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Carboxymethylated ι-carrageenan conjugated amphotericin B loaded gelatin nanoparticles for treating intracellular *Candida glabrata* infections

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ABSTRACT

Intercellular *Candida glabrata* infections are difficult to treat due to poor penetration of drugs into the fungal niche. Delivering amphotericin B (Amp B) into the macrophages where the pathogen inhabits is an effective solution. We are studying the macrophage targeting proficiency of ι-carrageenan for the delivery of Amp B using gelatin A nanoparticles (GNPs). The choice of gelatin A was the outcome of *in silico* inspections where the amino functionalized polymer having the best docking score with Amp B was selected. We prepared a sustained release formulation of amp B loaded carboxymethyl ι-carrageenan conjugated gelatin nanoparticles (CMC-Amp B-GNPs) with size 343 ± 12 nm and −25 ± 5.3 mV zeta potential. The formulations were found to be stable, biocompatible and non-haemolytic. Flow cytometry analysis showed 3 fold higher uptake of CMC-GNPs compared to the GNPs by RAW 264.7 cells. CMC-Amp B-GNPs showed enhanced antifungal activity than bare Amp B and Amp B-GNPs.

1. Introduction

Candida glabrata which accounts for 10–15% of invasive candidiasis in the USA and Europe, evade the immune system by persisting inside the macrophages [1]. The resistance of this haploid yeast towards the azoles made Amphotericin B (Amp B) the mainstream among antifungals [2]. Amp B molecule binds with ergosterol resulting in the formation of permeable channels in the cellular membrane of the pathogen, ensuing in disturbance of membrane integrity and eventually cell death [3]. Regardless of broad-spectrum antifungal action, the medical use of Amp B is limited by nephrotoxicity, and lasting renal impairment, particularly if taken with other nephrotoxic drugs. Numerous strategies have been introduced to impoverish nephrotoxicity including formulations with alternate-day dosing, by sustained release nanoparticle drug delivery and by liposomes [4,5]. Delivering the drug specifically to the macrophages using nanoparticles can be an effective solution to reduce the toxicity and ensuring sustained release.

The mannose receptor (MR) is an endocytic protein found on macrophages and dendritic cells. The extracellular regions comprise three domains: (i) Carbohydrate recognition domain (CRD), (ii) fibronectin type 2 domain and (iii) cysteine rich domain (CysD). The CRD binds mannose and *N*-acetyl-glucosamine, whereas the CysD binds to sulfated polysaccharides such as carrageenan. Unlike the CRD, the binding of ligands to CysD is calcium independent. Numerous studies are reported where drug delivery systems are targeted to the MR using ligands such as dextran, mannose, fuciodan *etc* [6–8].

Carrageenan, a sulphated linear polysaccharide [9] of D-galactose and 3,6-anhydro-D-galactose is extracted from red seaweeds of class Rhodophyceae. Carrageenan is widely stuided for its gelling properties in food industry as well as for its inhibition of papillomavirus and rhinovirus infections [10,11]. To our knowledge limited studies are done about the macrophage targeting potential of carrageenan. Hence the objective of the current study is to integrate the macrophage targeting potential of carrageenan to the NPs drug delivery methods. We hypothesize that ι-carrageenan will help in the enhanced uptake of Amp B loaded NPs to the *C. glabrata* infected macrophages.

Computational docking studies between the drug and nanocarrier could enhance the understanding of the development of

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the drug delivery complex systems. Such computational models can reinforce the perception of the stability of the drug, its loading and release potentials [12,13]. The selection of the adjuvant for Amp B drug is performed by computational docking studies using AutoDock software, where the polymer molecule with the best docking score (binding affinity) is the most suitable carrier for the Amp B drug. Various amino functionalized polymers are virtually screened (VS) according to their binding affinity with Amp B. The ι-carrageenan is modified by the method of carboxymethylation so that it can be easily conjugated to amino group of drug carrier. Molecular dynamics (MD) simulations is another efficient tool where one can virtually know the dynamic molecular level changes in the target-ligand complex systems. MD study provides a detailed dynamic picture of the drug and the carrier complex interactions, their stability as well as their energy with respect to the MD simulation time [14].

In the present study, we used *in silico* tools for the selection of carrier and *in vitro* tools to develop carrageenan conjugated Amp B loaded nanocarrier system, for targeting to macrophages by employing the sulfated polysaccharide-cysteine rich domain of macrophages interaction mechanism for the treatment of intracellular *C.glabrata* infections.

2. Materials and methods

2.1. Materials

Following chemicals were used for the experiments: Gelatin A 240 Bloom, amphotericin B, Sabouraud Dextrose (SD) broth and SD Agar from Himedia; ι-Carrageenan C-1805 – viscosity average molecular weight: 78940.23 from TCI chemicals (India) Pvt Ltd; Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium (RPMI), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), 2-Morpholineethanesulfonic acid (MES) buffer from Sigma Aldrich; Alamar blue and Fluorescein isothiocyanate from Invitrogen; Fetal Bovine Serum (FBS) from Gibco (India); and Acetone from Sd Fine Chem Ltd. Macrophage murine RAW 264.7 (ATCC TIB-71) and fibroblast L929 (ATCC CCL-1) cell lines were purchased from National Centre for Cell Sciences, Pune, India. *C. glabrata* (clinical isolate) was obtained from Microbiology Department of Amrita Institute of Medical Sciences, Kochi, India.

2.2. Structure generation of different polymers, Amp B and MR

2D chemical structures of two strands each of amino functionalized polymers of eight different polymers: Poly (L-lysine), Poly (ethylene amine), Poly (amino styrene), Poly (allyl amine), Poly (ethylene glycol bis-amine), Poly (vinyl amine), chitosan, gelatin A; carboxymethylated ι-carrageenan (CMC) and gelatin A conjugated carboxymethylated ι-carrageenan (CMC-GNP) were prepared using ChemBioUltra software [15]. The size of the amine functionalized polymer was restricted to 2 strands, due to the size limitation of the polymeric entity and computational cost. The chemical structure of Amp B drug was downloaded from pubchem (CID: 5280965). The structure of the drug and different polymers were then optimized by MMFF94 minimization method using ChemBioUltra software. Crystal structure of the CysD of the MR (PDB ID: 1FWU) [16] was downloaded from Protein Data Bank.

2.3. Molecular docking studies of Amp B drug with different polymers

Molecular docking technique was used for computing the binding affinity of Amp B drug with seven different polymers as mentioned above using AutoDock vina software [17]. Binding energy of Amp B with each of these polymers was compared, the best binding pose and energy between Amp B and the polymer were selected for future studies.The interactions between these complex systems were studied using the PyMoL visualization software [18].

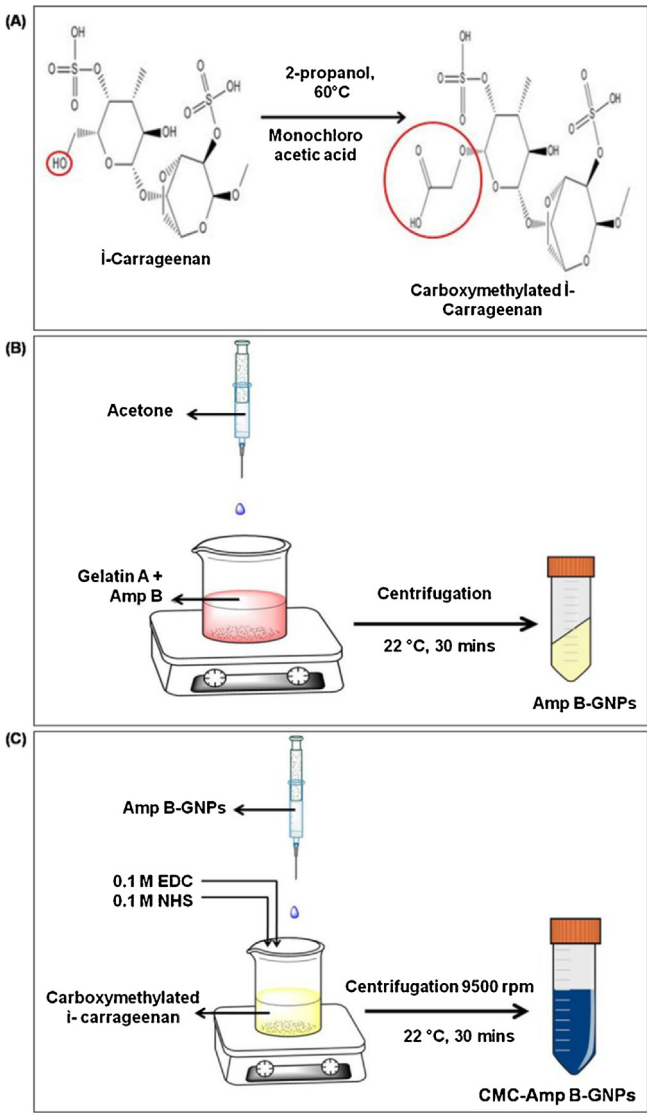

Fig. 1. Schematic representations of (A) Carboxymethylation of ι-carrageenan (B) Preparation of Amp B-GNPs and (C) Preparation of CMC-Amp B-GNPs.

2.4. MD simulations

MD simulation of the CMC-Amp B-GNP complex system was performed for 10 ns time interval using Discovery studio 4.0 program [19]. Initially, the complex structure was minimized using CHARMm force field to evade van der Waals clashes and bad geometry. Next the complex system is solvated followed by heating for 2 ps, equilibration and production run for 10 ns time interval. The MD simulation was carried out with a dielectric constant of 79 corresponding to PBS. MD trajectory was saved every 2 ps time interval for further analysis.

2.5. Carboxymethylation of ι-carrageenan

Carboxymethylated ι-carrageenan was prepared as described previously by Leong et al. [20]. Briefly, 200 mg of ι-carrageenan