

Enhanced antimicrobial effect of berberine in nanogel carriers with cationic surface functionality

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We report a strong enhancement of the antimicrobial action of berberine encapsulated into polyacrylic acid-based nanogels followed by further surface functionalisation with a cationic polyelectrolyte (PDAC). Due to the highly developed surface area, the nanogel carrier amplifies the contact of the berberine with the microbial cells and increases its antimicrobial efficiency. We show that such cationic nanogel carriers of berberine can adhere directly to the cell membranes and maintain much high concentration of berberine directly onto the cell surface. We demonstrated that the antimicrobial action of the PDAC-coated nanogel loaded with berberine on *E.coli* and *C.reinhardtii* is much higher than that of the equivalent solution of free berberine due to the electrostatic adhesion between positively charged nanogel particles and the cell membranes. Our results also showed a marked increase of their antimicrobial action at shorter incubation times compared with the non-coated nanogel particles loaded with berberine at the same conditions. We attribute this boost in antimicrobial effect of these cationic nanocarriers due to their increased concentration on the cell membranes which sustains high concentration of released berberine causing cell death within much shorter incubation times. This study can provide a blueprint for boosting the action of other cationic antimicrobial agents by encapsulating them in nanogels carriers functionalised with a cationic surface layer. Our nanotechnology approach would lead to developing more effective wound dressings, disinfecting agents, antimicrobial surfaces, antiseptic and antialgal/antibiofouling formulations.

Introduction

Engineered nanomaterials have been recognised for their potential to solve many challenges in the fields of environmental remediation,¹ agriculture,² food and health care.³ Drug delivery using nanocarriers is a rapidly developing field focused on transmitting optimal amounts of drugs to the desirable sites such as diseased/infected tissues, tumors, etc. while reducing unwanted side effects of the drugs on other tissues.^{4,5} The nanomaterials for drug delivery must be biocompatible, nontoxic, biodegradable and non-immunogenic. The most common drug delivery nanocarriers include liposomes, polymeric micelles and dendrimers, artificial DNA structures, and biodegradable scaffolds.⁶ Nanogels are cross-linked polymeric nanoparticles⁷ produced by physically or chemically cross-linking polymer networks that swell in an aqueous solutions due to the absorption of water facilitated by the presence of hydrophilic groups such as ether, sulphate, hydroxyl and carboxyl groups which are present in polymer chains.⁸ Good biocompatibility is vital for nanocarriers used for drug delivery to avoid adverse biological responses at the molecular, cellular or organ levels when utilized.^{9–11}

Berberine (BRB) is a isoquinoline quaternary alkaloid extracted from many kinds of medicinal plants such as *Hydrastis*

canadensis, *Berberis aristata*, *Coptis chinensis*, *Coptis rhizome*, *Coptis japonica*, *Phellodendron amurense*, *Phellodendron Chinese schneid*.^{12,13} It has attracted wide attention as a therapeutic agent against hyperlipidemia, diabetes, metabolic syndrome, polycystic ovary syndrome, obesity, fatty liver disease, coronary artery disease¹⁴ gastroenteritis and has antimicrobial, antidiabetic and anti-inflammatory properties.^{13,15,16} Berberine employs several antimicrobial effects being a NorA substrate capable of gathering in bacterial cells and of binding both single and double-stranded DNA, thus causing bacterial death by DNA damage.¹² It shows a weak activity against Gram-negative bacteria,¹² but has more powerful effect against Gram-positive bacteria, such as *Mycobacterium tuberculosis* and MRSA (Methicillin-Resistant *S.aureus*), by the multi drug resistant (MDR) pump NorA inhibition.^{12,17,18} It also shows antifungal activity on *Aspergillus*, *Penicillium*, *Candida* and *Cryptococcus*^{13,16} and has an anti-inflammatory activity.¹⁹ Several studies have been reported where berberine has been encapsulated in nanoparticles to form (berberine chloride-O-Hexadecyl-Dextran NPs) (BC-HDD NPs). Upon incubation with glucose stressed hepatocytes, a decrease in reactive oxygen species (ROS) generation, oxidative stress and lipid peroxidation was detected in BC-HDD NPs treated cells in comparison with bulk berberine solution.²⁰ Tsai et al. entrapped berberine into a chitosan hydrogel to design a transdermal delivery system for the treatment of cutaneous leishmaniasis.²¹ Chang et al. developed a novel nanocomposite with a heparin shell for berberine delivery to treat *H. pylori*.²²

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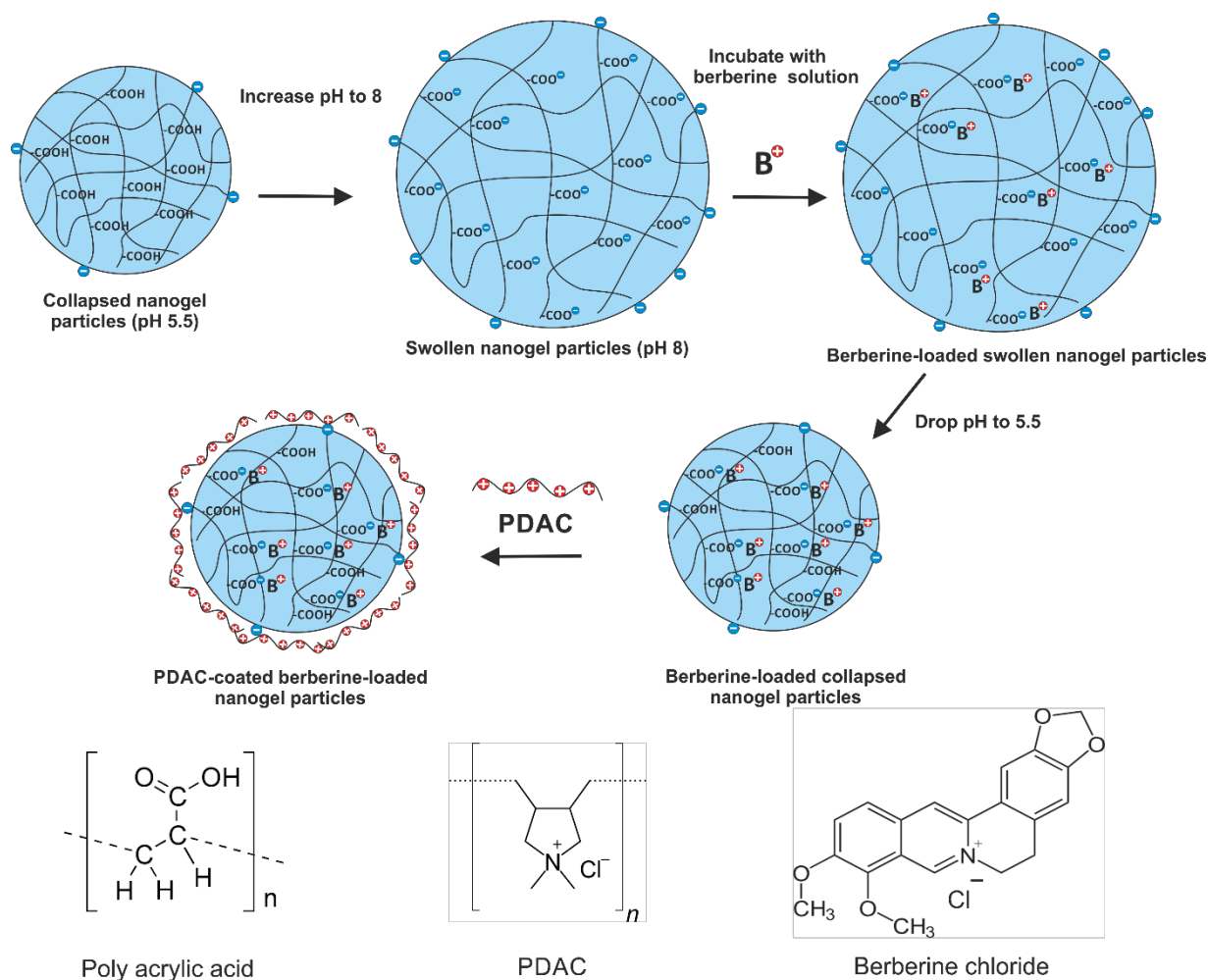


Figure 1. The schematic diagram for the encapsulation of berberine (from berberine hydrochloride) into polyacrylic nanogels by using swelling/deswelling cycle of the nanogel in the basic (pH 8) and acidic (pH 5) medium, respectively. The berberine-loaded nanogel was surface-functionalised with a cationic polyelectrolyte (PDAC). The chemical formulas of the carbomer nanogel (partially crosslinked polyacrylic acid), the PDAC and the berberine are also presented.

Lin *et al.* used berberine-loaded fucose-chitosan/heparin nanoparticles to obtain an enhanced suppressive effect when they interact with *H. pylori* and effectively reduce gastric inflammation in an *H. pylori*-infected animal study.²³ Recently, a new anhydrous reverse micelle (ARMs) delivery system was successfully prepared for berberine for enhanced drug oral bioavailability and anti-diabetic efficacy.²⁴ Berberine has also been shown to have enhanced anticancer efficacies through a solid lipid nanoparticle (SLN)-based delivery system demonstrated on MCF-7, HepG 2, A549 cancer cells²⁵ and human hepatic carcinoma.²⁶ In other studies, PLGA (polylactide glycolic acid) nanoparticles were prepared and loaded with berberine using single emulsion as well as multiple emulsion solvent evaporation techniques.²⁷ Yeast cells of *S. cerevisiae* have also been used for encapsulating berberine as novel carriers for the food and drug industries.²⁸ Likewise, berberine hydrochloride was encapsulated into alginate microspheres via an emulsification/gelation method.²⁹ Other authors report the development of berberine-loaded chitosan/fucoidan-taurine

nanoparticles as an epithelial protective material to prevent redistribution of TJ protein caused by bacterial endotoxin (LPS).³⁰ Hydrogel composites also were prepared using a cross linking method with variable amounts of gellan gum and polyvinyl alcohol loaded with berberine and its functionalization to gold nanoparticles.³¹

Recently, development of nanocarriers based on biodegradable materials attracted a lot of attention following concerns about the post-use fate of the nanocarrier formulations. Frangville *et al.*³² developed biodegradable nanoparticles of lignin using a solvent and pH change from Kraft lignin which showed no measurable toxicity against proxy organisms like yeast and microalgae. Richter *et al.*^{33,42} utilised this biodegradable nanocarrier to propose a silver ion delivery system based on silver ion-loaded lignin nanoparticles which were further coated with cationic polyelectrolyte. The produced silver ion delivery vehicles proved to more potent antimicrobial agent for a range of bacteria than metallic silver nanoparticles. A range of other antimicrobial particles have recently been developed.⁴³ Al-

Awady *et al.*⁴⁴ showed that the nanotoxicity of polyelectrolyte-coated titania nanoparticles alternates with the surface charge with the particles with cationic outer layer (or bare titania nanoparticles) being much more toxic than the ones with an outer layer of anionic polyelectrolyte.

In the present work, we expand these ideas to encapsulate berberine for antibacterial and antialgal applications by using Carbopol Aqua SF1 nanogels based on a partially cross-linked polyacrylic acid polymer. We study the stability of the berberine-loaded nanogel, its encapsulation efficiency, the controlled release of encapsulated berberine and the toxicity of the formed berberine-nanogel complexes and their cationic surface functionalised versions. Carbopol Aqua SF1 is an aqueous dispersion approximately 30% w/v of a lightly cross-linked and highly hydrophilic polyacrylic nanogel which does not exhibit toxicity towards animal organs and a range of microorganisms.³⁴

Figure 1 explains the encapsulation steps of berberine into Carbopol Aqua SF1 and the surface functionalisation of the produced nanoparticles with a cationic polyelectrolyte. The conjugation of the nanogel and the berberine is based on electrostatic interaction with non-covalent bonding to ensure further releasing of the antimicrobial agent upon incubation of the loaded nanogel particles with the tested microorganisms. We characterised the produced berberine-loaded Carbopol nanogels (BLC) and detailed results are presented and discussed in the Electronic Supplementary Information (ESI). The main paper is concerned with the antibacterial and antialgal action of BLC on two proxy microorganisms, *E. coli* and *C. reinhardtii* respectively. We also explore the link between the berberine release kinetics and the boost of its antimicrobial action after coating of the BLC nanocarriers with a cationic polyelectrolyte. This approach has the potential to boost the action of other antimicrobial agents when encapsulated in cationic-surface functionalised nanocarriers.

Materials and Methods

Materials

Deionised water purified by reverse osmosis and ion exchange from a Milli-Q water system (Millipore, UK) was used in all our studies. Its surface tension was 71.9 mNm^{-1} at 25°C , with measured resistivity less than $18 \text{ m}\Omega \text{ cm}^{-1}$. Carbopol Aqua SF1TM aqueous suspension (30 wt%) was supplied by Lubrizol, USA. Poly(diallyldimethylammonium chloride) (PDAC) was supplied from Sigma Aldrich, UK. Berberine hydrochloride (98%) and fluorescein diacetate (FDA, 98%) was delivered by Fluka, UK. *Chlamydomonas Reinhardtii* (cc-124 strain) was sourced from Flickinger's group at North Carolina State University, USA. This microalgae culture was grown in Tris-Acetate-Phosphate (TAP) culture medium with an incubation temperature of 30°C . The culture media for *C. reinhardtii* consisted of TAP salts (NH_4Cl ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), phosphate buffer solution (PBS) and Hutner's trace elements solution (EDTA disodium salt, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, H_3BO_3 , $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, all purchased from Sigma-

Aldrich, UK. We used two different batches of cationic and anionic polyelectrolytes: poly(sodium 4-styrene sulfonate) sodium salt (PSS), M.W. 70 kDa and 10 kDa, respectively and poly(allylamine hydrochloride) (PAH), M.W. 15 kDa and 65 kDa, respectively, all purchased from Sigma Aldrich, UK. The microalgae batch was grown in the TAP media at pH 7 while being illuminated for 72 hours with a white luminescent lamp with a light intensity of 60 W m^{-2} under constant stirring with a magnetic stirrer.^{33,36} The stock cultures of *C. Reinhardtii* which were used for testing were with typical concentration 3.7×10^5 cells per mL determined by automatic cell counter (Cellometer) and the *E. coli* bacterial culture stock was with approximately 4.7×10^7 cells per mL. We found that the results from the antimicrobial testing of our nanocarriers were not sensitive to the exact cell concentration in the stock used over a wide range of values as the cell viability is determined as a relative value to the viability of the control system.

Encapsulation of berberine into polyacrylic nanogel particles

This procedure for preparation of BLC was done using a swelling/deswelling technique. Briefly, a 0.1 wt% Carbopol Aqua SF1 nanogel suspension was prepared by weighing 0.1 g of stock suspension and dispersed in a 20 ml Milli-Q water, then adjusted to pH 8 using 2 drops of 0.25 M NaOH. 75 ml of 0.2 wt% of berberine hydrochloride solution was added to the nanogel suspension to prepare 0.15 wt% berberine in 0.1 wt% carbopol suspension. The produced BLC dispersion was stirred for 30 min and the pH was decreased to pH 5.5 using 2 drops of 0.25 M HCl while stirring. After that the solution was centrifuged for 30 minutes at 8500 rpm. The supernatant was taken to measure the encapsulation efficiency of berberine in the nanogel and the BLC precipitate was washed twice in Milli-Q water. Afterwards, the precipitate was dispersed in Milli-Q water and the pH of the solutions was increased to pH 8 using 2 drops of 0.25 NaOH solution. The resulting solution was stirred overnight to make sure all the aggregated Carbopol solution had been allowed to swell at pH 8 and then the pH of the solution was decreased to pH 5 using acetate buffer solution. The total volume of BLC suspension was kept at 100 ml.

Release kinetics of berberine from BLC

It was investigated the percentage of *in vitro* berberine release using aliquots of equivalent concentrations of berberine-loaded 0.1 wt% Carbopol. This formulation had already been optimized in terms of the encapsulation stability with particle size and zeta potential of the colloidal nanogel was 135 nm and -40 mV at pH 5.5, respectively. The berberine-loaded nanogel suspension was placed into a dialysis cassette (10K MWCO, Thermo Fisher, UK) which allowed the berberine released from the nanogel to diffuse through its membrane pores. The dialysis device was placed into: (i) a beaker which was already pre-filled with acetate buffer solution (pH 5.5) or (ii) a beaker with a phosphate buffer at pH 7.5 in order to monitor the amount of released berberine at a specific pH. After that, the concentration of the released berberine *in vitro* was measured using UV-Visible spectrophotometer. All release experiments were carried out

in triplicate. The percentage of cumulative drug release was calculated.

Functionalisation of the nanogel particles with cationic polyelectrolytes

Cationic polyelectrolyte was used to reverse the surface charge of Carbopol Aqua SF1 nanogel. PDAC solutions with a range of different concentrations (0.001–0.01 wt%) were mixed separately with a fixed concentration of the nanogel (0.1 wt%) at pH adjusted in the range to 4.75–5.00 using acetate buffer with the final volume of the suspension being 10 ml. The zeta potential was measured for each sample to find out the optimum concentration of both the cationic polyelectrolyte and the nanogel which in turn give stable suspension.

Surface functionalization of BLC with PDAC

A 1.10 ml aliquot of 0.2 wt% PDAC solution was rapidly added to a suspension of 10 ml of the BLC suspension with vigorous shaking and then diluted to 20 ml to form PDAC-coated 0.0075 wt% berberine-loaded 0.05 wt% Carbopol Aqua SF1 nanogel dispersion (PDAC-coated BLC). The resulting solution was used as a stock to study its antimicrobial activity on *C.reinhardtii* and *E.coli*.

Cytotoxicity test of the non-coated nanogel particles

A cell viability assay was performed to determine the cytotoxicity of the nanogel on both of *C.Reinhardtii* and *E.coli*. A 0.3 wt% dispersion of Carbopol Aqua SF1 was prepared in a 100 ml volumetric flask by dilution of an aliquot of the supplied stock with milli-Q water. The pH was adjusted to 5.5 using acetate buffer. 5 ml aliquots the cell suspension was washed from the culture media and incubated with a series of 5 ml aliquots of the nanogel dispersion of concentrations (0.01–0.15 wt%) and tested at different incubation times. The control sample was treated in the same way without incubation with the nanogel. The cell viability was examined as described below.

Cell viability assay protocol for *C. Reinhardtii*

1 ml aliquots of the cell suspension were taken from each treated sample, centrifuged for 4 minutes at 3000 rpm and washed with Milli-Q water to remove the excess of the nanogel. The cells were re-suspended in 1 ml of Milli-Q water and treated with FDA cell viability assay as follows. Fluorescein diacetate (FDA) is a non-polar esterified compound which easily diffuses through the cell membranes.^{37,38} If the cell is viable and its membrane is intact the hydrolysis of the non-fluorescent precursor (FDA) by intracellular esterase leads to accumulation of fluorescein.³⁹ We used 0.5% w/v FDA dissolved in acetone. 20 μ L of this solution was used per mL of cell sample. The tube with the mixture was shaken for 10 minutes using a vortex at 1500 rpm in dark conditions to avoid the photobleaching of the produced fluorescein inside the viable cells. The cells were washed three times with Milli-Q water using a centrifuge (Eppendorf Minispin plus) at 3500 rpm for 5 minutes in order to remove any extracellular fluorescein. The microalgae cell viability tests were carried out using an automatic cell counter.

Antimicrobial assay for aqueous solutions of free berberine

We studied the biological activity of free berberine upon incubation with *C.Reinhardtii* and *E.coli*. 5 ml aliquots of the suspensions (washed from the culture media) were incubated with 5 ml aqueous solution of berberine hydrochloride of a series of concentrations (0.005–0.15 wt%) prepared and diluted with autoclaved Milli-Q water from 0.2 wt% stock solution at various incubation times. The control sample was kept under the same conditions but without berberine. After that, 1 ml aliquots of the suspended cells were taken from each treated sample, centrifuged for 4 minutes at 3000 rpm, washed to remove the supernatant and then re-suspended in 1 ml Milli-Q water. FDA live/dead viability assay was used as described above for *C.Reinhardtii*. *E.coli* cell viability was tested using BacTiter-Glo reagent followed by measurement of the relative luminescence of the sample (see below).

Antialgal activity of BLC on *C. Reinhardtii*

The activity of BLC towards *C.reinhardtii* was tested in suspension culture as follows. A 100 ml aliquot of 5×10^6 cells ml^{-1} of the *C. reinhardtii* culture was centrifuged out of the culture media, washed three times and re-dispersed with 50 ml Milli-Q water. 5 ml aliquots of 1×10^7 cells ml^{-1} suspension of washed *C. reinhardtii* microalgae cells were then incubated with a series of 5 ml aliquots of different equivalent concentrations of 0.15 wt% berberine-loaded 0.1 wt% Carbopol Aqua SF1 suspension (0.01, 0.025, 0.05, 0.075, and 0.1 wt%). The cell viability was measured at different incubation times up to 6 hours using the FDA live/dead assay.

Antimicrobial activity of BLC on *E.coli*

The activity of BLC towards *E.coli* was tested in suspension culture as follows. 50 ml of dispersed *E.coli* cells cultured in LB medium were washed three times and redispersed with 50 ml autoclaved Milli-Q water in 50 ml centrifuge tube. The relative luminescence unit for the culture media free cells was found to be 140 a.u. 5 ml aliquots of this cell dispersion were incubated with 5 mL BLC suspension of different equivalent overall concentrations of berberine. After each incubation, 1.0 ml of each *E.coli* suspension sample was washed and re-suspended in 1 ml Milli-Q water. Then 100 μ L of culture media-free *E.coli* cells was incubated with 100 μ L of BacTiter-Glo microbial cell viability reagent in white opaque 96-well microplate solid flat bottom. The relative luminescence intensity was measured as a function of incubation time to find out the cell viability upon incubation with nanogel loaded with different concentrations of encapsulated berberine.

Cytotoxic Effect of PDAC on *C. Reinhardtii* and *E.coli*

We tested the toxicity of the cationic polyelectrolyte (PDAC) towards both *C.reinhardtii* and *E.coli*. A series of aqueous solutions of PDAC (0.0045–0.05 wt%) were incubated separately with 5 ml of each microorganisms culture dispersion and the cell viability was measured at different incubation times as described above.

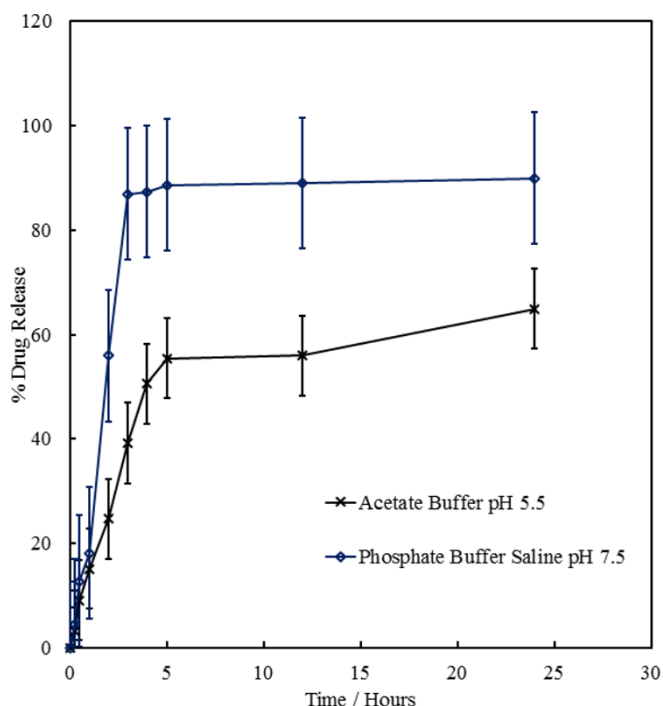


Figure 2: The percentage of berberine release as a function of time for 0.15 wt% berberine-loaded in 0.1 wt% Carbopol (BLC).

Cytotoxic effect of PDAC-coated nanogel on *C. Reinghardtii* and *E. coli*

This procedure explains the cytotoxic effect of the PDAC-coated nanogel on *C.reinghardtii* and *E.coli*. 5 ml aliquots of PDAC-coated nanogel suspension of each concentration (0.0018–0.018 wt%) were incubated individually with 5 ml each suspension of *C. Reinghardtii* or *E. coli*. Then the cell viability was measured using the same assay. This experiment was done as a control to evaluate the antimicrobial activity of the PDAC-coated nanogel carrier without a berberine load.

Antimicrobial activity of PDAC-coated BLC nanogel

We investigated the antimicrobial action of PDAC-coated BLC nanogel suspension on *C.reinhardtii* and *E.coli* as follows. 50 ml aliquots of each cell culture were centrifuged from the culture media, washed three times and re-dispersed in 5 ml autoclaved purified water. 250 μ l aliquots of the washed cells were then incubated with 5 ml of series of diluted suspensions of the PDAC-coated BLC stock dispersion (PDAC-coated 0.0075 wt% berberine-loaded 0.05 wt% Carbopol Aqua SF1) whereby the cell viability was measured according to the protocols described above. For *C.reinhardtii*, we varied the PDAC-coated BLC concentrations from 0.0018 wt% PDAC-coated 0.0015 wt% BLC suspension to 0.0045 wt% PDAC-coated 0.00375 wt% berberine-loaded nanogel complex. For *E.coli* the concentration range was from 0.0022 wt% PDAC-coated 0.0015 wt% BLC to 0.0088 wt% PDAC-coated 0.006 wt% BLC nanogel.

Protocol for SEM imaging of the treated cells

The cells (microalgae or *E.coli*) were washed from their culture media using PBS, adhered to Aclar film, fixed in 2.5 wt% glutaraldehyde in 0.1M cacodylate buffer pH 7.2 for 2 hours at room temperature, rinsed three times with cacodylate buffer, post fixed in 1 wt% osmium tetroxide in cacodylate buffer for one hour, rinsed in cacodylate buffer, dehydrated in series of ethanol-water solution with decreasing water content up to 100% ethanol and then dried using liquid carbon dioxide in a critical point dryer.

Results and Discussion

Encapsulation of berberine into Carbopol Aqua SF1 nanogel

The strategy behind the swelling/deswelling technique is to allow berberine cations to infuse the core of the nanogel particles through increasing pH of the nanogel suspension up to 8. At this pH, the nanogel particles are swollen and the carboxyl groups of the polyacrylic acid more deprotonated which allows the berberine cations to interact with them. The pH drop to 5.5 causes shrinkage of the BLC nanogel. The berberine-loaded nanogel at pH 5.5 was centrifuged to remove the free berberine. To avoid precipitate in this process, the pH of the berberine-loaded nanogel was raised to pH 8 so that the particles swell and disperse again followed by de-swelling at pH 5.5 using acetate buffer. This procedure yielded fully dispersed and stable suspension of berberine-loaded Carbopol nanogel. In the ESI we present a detailed study of the swelling behaviour of the Carbopol nanogel before and after loading with berberine. The collapsed (not-loaded) nanogel particles have an average diameter of about 100 nm at a pH below 6.8 and swell very sharply to approximately 330 nm just above pH 6.8. After loading with berberine at pH 8 and then collapsing at pH 5, the size of the BLC particles is about 130 nm (c.f. Figures S3 and S9). The overall swelling is attributed to the increase of the dissociation of the carboxyl groups of the nanogel which in turn caused electrostatic repulsion among them and expands the nanogel matrix. This facilitates the release of the previously entrapped berberine out in the surrounding medium. Both the non-loaded Carbopol nanogel and the BLC have a negative surface charge in their collapsed state which is preventing them from adhering to the negatively charged cell walls. In the ESI we present a range of UV/vis and FTIR spectroscopy studies of the BLC nanogel which confirm the presence of approximately 10% berberine in the particle cores. We present data on the BLC particle size upon coating with different cationic polyelectrolytes (see Figures S10 and S13).

BLC can slowly release berberine upon dilution (see Figure 2). It can be observed that the berberine release depends on the pH of the medium. At pH 5.5, approximately 25% of the encapsulated berberine was released in 2 hours and then the 55 % was released after 5 hours. After this the % released levelled off with only a slight increase of 65% after 24 hours. The slow release at pH 5.5 occurred due to the high zeta potential value of the anionic Carbopol nanogel at that pH which allowed delay in the release of the berberine cations out of the nanogel.

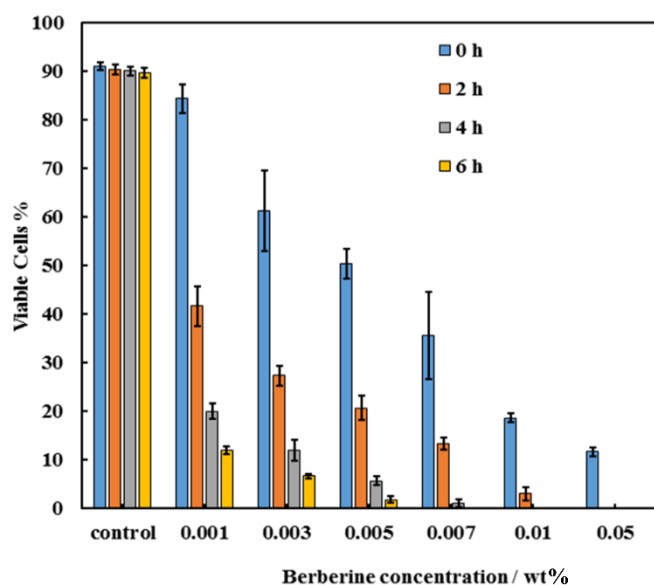


Figure 3. The viability of *C. reinhardtii* cells incubated with aqueous solutions of different concentrations of free berberine hydrochloride at different incubation times. The pH of the solution was adjusted with acetate buffer solution (pH 5.5).

At pH 7.5, there is a more dramatic release of 86 % berberine up to 3 hours and 90% after 24 hours. This due to the decrease in the zeta potential value at pH 7.5 which means the berberine becomes unconjugated from the carboxyl groups of Carbopol. One possible explanation of this result is that at pH 5.5 the berberine is trapped inside the collapsed nanogel particles which ensure slower release than at pH 7.5 where the nanogel is swollen. At pH 5.5 only ~60% of the berberine is released over 6 hours due to the strong interaction between the entrapped berberine cations and the anionic carboxyl groups of the nanogel. At higher pH, the percentage of the released berberine is significantly higher as the nanogel particle swell.

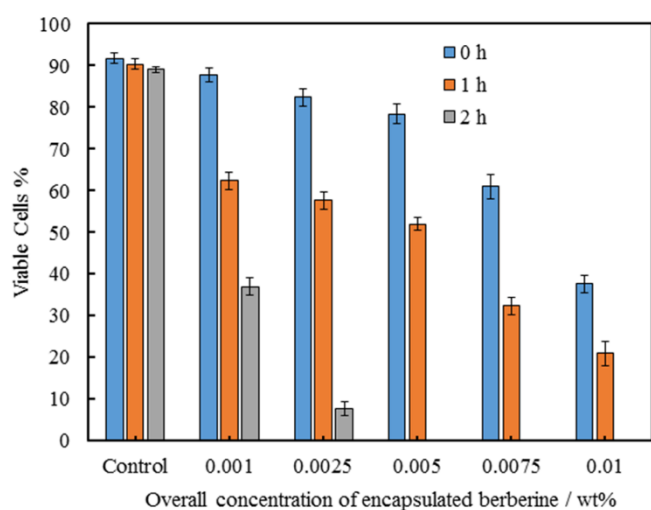


Figure 4. The viability of *C. reinhardtii* cells incubated with solutions of series of different overall concentration of BLC after different incubation times.

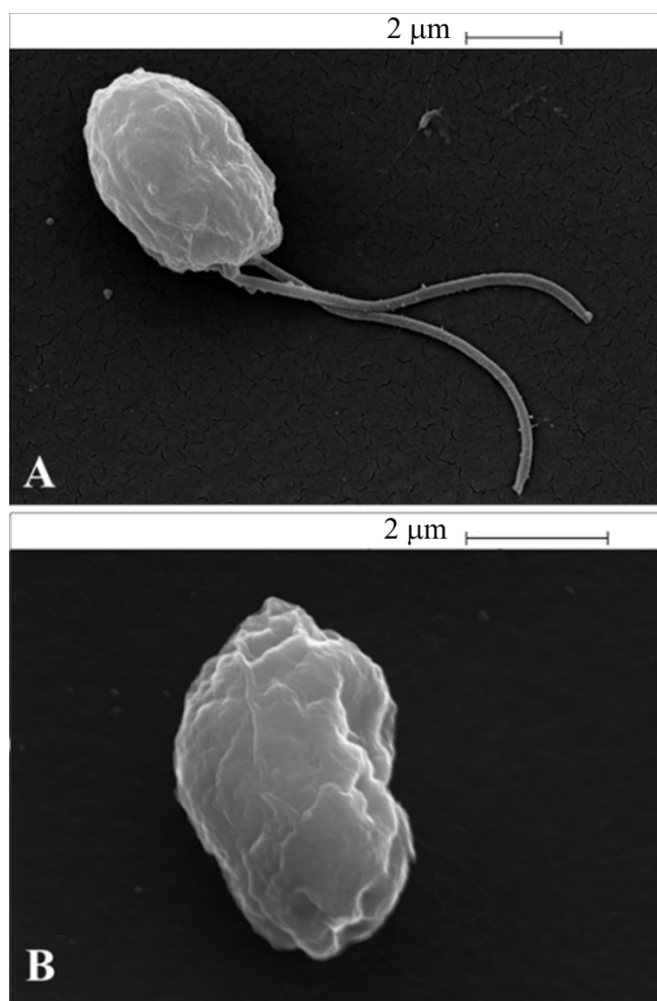


Figure 5. SEM images of *C. reinhardtii* cells after incubation with 0.01 wt% BLC whereby (A) represents the control sample and (B) show a typical image of an incubated cell with 0.01 wt% BLC after 2 hours.

Cytotoxicity assay of the non-coated nanogel carrier

Cytotoxic assays were conducted through the incubation of suspensions of different concentrations of nanogel particles with two test microorganisms: *C. reinhardtii* (microalgae) and *E. coli*. The cells were removed from the culture media to avoid any interaction between the nanogel particles and the components of culture media. Our data presented on Figure S20 (ESI) show no cytotoxic effect of Carbopol Aqua SF1 nanogel without any antimicrobial additives on *C. reinhardtii* and upon for a wide range of Carbopol Aqua SF1 concentrations at room temperature and up to six hours of incubation. Figure S21 (ESI) also shows no pronounced cytotoxic effect of the bare nanogel particles on *E. coli* cells for up to 24 hours. These results indicate that the nanogel particles do not affect the viability of these microorganisms over the duration of the experiments. Our results are also in agreement with the technical data sheet provided by the manufacturer for other microorganisms.³⁴

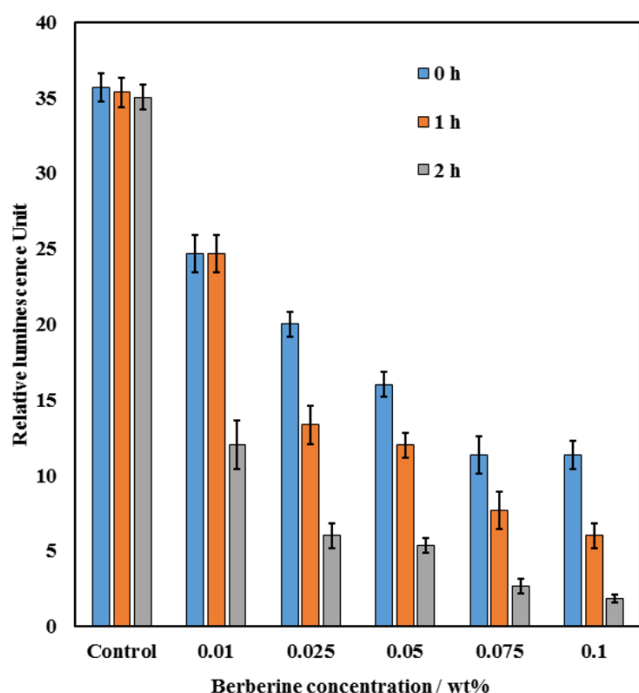


Figure 6. The relative luminescence intensity representing the *E. coli* viability of as a function of the concentration free berberine hydrochloride for 2 hours incubation time.

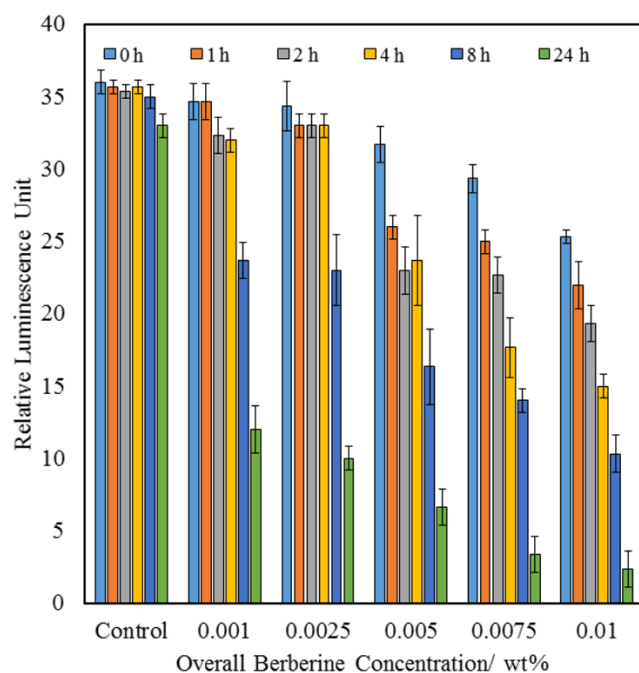


Figure 7. The relative luminescence intensities representing the viability of *E. coli* cells as a function of overall concentration of BLC for different incubation times.

The morphology of *C. reinhardtii* after incubation with the nanogel was studied by TEM. Figure S22 in ESI shows the TEM images of microalgae cells incubated for 24 hours with 0.1 wt% Carboxypol nanogel at pH 5.5. As seen in Figure S22B, the cell

organelles of the microalgae seem more or less intact without visible disruption in the cell microstructure compared with the control sample as shown in Figure S22A. These results also suggest that nanogel does not have detectable effect at concentrations up to 0.1 wt% on the microalgae.

Antialgal activity of BLC on *C. reinhardtii*

We compared the antialgal activity of free berberine and the BLC on *C. reinhardtii*. The microalgae cells were separated from the culture media before incubation with series of solutions of free berberine and BLC of different concentrations. The cell viability was assessed after a range of incubation periods followed by removal of the cells from the tested solution. Figure 3 shows the cell viability of the microalgae as a function of free berberine concentration. The viability of the microalgae immediately upon incubation (0 hours) gradually declined from low to high berberine concentrations, (85% at 0.001 wt% to around 10% at 0.05 wt%). Zero incubation time corresponds to the cells mixed with the berberine hydrochloride solution followed by immediate washing (within 5 min) and viability testing with FDA assay. After 2 hours of incubation the cells viability sharply decreased in comparison with the control sample with 0.05 wt% being the minimal concentration of free berberine hydrochloride which kills all the cells, 5% viable algal cells were found at a concentration of 0.01 wt% berberine after 2 hours incubation time. After 4 hours of incubation, the viability was reduced to 20% at 0.001 wt% berberine concentration and by 6 hours incubation time, no viable cells were observed at concentrations of berberine hydrochloride from 0.007 wt% to 0.05 wt%. The cell viability went down to 15% at 0.001 wt% berberine. This demonstrates that free berberine hydrochloride tends to be an effective antialgal agent at moderate concentrations over a sufficiently long incubation. Figure 4 shows the viability of the *C. reinhardtii* after incubation with BLC dispersions of different concentrations at room temperature for up to 2 hours.

One can see that the cell viability decreased slightly up to 0.005 wt% BLC and then gradually declined from 0.0075 wt% to 0.01 wt% of BLC at zero incubation time. After 1 hour incubation time the cell viability was reduced progressively from 60% to 20% at 0.01 wt% average encapsulated berberine. After 2 hours, however, the cell viability decreased sharply from 37% at 0.001 wt% to 7% viability at 0.0025 wt% BLC, respectively. At BLC concentrations above 0.01 wt% the cells complete lost their viability. The accumulation of free berberine cations at the cell membrane causes its opening and leads to the cell death. Comparing the activity of free berberine hydrochloride (Figure 3) to this for BLC (Figure 4) one may conclude that the berberine encapsulation in the non-surface functionalised nanogel appeared to slightly decrease the antialgal activity of berberine. The encapsulated berberine in BLC is not completely released in 1-2 hours (see Figure 2), therefore there is lower overall concentration of released berberine cations around the cell walls compared with case when the cell are treated with the same overall concentration of free berberine. In this case the BLC is negatively charged and does not adhere to the negatively

charged the cell walls, hence they are targeted only berberine released in the bulk of the solution.

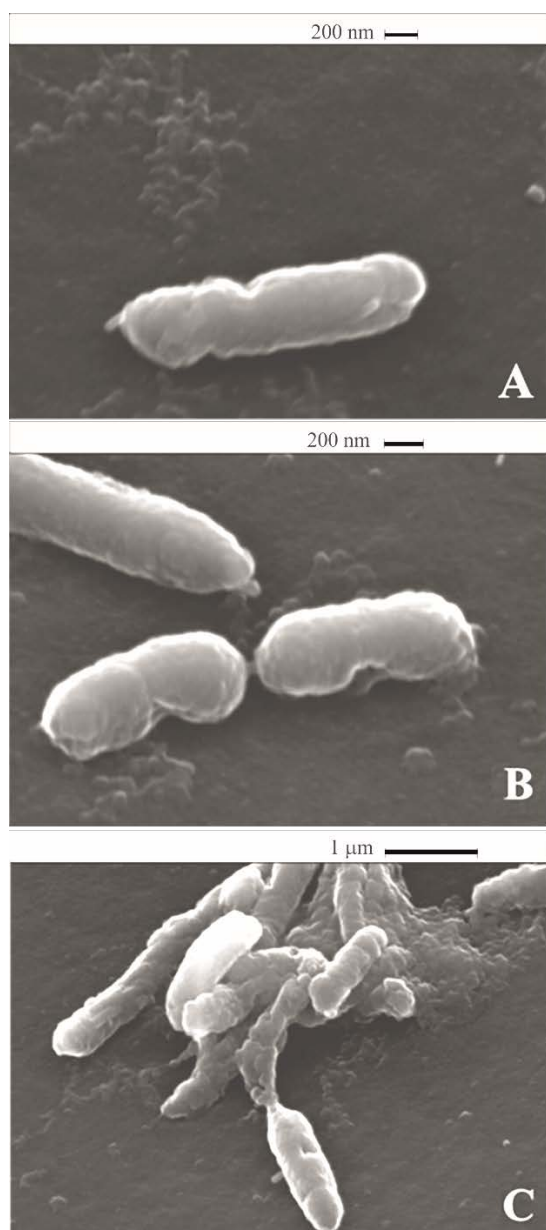


Figure 8. SEM images for *E. coli* cells incubated with a suspension of 0.01 wt% BLC. (A) Control sample and (B, C, D) represent *E. coli* cells incubated with 0.01 wt% BLC at room temperature after 24 hours incubation time.

Figure 5 shows SEM images of *C. reinhardtii* cells taken after incubation with 0.01 wt% BLC for 2 hours. It can be seen that the morphology of the cells had changed significantly and irregular membrane shapes as well as the detachment of the flagella for the treated microalgae cells appear (see **Figures 5B, 5C, and 5D**) in comparison with the control sample (**Figure 5A**). Other authors have also reported similar change in the cell morphology for different anti-algal agents which in our case could potentially be attributed to the interaction between the cell membrane and the BLC as endocytic cargos using

clathrin/actin mediated-endocytosis.⁴⁰ Note the size of the dimples left on the membrane of the treated cells corresponds approximately to the size of the collapsed BLC nanogel particles. However, TEM and SEM imaging of these samples show no experimental evidence of internalisation of the BLC particles by endocytosis.

Antimicrobial activity of BLC on *E. coli*

We also studied the antimicrobial activity of free berberine and BLC on *E. coli* at various incubation times. The data presented in **Figure 6** show that free berberine also has a strong effect on the viability of the *E. coli*. The cell viability decreased at free berberine concentrations from 0.01 wt% to 0.1 wt% in the moment of addition of the cells to the solutions (0 hour incubation time). After 1 hour of incubation, the *E. coli* viability became much lower in comparison with the control sample. Similarly, at 2 hours incubation time about 95% of the *E. coli* cells were killed at 0.075 wt% and 0.1 wt% free berberine, respectively. The results for the antimicrobial activity of BLC on *E. coli* are shown in **Figure 7**. Surprisingly, BLC showed lower antimicrobial activity against *E. coli* than free berberine at the same overall berberine concentration. One possible reason could be the thicker cell wall of *E. coli* which makes it less sensitive to berberine than *C. reinhardtii* (c.f. **Figures 3 and 4**). For up to 4 hours incubation BLC showed very low antibacterial activity. For incubation times up to 8 hours, the antimicrobial activity increased from 30 % at 0.001 wt% to 70% at 0.01 wt% of overall encapsulated berberine (in BLC). After 24 hours of incubation, the viability of the *E. coli* sharply decreased to less than 5% at 0.01 wt% of overall concentration of BLC. The difference in the activity of free berberine and BLC is attributed to the slower rate of berberine release from the BLC. In addition to that, the collapsed nanogel particles at pH 5.5 have a relatively high negative zeta potential which retains the cationic berberine. In addition, the negatively charged BLC particles is likely to repel from the negatively charged surface of the *E. coli* cell wall which seems to reduce the effectiveness of the local berberine release in the cells near vicinity. SEM images of *E. coli* cells after incubation with 0.01 wt% BLC suspension for 24 hours (**Figure 8**) show that the cells have some dimples and cavities on their surface which are absent on non-treated cells. This is due to the interaction between the cell membrane and berberine cations released from BLC particles which have been in their vicinity. Part of the BLC particles may also be internalized into the cells *via* endocytosis⁴¹ where this pathway is similar to that discussed for *C. reinhardtii*.

Antimicrobial activity of the PDAC-coated nanogel

Our experiments on the antimicrobial action of encapsulated berberine on *E. coli* showed that BLC has lower antimicrobial activity in comparison with free berberine hydrochloride. The main reason for this is that the nanogel particles retain their negative surface charge even after the encapsulation of the berberine in their cores. Our characterisation study presented in the ESI show that only one berberine molecule per 33 carboxylic groups is absorbed in the core of the nanogel particles. The result of this is that the BLC has a negative zeta

potential (-40 ± 5 mV) similar to the non-loaded nanogel (see Figure S2 in ESI for more details).

polyelectrolyte PDAC in aqueous solution has an extremely strong antimicrobial effect in a wide range of concentrations ranging from 0.0045 wt% to 0.05 wt%.

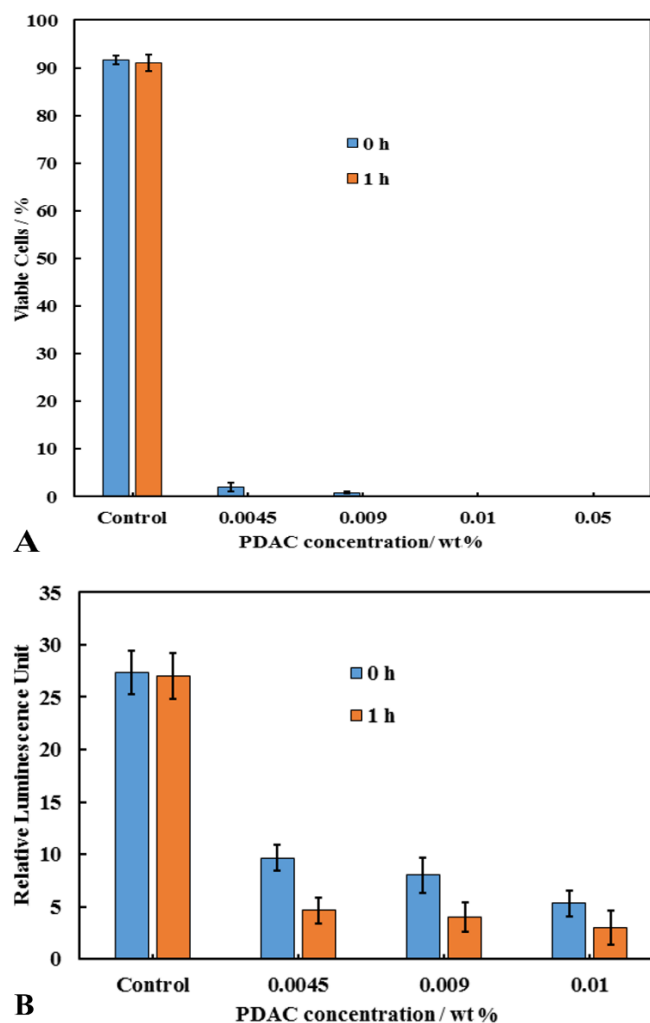


Figure 9. The cytotoxic effect of solutions of different PDAC concentration on (A) *C. reinhardtii* and (B) *E. coli* for up to 1 hour incubation time at room temperature. Solutions of different PDAC concentrations were incubated with a fixed amount of *E. coli*.

The electrostatic repulsion of the BLC particles with the negatively charged cell surface does not allow them to accumulate in near vicinity of the cell membrane and release the encapsulated antimicrobial agent. To overcome this problem, the nanogel particles were coated with a cationic polyelectrolyte PDAC to reverse their surface charge to positive which was expected to increase their adhesion to cells and potentially boost their antimicrobial action.

To differentiate between the cytotoxic action of the berberine and this of PDAC-coated carrier we studied the effect of the PDAC alone on *C. reinhardtii* and *E. coli*. This was achieved by incubating *E. coli* with solutions of different concentrations of PDAC to determine its threshold of cytotoxicity on these microorganisms. Figures 9A and 9B show the effect of aqueous solutions of PDAC of different concentrations on *C. reinhardtii* and *E. coli*, respectively. Note that the free cationic

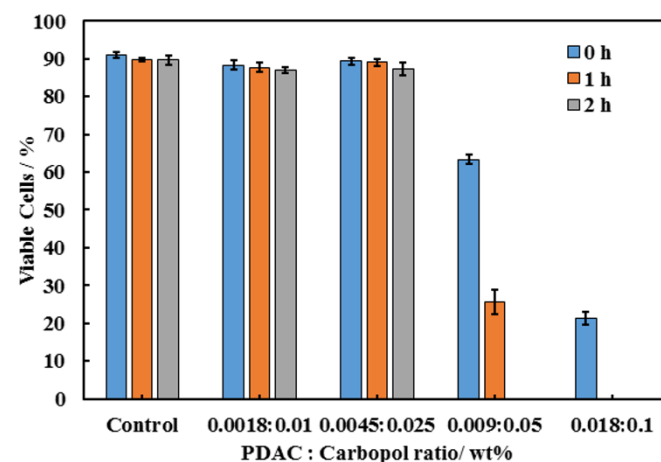


Figure 10. Cytotoxic effect of PDAC-coated nanogel upon incubation with microalgae cells for 2 hours at room temperature.

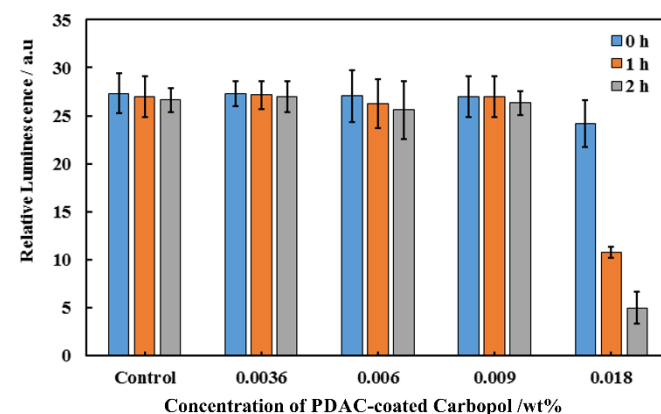


Figure 11. The cytotoxic effect of dispersions of various concentrations of PDAC-coated nanogel (without berberine) on *E. coli* for 2 hours incubation at room temperature. The relative luminescence is proportional to the cell viability.

Cytotoxicity of PDAC-coated nanogel on *C. reinhardtii* and *E. coli*

In order to measure the antimicrobial activity of PDAC-coated and BLC on microalgae and *E. coli*, the cytotoxic effect of PDAC coated-Carbopol Aqua SF1 (without berberine) towards these microorganisms was investigated. The free PDAC was removed from the nanogel suspension before incubation with cells to differentiate between the cytotoxic effect of PDAC and the PDAC-coated nanogel. A fixed amount of each cell culture was incubated at room temperature with suspensions of varying concentrations of PDAC-coated Carbopol Aqua SF1 nanogel for several incubation times. Figure 10 shows the results for *C. reinhardtii* where there is little effect until 0.009 wt% to 0.018 wt% at which point the viability is strongly affected. In this range even a 0h (5 min) incubation time, the PDAC-coated nanogel particles appear to be toxic for the thin cell membrane

of this microalgae without any loaded berberine. The cytotoxic effect of PDAC-coated nanogel particles was also examined on *E.coli*.

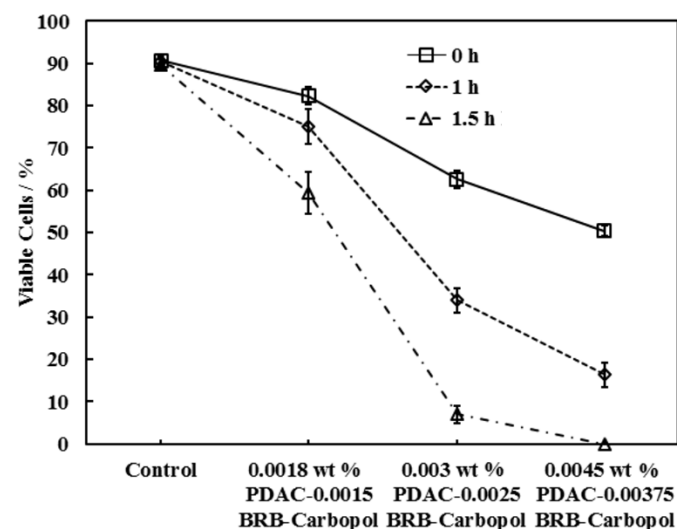


Figure 12. The viability of *C. reinhardtii* upon incubation with solutions of different concentrations of PDAC-coated BLC. The solutions were prepared depending on dilution from stock solution with the concentration 0.009 wt% PDAC-0.0075 wt%-0.075 wt% BLC suspension for different incubation times (5 min, 1h and 1.5h). The cells are washed from the nanogel suspension and their viability determined by the FDA live/dead cell assay.

Figure 11 shows that PDAC-coated nanogel in the concentration range 0.0036-0.009 wt% was not toxic with up to a 2 h incubation time while at 0.018 wt% PDAC (coated on the nanogel) concentration there was a cytotoxic effect on *E.coli*. Therefore, for our experiments with PDAC-coated BLC we used PDAC concentrations from 0.0036 wt% to 0.009 wt% which by themselves are not-toxic for both microorganisms. This allowed us to evaluate the effect of the encapsulated berberine when the nanogel particle had been converted to cationic (positive zeta-potential) due to the PDAC coating.

Antimicrobial activity of PDAC-coated BLC on *C.reinhardtii*

We studied the antimicrobial activity of BLC additionally coated with PDAC to enhance the electrostatic adhesion between the negatively charged cell membrane and positively charged PDAC-coated BLC particles. **Figure 12** shows the viability of *C.reinhardtii* incubated with suspensions of different concentration of PDAC-coated BLC. Here and hereafter the overall concentration of encapsulated berberine in the BLC suspension is quoted in the formulations. At 0 hours incubation time (instant exposure), the viability of the microalgal cells decreased from 82% at 0.0018 wt% PDAC-0.0015 wt% BLC concentration to 50% at 0.0045 wt% PDAC-0.00375 wt% BLC formulation and then sharply declined to 15% at 0.0045 wt% PDAC-0.00375 wt% BLC. Upon increasing the incubation time to 1.5 hours, all the cells died for 0.0045 wt% PDAC-0.00375 wt% BLC formulation while the viability was 7% for the 0.003 wt% PDAC-0.0025 wt% BLC. However, 40% of algal cells were viable after 1.5 hour for the 0.0018 wt% PDAC-0.0015 wt% BLC.

Therefore, the combination of 0.0045 wt% PDAC-0.00375 wt% BLC was the most effective concentration to kill algal cells after 1.5 hour incubation time.

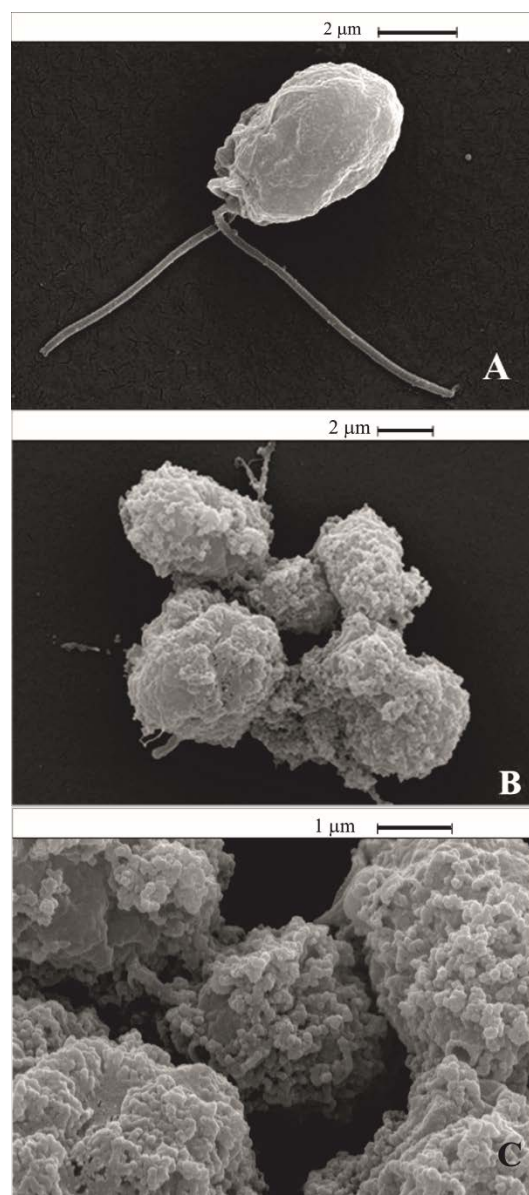


Figure 13. SEM images of *C.reinhardtii* microalgae cells. (A) Control sample of the microalgae cells. (B, C, D and E) *C.reinhardtii* cells incubated with 0.0045 wt% PDAC-coated 0.00375 wt% BLC after one hour incubation time at room temperature. The experiment was conducted through incubation a suspension of microalgae cells with solution of PDAC-coated 0.00375 wt% BLC particles.

Figure 13 shows SEM images for *C.reinhardtii* cells that had incubated with 0.0045 wt% PDAC-coated 0.00375 wt% BLC for 1 hour. The images on **Figure 15B**, **15C**, and **15D** suggest that the microalgae cells were completely damaged by this cationic surface-functionalised berberine-nanogel complex and acquire an irregular shape in comparison with the control sample (see **Figure 13A**). The reason for this is due to the electrostatic interaction between the cationic PDAC-coated BLC and the

anionic cell membrane allows the adhering particles to release berberine in the local vicinity of the cell surface causing the membrane disruption.

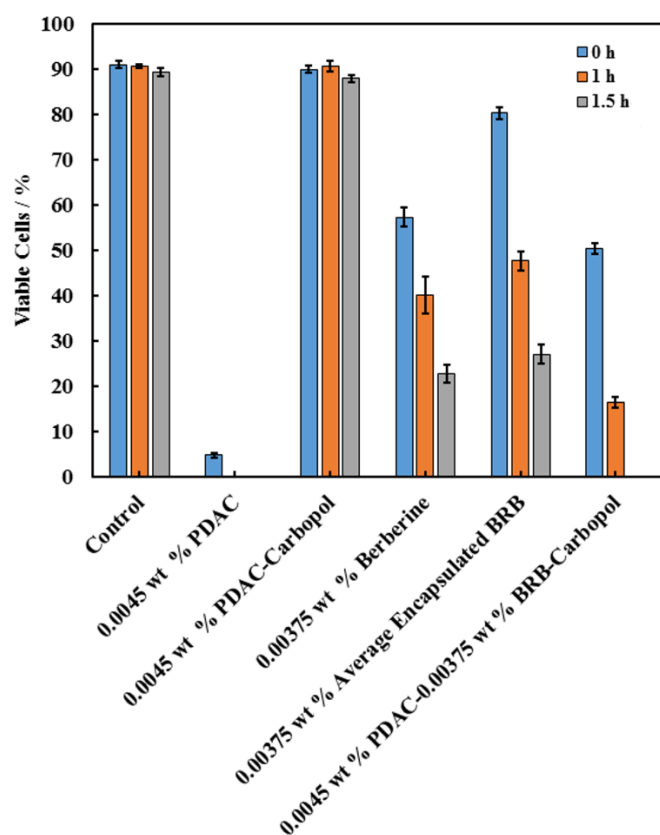


Figure 14. The viability of *C. reinhardtii* microalgae cells upon incubation with 0.0045 wt% PDAC, 0.0045 wt% PDAC-coated Carbopol, 0.00375 wt% free berberine, 0.00375 wt% BLC, and 0.0045 wt% PDAC-coated 0.00375 wt% BLC suspension for different incubation times (5 min, 1h and 1.5h). After incubation, the cells were washed from the respective formulation and their viability determined by the FDA live/dead cell assay.

Figure 14 represents a comparison between the antimicrobial activities of PDAC-coated BLC, free berberine and BLC. More than 90% of algal cells were killed upon instant incubation (0 hours) with 0.0045 wt% PDAC. However, at the same concentration of PDAC which had been used to coat the, there was no toxic effect without a berberine load. The BLC showed lower antimicrobial activity than free berberine (0.00375 wt%) which kills the cells after 1.5 h of incubation time as its release is slow from the core of the nanogel particles. In the latter case there is also a repulsion between the BLC and the cell membrane as they both carry negative surface charge. There was, however, an increase in the antimicrobial activity for the encapsulated berberine in the PDAC-coated nanogel compared with the uncoated BLC. The PDAC-coated BLC are cationic particles and in aqueous media they are strongly attracted to the negatively charged cell walls, allowing high local concentrations of released berberine to disrupt the cell membranes and kill the cells.

Antimicrobial activity of PDAC-coated BLC on *E. coli*

The antimicrobial activity of PDAC-coated BLC with *E. coli* was investigated by incubation of solutions of various concentration of PDAC coated BLC with a fixed amount of *E. coli* cells removed from their culture media for two hours at room temperature.

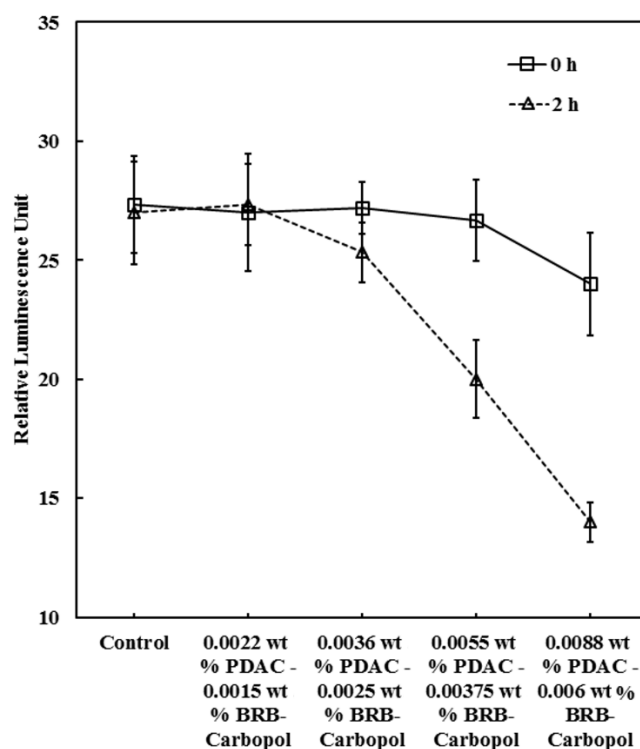


Figure 15. The antibacterial activity of suspensions of different concentrations of (PDAC-coated BLC) against *E. coli*. The solutions were prepared from the stock solution (0.011 wt% PDAC-coated 0.0075 wt% berberine-loaded 0.05 wt% nanogel).

Figure 15 shows that there was no noticeable antimicrobial activity for berberine concentrations from 0.0015 wt% to 0.0025 wt%. However, antimicrobial activity was seen for concentrations of 0.00375 wt% to 0.006 wt% berberine after two hours of incubation. Suspensions with 0.0088 wt% PDAC-coated 0.006 wt% BLC appeared to have high antimicrobial activity towards *E. coli*. This increase in the antimicrobial efficiency was attributed to the increase in the concentration of berberine encapsulated into the nanogel carriers as well as the electrostatic attraction between PDAC-coated BLC and the outer cell membrane of *E. coli*, similarly to the microalgae, as discussed earlier. This is confirmed by the SEM images of the *E. coli* in **Figure 16** that were incubated with 0.0088 wt% PDAC-coated 0.006 wt% BLC for two hours at room temperature. It can be seen from **Figures 16B and 16C** that particles of PDAC-coated BLC are clustered on the surface of the cell membrane of *E. coli* cells compared with control sample where there was no berberine involved (see **Figure 16A**). This aggregation occurs due to the electrostatic attraction between the cationic PDAC-coated BLC particles and the anionic cell membrane of *E. coli*

which contributed to the strong boost of the antibacterial activity of berberine in comparison with the same concentration of free berberine over the same period of incubation.

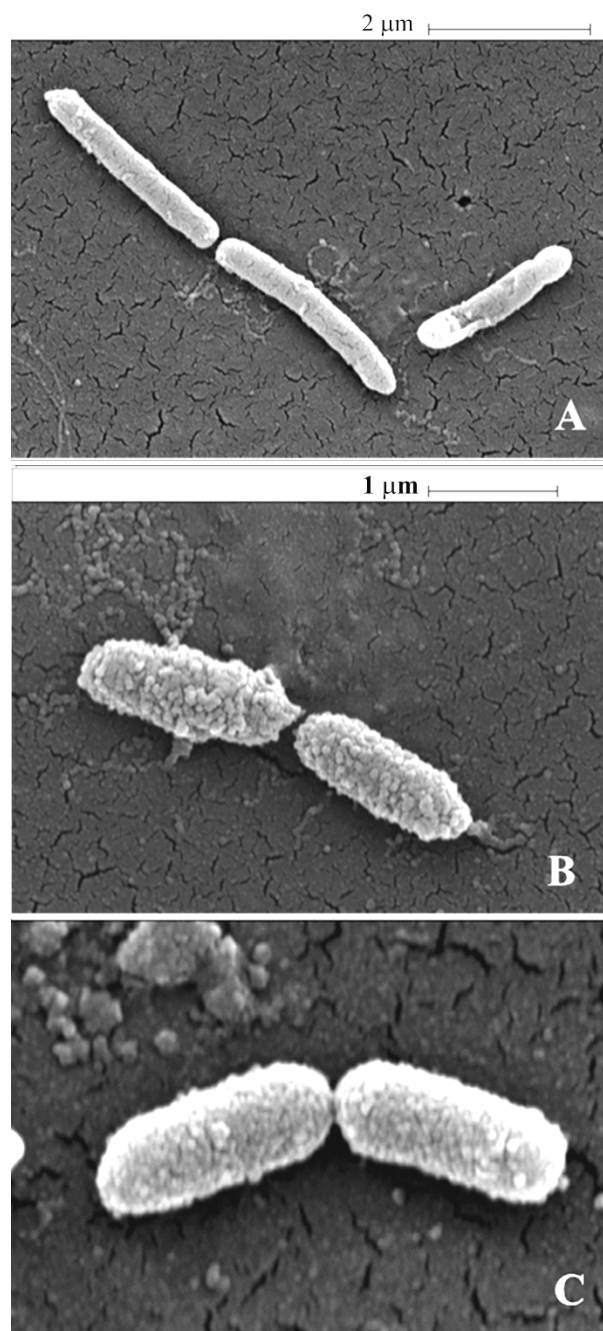


Figure 16. SEM images of *E. coli* cells. (A) Control sample of *E. coli* and (B, C, and D) incubation of *E. coli* cells with solution of 0.0088 wt% PDAC-coated 0.006 wt% BLC suspension.

Conclusions

We developed a novel surface functionalized nanocarrier for berberine by using polyacrylic acid based nanogel particles (Carbopol Aqua SF1) coated with a cationic polyelectrolyte

(PDAC) which shows a strong boost of the berberine antimicrobial action. The latter was studied on *C. reinhardtii* microalgae and *E. coli* for berberine-loaded nanogel (BLC) coated with the cationic polyelectrolyte (PDAC-coated BLC).

We demonstrated that the cationic coating of the nanogel increase strongly the antimicrobial action of the berberine against both *C. reinhardtii* and *E. coli* even for very short incubation times. The increase in the antimicrobial activity was attributed to the favourable electrostatic adhesion between the cationic PDAC-coated BLC and the anionic cell membranes allowing accumulation of BLC particles on the cell walls and local release of concentrated berberine causing the cell death. SEM images confirmed the aggregation of these cationic PDAC-coated berberine-loaded nanogel on the surface of the cell membranes for both *C. reinhardtii* and *E. coli*. This nanotechnology inspired approach can be potentially applied to boost the antimicrobial action for a range of low-molecular weight antimicrobial and antialgal agents by using cationic surface-functionalised nanogel carriers. Similar strategy could also find applications for enhancing the action of topical antibiotics and antifungal agents and may be used to bypass antimicrobial resistance.

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