed only once using ultra pure water to reduce the loss of 5FU entrapped. Drug loading efficiency was calculated by measuring the drug present in the supernatant after centrifugation and washing steps using reverse phase HPLC (RP-HPLC) (Shimadzu SCL-10A-VP System controller, SPD-M10A-VP Diode array detector, SIL-10AD-VP Auto Injector, LC-10AD-VP Liquid chromatograph) comprising a GL Sciences Inc., 5 μm, Inertsil ODS-3, $4.6 \times 250$ mm analytical column [36] and UV-Visible spectrophotometer (DU730, Beckman Coulter). Entrapment efficiency was calculated as the difference between the drug used for encapsulation and the free drug available in the supernatant [39].

### 2.6. Preparation of fluorescent MR/CH Nps

Two different nanoparticle suspensions were prepared with two different fluorochromes separately. In first set, MR was labeled with SR and secondly CH with FITC. 100 μl of SR solution was added directly to MR solution under constant stirring for 15–30 min in dark for fluorescent tagging. After which, unlabeled chitosan solution was added drop wise to this solution to form SR-MR/CH Nps spontaneously. In second set of experiment, FITC labeled CH was synthesized based on the reaction of the isothiocyanate group of FITC with primary amino group of CH [40]. 1 mg of FITC was dissolved in 1 ml of dehydrated methanol and mixed with CH solution under constant stirring. After 3 h of reaction in dark at ambient temperature, FITC labeled CH was added to unlabeled MR solution. SR and FITC labeled MR/CH Nps were washed and stored at dark for conducting cell uptake and imaging studies.

### 2.7. Characterization of MR/CH Nps

Morphology of drug loaded MR/CH Nps were characterized using scanning electron microscopy (SEM) (JEOL, JSM-7400F). The nanoparticle suspension was spread uniformly on a piece of Sn substrate and dried in a desiccator before the characterizations. Nps were analyzed using X-ray Photoelectron Spectroscopy (XPS) (KRATOS). Analysis was carried out under a basic pressure of $1.7 \times 10^{-8}$ Torr and the X-ray source used was anode mono-Al with pass energy of 40 (survey scan). XPS spectra for 5FU-MR/CH with peaks of C, N, O, S, P and F were obtained. Absorption spectra were