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Chitosan nanoencapsulation of flavonoids enhances their quorum sensing and biofilm formation inhibitory activities against an *E.coli* Top 10 biosensor

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ABSTRACT

Phytochemicals have been found to be promising alternatives to conventional antibiotic therapies for the control of bacterial infections, as they may entail less selective pressure and hence reduce the development of resistance. This study involved examining the inhibition of biofilm formation and of quorum sensing (QS), and the cytotoxicity on mammalian cells of two flavonoids, quercetin and baicalein, in free form and associated into chitosan-based nanocapsules. This was done by use of a transformed *E. coli* Top 10 biosensor strain, while the cytotoxicity was evaluated on MDCK-C7 cells. In free form, application both flavonoids exhibited slight inhibitory activity on the QS response and biofilm formation, a scenario that was improved positively upon encapsulation with chitosan (Mw ~115,000 g/mol and DA ~42%). The association efficiency of 99% (quercetin) and 87% (baicalein) was determined, and each formulation had an average diameter of 190 ± 4 and 187 ± 2 nm, and zeta (ζ) potential of +48.1 ± 2.03 and +48.4 ± 3.46 mV, respectively. Both types of systems were stable against aggregation in M9 and MEM media. The *in vitro* release kinetics data of both flavonoids seemed to be similar with only ~20% released over the first 5 h, or ~10% over the first 4 h, respectively, with subsequent sudden release increase up to ~40% in both cases. The free phytochemicals seemed to be cytotoxic to MDCK-C7 cells at higher doses, however, upon nanoencapsulation, a cytoprotective effect was evidenced. We have gained proof-of-principle of the advantages of encapsulation of two bioactive flavonoids.

1. Introduction

Bacterial quorum sensing (QS) is an area that currently has drawn increasing interest especially in bacterial pathogenicity given its responsibility to their phenotypic traits. QS is a cell-to-cell communication process that enables bacteria to assume a collective synchronous behaviour under the influence of chemical signalling molecules called autoinducers (AIs) which mediates interspecies or intraspecies behaviours [1]. As the cell density increases, AIs accumulate in the environment where they are sensed by protein receptors, which in turn act as transcriptional activators for the expression of specific genes; the products of these genes direct activities that are beneficial when performed by bacterial communities acting in synchrony [1]. Homoserine lactones (HSLs) produced by Gram-negative bacteria are the best studied AIs and possibly the most common group of bacterial QS AIs while Gram-positive bacteria use small peptides called AI2 [2,3]. Frequently, pairs of genes encoding the HSL synthase and the HSL-sensing transcriptional regulator are found close to each other in bacterial genomes [4]. This phenomenon was first discovered in the marine bacterium *Vibrio fischeri* [5] and the term *quorum sensing* was later coined by Fuqua et al. [6] referring to the acylated homoserine lactone (AHL)-mediated luxR/luxI regulated system.

Biofilm formation is one of the virulence traits associated to QS [7]. Bacteria in biofilms are not easily reached neither by antibiotic agents nor by the host immune system, as they act as diffusion barriers, hence, bacteria continue multiplying inside the extracellular matrix up to a point at which they disperse to start forming new biofilm communities [8]. Microbial biofilms readily form on indwelling medical devices and cause serious diseases that are hard to control since effective therapy is lacking. According with

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CDC and NIH, between 65%–80% of infections could be caused by biofilms, demonstrating the need to develop improved treatment options [9]. Therefore, targeting and blocking QS circuits in bacteria may represent a strategy to disarm their virulence and hence, making them more susceptible to elimination by the host immune system or low doses of antibiotics [10]. Anti-virulent agents possessing QS inhibitory activity might not pose selective pressure on bacterial pathogens and thus, may contribute to reduce the rapid emergence of so-called "superbugs", bacteria resistant to several antibiotics [11,12]. Disruption of QS-regulated processes has been accepted to reduce accumulation of virulence factors at the infection site, and dismantles the collective virulent power of pathogens. Such antiquorum sensing strategies are collectively called quorum quenching (QQ) [13].

Plant phytochemicals may be possessing anti-quorum sensing activities based on the various studies. Among them include, but are not limited to, zingerone [12], curcumin [14], cinnamon oil [15], pure *trans*-cinnamaldehyde [16,17], proanthocyanidins [18], and flavonoids like quercetin [19]. On the other hand, it is now known that the activity of anti-virulent agents or antimicrobials may improve upon encapsulating them in biopolymer-based materials [20,21]. This may be attributed to the overall increase in their bioavailability, lower dosage release for longer periods, to the overcoming physiological barriers resistances, among other, as compared to the free agents [20–22].

Different types of nanocarrier formulations have extensively been used for drug delivery [23,24]. Despite this, minimal information is available for their use in delivering QS inhibitory agents. However, biologically-derived materials such as polysaccharides and proteins can be used as components of nanoformulations as they are fully biodegradable, biocompatible and non-immunogenic. Hence, they are attractive for the development of innovative drug delivery vehicles [25]. Among the most interesting biopolymers in this area, is chitosan that has gained enormous traction. Chitosan are a family of amino linear heteropolysaccharides comprised by β (1–4) 2-acetamido-2-deoxy-β-D-glucopyranose (N-acetyl glucosamine) and 2-amino-2-deoxy-β-D-glucopyranose (D-glucosamine) units, randomly or distributed as blocks throughout the biopolymer [26]. Chitosan and its chemical derivatives are particularly attractive as a building blocks for drug delivery nanoformulations in light of its biocompatibility, biodegradability, bio– and mucoadhesivity, and hydrophilic character that facilitate the administration of poorly absorbable drugs across various epithelial barriers [27–31]. It can also be easily be manipulated chemically and hence forming a good vehicle for delivering both hydrophobic and hydrophilic drugs/agents to target sites [26].

Quercetin and baicalein (see supplementary data – S1) are both flavonoid lipophilic phytochemicals. They are common polyphenolic compounds in nature and are found ubiquitously in plants, including food products like onions, many fruits, or in herbs [32–35]. Previous studies have shown that quercetin and baicalein can act as competitive inhibitors for the signalling compound towards LasR/Rh1R and orphan regulator QscR receptors pathway commonly found with *P. aeruginosa* and can serve as novel QS-based antivirulence agents to manage such groups of pathogens [19,36–38]. In these studies, it was deduced that both flavonoids have both antivirulence and antibiofilm formation activity against *P. aeruginosa* PA01. However, only few of the available studies have reported on the role of plant extracts and phytochemicals as QS inhibitors, deal with the potential mechanisms of action [39] and even their activity upon nanoencapsulation. In this study, we report the AQS and antibiofilm activities of free and chitosan nanoencapsulated quercetin and baicalein against a bioengineered *E. coli* Top 10 biosensor that has a Lux R receptor pathway. Also their viability towards MDCK–C7 mammalian cell line was studied.

2. Materials and methods

2.1. Materials

The parent chitosan (Mw ~288,000 g/mol and DA 16%) was provided by Mahtani Chitosan Pvt. Ltd., India (Sample code 132; batch no. SCCF 20140609). The chitosan used for this study was derived from this parent sample as described in previous studies [40,41]; the Mw was ~115,000 g/mol as determined by HPSEC-MALLS and the DA of ~42% as measured by ^{1}H NMR spectroscopy according with the method by Lavertu et al. [42]. Lecithin was a kind gift from Cargill (Epikuron 145 V, Cargill Deutschland GmbH & Co. KG, Hamburg, Germany); Miglyol 812 N was from Sasol GmbH (Witten, Germany). 3OC_6HSL and all other chemicals were of analytical grade and unless otherwise stated were from Sigma (Sigma-Aldrich, Hamburg, Germany).

2.2. Baicalein and quercetin nanocapsules formulation

CS-based nanocapsules of both baicalein (Mw- 270.24 g/mol) and quercetin (Mw- 302.24 g/mol) were prepared according with the protocol originally developed in Prof. Maria J. Alonso's laboratory (University of Santiago de Compostela, Spain) with slight modifications [25,43]. Briefly, due to the hydrophobic nature of the two phytochemicals 1 mg of each was dissolved in 1 mL of absolute ethanol. Then 256 μL of the each of the phytochemical was taken, 250 μL of lecithin (100 mg/mL of lecithin in ethanol) and 62.5 μL of Miglyol 812® were added to a beaker with 4.75 mL of absolute ethanol. Immediately afterwards, this organic phase was poured into of 10 mL of CS solution (0.5 mg/mL) which turned milky. Ethanol and a portion of the volume of water were evaporated in a rotavapor at 40 °C for ~8–15 min to a final volume corresponding with one third of the original one. The blank NCs were also made according with the same protocol but no phytochemicals were added in the organic phase. All preparations were made in triplicate.

2.3. Physicochemical properties of the nanocapsules

The size distribution of the nanoformulations was determined by dynamic light scattering with non-invasive back scattering (DLS-NIBS) with a measurement angle of 173°. The zeta (ζ) potential was measured by mixed laser Doppler velocimetry and phase analysis light scattering (M3–PALS). A Malvern Zetasizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK) fitted with a red laser light (λ = 632.8 nm) was used for both methods. The samples were diluted 1:100 in acidified water before measurement.

2.4. Encapsulation/association efficiency

It was determined as per the method used by Kaiser et al. [25]. Briefly, aliquots of 500 μL of the flavonoid-loaded nanoformulations were pipetted into Vivaspin® 500 ultrafiltration spin columns and then were centrifuged by centrifuge (Mikro 220 R, Hettich GmbH & Co. KG, Tuttlingen, Germany) at 16,000 rpm for 1 h at 15 °C. Free baicalein and quercetin content of the subnatant were determined by UV spectroscopy at λ = 324 and 374 nm, respectively, using the corresponding calibration curves registered either in absolute ethanol or upon dilution in M9 medium (supplementary data S2 and S3, respectively). The association efficiency was calculated as the difference between the total amount of the flavonoid incorporated in the formulation and the amount present in the subnatant. The association of quercetin was also determined by resuspending the creamy layer with distilled water up to 1 mL and then 25 μL were taken and mixed with 475 μL of absolute ethanol