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Epigallocatechin gallate-loaded polysaccharide nanoparticles for prostate cancer chemoprevention

Aims: Polysaccharide nanoparticles were studied as drug delivery vehicles for chemopreventive agents. **Materials & methods:** Green tea polyphenol epigallocatechin-3-gallate (EGCG) was incorporated into a carbohydrate matrix of gum arabic and maltodextrin with an encapsulation efficiency of approximately 85%. **Results:** Encapsulated EGCG retained its biological activity, reducing the cell viability and inducing apoptosis of Du145 prostate cancer cells. Clonogenic assay demonstrated that encapsulation of EGCG enhanced its inhibitory effect on cell proliferation (10–20%) at lower concentrations (1–2 μM), compared with free EGCG. **Conclusion:** This study highlights the use of polysaccharide nanoparticles in chemoprevention as they can be used to deliver natural antioxidants capable of inhibiting steps of the tumorigenesis process.

KEYWORDS: carbohydrate nanoparticles ▪ chemopreventive agent ▪ encapsulation ▪ green tea catechins ▪ gum arabic ▪ maltodextrin ▪ prostate cancer cells

Sandra Rocha[†1], Roman Generalov[2], Maria do Carmo Pereira[1], Ivone Peres[1], Petras Juzenas[2] & Manuel AN Coelho[1]

[1]Laboratory for Process, Environmental & Energy Engineering, Chemical Engineering Department, Faculty of Engineering, University of Porto, Rua Roberto Frias, 4200-465 Porto, Portugal
[2]Department of Radiation Biology, Institute for Cancer Research, Norwegian Radium Hospital, Oslo University Hospital, Montebello 0310, Oslo, Norway
[†]Author for correspondence:
Tel.: +351 225 081 589
Fax: +351 225 081 449
sandra.rocha@fe.up.pt

Nanochemoprevention has recently been proposed for cancer control [1]. The concept is based on the delivery of chemopreventive agents using nanoparticles (NPs), such as polylactic acid (PLA)–polyethylene glycol (PEG) or gelatin NPs [1,2]. Natural antioxidants, such as catechins (flavan-3-ols), the most abundant flavonoids in food (red grapes, apples, tea and cocoa) [3], provide protective effects against cancer (reviewed in [4]). Protection by epicatechin of DNA against γ-radiation induced strand breaks has been reported [5]. On the other hand, the combined effect of x-ray radiation with antioxidants from tea extracts is known to enhance apoptosis of cancer cells [6]. Another antioxidant, theanine, enhanced anti-tumor activity of chemotherapeutic agents [7] and, by contrast, attenuated adverse oxidative damage induced by doxorubicin [8]. The chemopreventive effects of epigallocatechin-3-gallate (EGCG) have been well studied, particularly in prostate cancer [4,9]. EGCG causes a reduction in prostate cell number of both androgen-dependent (LNCaP) and-independent (DU145) prostate cancer cells [10]. Animal studies using athymic mice inoculated with both type of cells proved that EGCG significantly halts the growth of tumors [11]. Studies on transgenic adenocarcinoma of the mouse prostate (TRAMP) show an inhibition of tumor growth in mice treated with green tea polyphenols, suggesting that these phytochemicals may also have chemotherapeutic properties [4,12]. The fact that antioxidants have been reported to display dual action (oxidant and pro-oxidant capabilities) warrants further investigation of catechins. In this study, we have encapsulated EGCG in maltodextrin-gum arabic NPs with high efficiency (>80%) and assessed its activity on androgen-independent Du145 human prostate cancer cells. Its cytotoxic efficacy was evaluated and compared with free EGCG. Both gum arabic and maltodextrin are nontoxic molecules widely used in the food and pharmaceutical industries. EGCG is relatively stable at acidic pH (2.0–5.5), but is unstable at pH 7.4 and alkaline medium as in the human intestine [13,14]. Its bioavailability is less than 2% of the oral dose administered in rats and less than 20% in mice [15–17]. The poor bioavailability is related to poor permeability, efflux mechanisms and fast rate of glucuronide conjugation, the process of converting chemical compounds to glucuronides to assist excretion of toxic substances, drugs or other molecules that cannot be used as energy sources. It has also been demonstrated that galloylation (esterification with gallic acid) of catechins reduces their absorption and they are rapidly eliminated by preferential excretion in bile [18]. Nanotechnology is being applied to cancer treatment to improve the stability and minimize the toxicity of chemotherapy drugs, but, by now, such an approach is being explored less in cancer prevention [19–21]. The encapsulation of chemopreventive agents in NPs may solve problems inherent to their limited use in cancer from the inefficient systemic delivery and low bioavailability [1].

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Material & methods

Chemicals

Gum arabic from acacia tree (grade Ph. Eur.), dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS; 0.01 M, 0.0027 M KCl, 0.137 M NaCl, pH 7.4) were purchased from Fluka (Germany). Maltrin® maltodextrin DE 16.5–19.5 (US FDA GRAS [generally regarded as safe]) and sunphenon EGCG, obtained from the leaf of green tea (EGCG >90%; caffeine <1%), were kindly provided by Grain Processing Corporation (USA) and Taiyo Kagaku Co., Ltd. (Japan), respectively. Methanol was from Merck (Germany). RPMI-1640 medium, L-glutamine (G7513), penicillin-streptomycin solution (P0781), protein standard (2 mg BSA/ml; P0834), trypsin-EDTA solution 1 × (T3924), (MTT reagent) 3-[4,5-dimethylthizaol-2-yl]-2,5-diphenyl tetrazolium bromide, caspase-3 assay kit (CASP3C) and Fluka protein assay reagent (57697) were purchased from Sigma Aldrich AS (Norway). Fetal bovine serum (standard quality, A15–101) was received from PAA Laboratories GmbH (Austria).

NP preparation

Polysaccharide-based NPs were prepared by firstly dissolving the gum arabic (38%) in ultrapure water at 50–60°C, then adding the maltodextrins (57%). EGCG (5%) was then added to the solution at room temperature, with constant homogenization at 9500 rpm with a dispersing device IKA® DI25 Basic. The mixture was spray-dried in an atmospheric pilot spray-dryer (designed by Niro A/S) operated at an air inlet temperature of 160 ± 5°C and outlet 60 ± 5°C to obtain the formulation in powder form. Void NPs were prepared by the same method.

EGCG loading efficiency

First, the NPs were washed with methanol to quantify the EGCG adsorbed at their surface, as maltodextrin-gum arabic matrix precipitates in that solvent, whereas EGCG is highly soluble. After centrifugation at 14,000 g for 10 min, the supernatant was filtered through a 0.2-µm syringe filter and analyzed by UV-vis spectroscopy (Shimadzu UV-1700). The sedimented NPs were then resuspended in aqueous solution (buffer or ultrapure water) for EGCG to be extracted with methanol several times. EGCG concentration was assayed spectrophotometrically at 274 nm to determine the loading efficiency.

Dynamic light scattering & zeta potential

Size distribution and zeta potential of NPs suspended in PBS buffer (0.01–0.5%) were determined by dynamic light scattering and laser doppler velocimetry, respectively, using a Zetasizer Nano ZS (Malvern Instruments Ltd, UK). Size measurements were performed at 25°C at a scattering angle of 173°.

In vitro release studies

Nanoparticles (46 mg/ml) were suspended in PBS, pH 7.4, and incubated at 37°C with constant magnetic stirring in regenerated cellulose membrane (molecular weight cut-off [MWCO]: 1000; Spectrum Labs Europe BV, The Netherlands). At fixed time intervals, an aliquot of the dialysate buffer was withdrawn and EGCG concentration was determined spectrophotometrically at 274 nm. The results were normalized to free ECGG, which is able to cross the membrane used as a control at the same time intervals.

Electron microscopy

Nanoparticles were suspended in PBS (46 mg/ml) and 5 µl was placed on glow discharged formvar-coated Ni grids and stained with 1% filtered uranyl acetate solution. The grids were analyzed by transmission electron microscopy (TEM, Zeiss microscope operated at 60 kV) and scanning electron microscopy (SEM, FEI Quanta 400) operated in backscattered electron and secondary electron imaging modes at 25 kV.

Cell line culture

Human prostate carcinoma Du145 cells were grown in surface-treated Nunclon culture dishes (Nunc AS, Roskilde, Denmark) using a standard RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% (w/v) penicillin and streptomycin, and 1% L-glutamine. All cell culture dishes were maintained at 37°C in 95% humidified atmosphere and 5% CO_2. For subculturing, approximately 5×10^5 cells were seeded per 25 cm^2 culture flask and split twice a week. Viable cells were counted using an automated Coulter counter (Vi-Cell XR, Beckman-Coulter Inc., Miami, FL, USA). For cytotoxicity experiments, the cells were harvested from the splitting culture and seeded in six-well plates (clonogenic assay) or in 25 cm^2 flasks (viability and apoptotic assays) at a density of 200 cells/well and 0.15×10^6 cells/flask, respectively.

Nanoparticles were incubated with cells at a concentration of 0.9 mg/ml (for loaded NPs the mass corresponds to 100 µM EGCG), for 64 h at 37°C for viability and apoptotic assays, and at concentrations from 0.009 to 0.9 mg/ml (1–100 µM EGCG) for 9 days at 37°C for clonogenic assay. The medium was not changed or replenished during the incubation time. After that, the cells were washed with RPMI medium and the assays were carried out. Only the overall end point effects (cell viability and clonogenic potential) of both encapsulated and free EGCG were studied to compare their activity.

Cell viability assay

Cell viability was determined by the MTT reduction assay, which relies on the reduction of MTT by mitochondrial dehydrogenases. MTT reagent (50 µl/ml of 2 mg/ml MTT) was added to each flask, which was incubated at 37°C for 4 h. The cells were then washed twice with ice-cold PBS solution. The crystallized formazan was subsequently dissolved in DMSO. The absorbance of the samples was measured at 570 nm using a plate reader (Multiscan EX, type 355, Labsystems, Helsinki, Finland). Background absorbance values, as assessed from cell-free wells, were subtracted from the absorption values of each test sample. Percentage of MTT reduction was calculated by dividing absorbance of the sample by the absorbance of a control (cells treated in an identical manner but without EGCG or particles). Averages from three replicates were used for each sample and control.

Caspase-3 activation assay

Du145 cells were grown as previously described. EGCG carbohydrate NPs were dissolved in PBS buffer and incubated with cells at an EGCG final concentration of 100 µM for 64 h. Toxicity of the samples was evaluated by activation of caspase-3, measured using the CASP3C colorimetric 96-well plate assay kit (Sigma, MO, USA), following the manufacturer's instructions. Briefly, confluent cells were incubated with EGCG (either free or NP loaded) and unloaded NPs for 64 h. Subsequently, cells were trypsinized, concentrated by centrifugation and the cell pellet was lysed in 100 µl hypotonic lysis buffer (Promega); 40 µl of each cell lysate were used in duplicate to determine caspase-3 activation. The remaining cell lysate was used to measure total cellular protein concentration with the Fluka protein reagent absorbing at 595 nm, using bovine serum albumin as standard. Values shown are the mean of three independent assays.

Clonogenic assay

Epigallocatechin-3-gallate, free or encapsulated in carbohydrate NPs, was dissolved in PBS buffer and added to the cells at different concentrations (1, 2, 5, 10, 25, 75 and 100 µM in EGCG content). After the incubation period (9 days), survival and proliferation of the cells were determined by methylene blue staining. The medium was discarded and cells were washed with 0.9% NaCl and fixed with 70% ethanol. The cells were then stained with methylene blue solution (10% in 0.01% w/v NaOH). The number of colonies with 50 or more cells/colony was determined. The surviving fraction was calculated by the number of colonies counted divided by the number of colonies plated with a correction for the plating efficiency (PE), defined as the number of colonies observed/the number of cells plated of a control sample:

$$\textit{Surviving fraction} = \frac{\textit{Number of colonies}}{\textit{Number of cells seeded} \times PE}$$

Statistical analysis

Values are reported as mean ± STD. Student's t-test statistical analysis was used to determine statistical significance between cells exposed to assay media and cells exposed to EGCG and NPs.

Results

EGCG-loaded NPs

Epigallocatechin-3-gallate/nanoparticles were characterized after suspension in PBS buffer to be closer to the conditions used in the cells

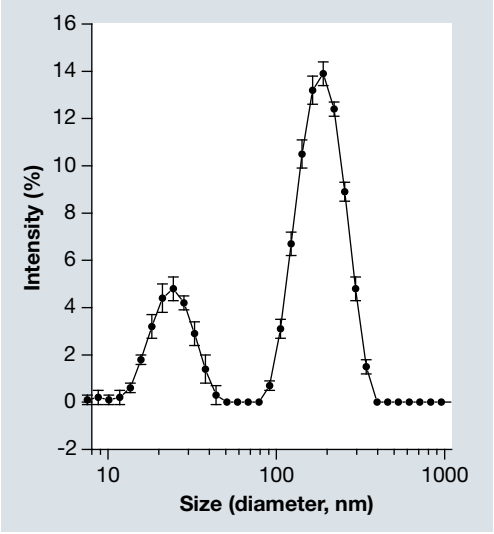

Figure 1. Size distribution of epigallocatechin-3-gallate/nanoparticles, as determined by dynamic light scattering.

Table 1. Characterization of maltodextrin–gum arabic nanoparticles.

System	Mean diameter (nm)	Polydispersity index	Zeta potential (mV)	Loading efficiency (%)
GA:MD	100 ± 10	0.42 ± 0.20	-9.6 ± 0.2	-
EGCG/GA:MD	120 ± 28	0.45 ± 0.23	-12.3 ± 0.8	85 ± 3

EGCG: Epigallocatechin-3-gallate; GA: Gum arabic; MD: Maltodextrin.

(cell medium). The hydrodynamic diameter and zeta potential of NPs are summarized in Figure 1 & Table 1. Two particle size populations with average diameters of approximately 30 and 200 nm were observed (Figure 1), indicating high sample polydispersity (PdI; 0.4; Table 1). Similar results were obtained in ultrapure water [22]. The zeta potential was negative for both loaded and unloaded NPs (Table 1). Figure 2A–C shows SEM back-scattered electron images of NPs stained with uranyl acetate. When an electron beam hits the sample, back-scattered electrons, among other signals, are emitted depending on its atomic number. Uranyl acetate, which interacts with anionic groups of the polysaccharide complex and polyphenol, back scatters electrons and the structure appears brighter. The images reveal that NPs have a tendency to aggregate, which caused the loss of their spherical shape (Figure 2C & 2D). The brighter areas in Figure 2B on the surface of the larger particle correspond to NPs of approximately 30 nm. The secondary electron imaging shows that the surface of the NPs is smooth (Figure 2D). TEM analysis confirmed the size polydispersity and the morphology of the NPs (Figure 3).

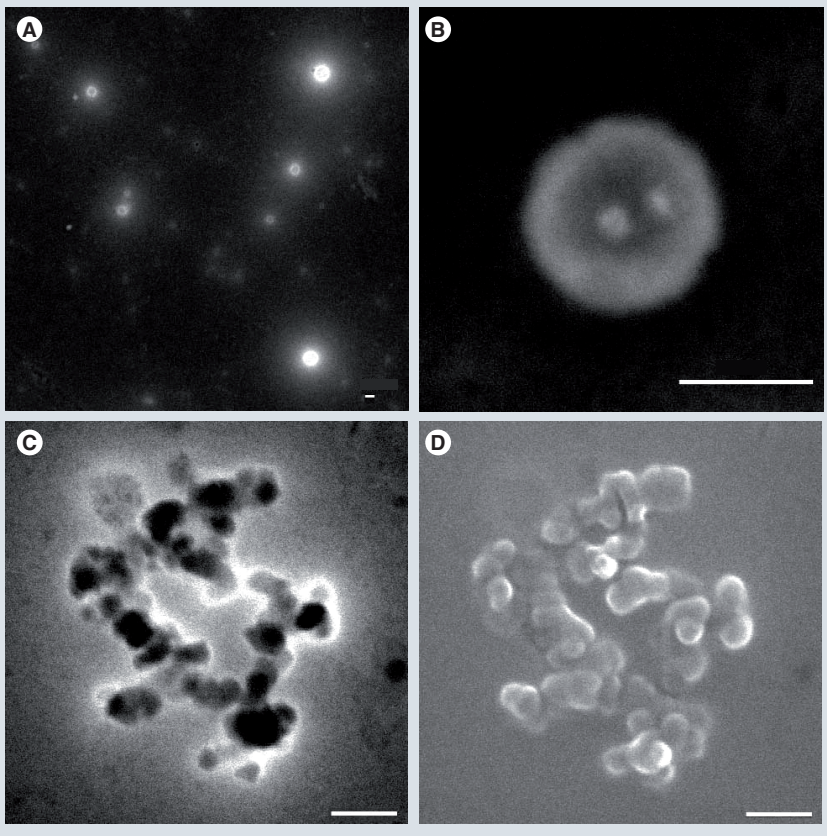

Figure 2. Scanning electron microscopy analysis of epigallocatechin-3-gallate/nanoparticles: backscattered electron (A–C) and secondary electron images (D). The scale bar is 100 nm.

The encapsulation efficiency was determined by UV-vis spectroscopy after extraction of EGCG with methanol. The concentration of EGCG adsorbed at the NP surface was 8 ± 1%. The concentration of EGCG incorporated in the inner space of NP was 85 ± 3% of the initial concentration, indicating an encapsulation efficiency higher than 80% (Table 1). Similar results were obtained by attenuated total reflection fourier transform infrared spectroscopy (data not shown).

The *in vitro* release profile of EGCG at pH 7.4, studied using regenerated cellulose membranes, is shown in Figure 4. The amount of EGCG released based on total entrapped catechin was 46 and 100% after 10 min and 3 h, respectively. Encapsulated catechin exhibits a rapid initial release from NPs, with 65% released within the first 30 min. After this burst release, the rate follows a sigmoidal profile, which EGCG release is slow and then progresses to a more rapid release phase initially before leveling off.

Cell metabolic activity & apoptotic response

The cytotoxicity of encapsulated EGCG to Du145 cells was evaluated at a concentration of 100 µM by MTT reduction and caspase-3 activation assays, and compared with the activity of nonencapsulated antioxidant. This concentration and long incubation period (~3 days) are normally the conditions used to study EGCG inhibitory effect because the uptake of free EGCG is concentration dependent [23].

As shown in Figure 5, Du145 cell line showed significantly lower metabolic activity in the presence of EGCG/NPs, compared with untreated control cells. After incubation for 64 h, encapsulated EGCG caused similar reduction of cell viability (38%) as free EGCG (34%). Unloaded NPs did not induce significant toxicity on the cells.

Encapsulated EGCG induced considerable caspase-3 activation similar to free EGCG, when compared with nontreated cells and cells treated with unloaded NPs (Figure 6).

Survival & proliferation assay

Clonogenic assay, a standard analysis for screening cytotoxic activity of drugs, was used to study the effect of catechin-loaded NPs at different concentrations on prostate cancer cells. This assay is much more sensitive than that previously described due to very low cell-seeding density. Treatment of Du145 cells with increasing concentrations of encapsulated EGCG from 0 to 10 µM resulted in a concentration-dependent decline in cell survival, as evidenced by the reduction in surviving fraction (Figure 7A). The effectiveness of encapsulated EGCG was higher when compared with free EGCG at low concentration (1 and 2 µM). At concentrations of 25 µM or higher, all cells died. The surviving fraction of cells treated with unloaded NPs was similar or higher to the control sample (no treatment), confirming that the carbohydrate matrix is not toxic to Du145 cells (Figure 7B). Statistical analysis established that unloaded NPs at 1 mg/ml significantly increase the surviving fraction, compared with the control ($p < 0.05$).

Discussion

Epigallocatechin-3-gallate, the most abundant and active catechin, has been extensively studied for its inhibitory effect against tumorigenesis [24]. However, its use in cancer prevention and treatment is poorly implemented. This difficulty may derive from chemical instability and low bioavailability of the molecule [3,17]. In addition, catechins are usually not formulated in the pure forms but rather as plant extracts, which makes their delivery by conventional dosage forms, such as tablets and liquid orals, extremely difficult [17]. The effectiveness of chemopreventive agents, such as EGCG, is believed to improve if delivered by NPs that passively target the tumor tissue through enhanced permeation (the effect of increased fenestration of pathological vasculature) [25] and meliorated retention improved by NPs, without altering their chemical nature or biological action [17,21,26,27].

Our data show that the biodegradable polymeric matrix provides high encapsulation efficiency (>80%). Both maltodextrin and gum arabic are water-soluble materials that protect encapsulated molecules from oxidation [28]. NMR studies provided a strong indication of an interaction between EGCG and the polysaccharide matrix [22] and an increase in EGCG stability in aqueous medium over time (study performed in ultrapure water; data not shown). The two size particle populations (mean size diameters of 30 and 200 nm) are formed possibly due to the heterogeneity of gum arabic: approximately 80% of the material consists of highly branched arabinogalactan units and the other fraction is composed of heterogeneous arabinogalactan–protein complexes of high molecular weight, which have a globular conformation [29]. Particle bridging may also be occurring due to the rubbery-like nature of the polymers. The NP negative zeta-potential values are attributed to the carboxyl groups of uronic acid of gum arabic that are deprotonated in its normal ionized form near neutral pH values. The lower zeta-potential value of EGCG/NPs might be explained by the presence of the polyphenol that has an apparent pKa value between 3 and 4 [30]. Particles sterically stabilized, particularly small ones, are described to preferentially localize in tumors and sites of infection

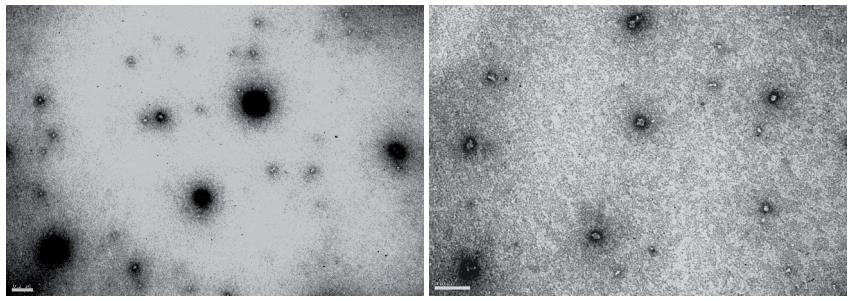

Figure 3. Transmission electron microscopy images of polysaccharide nanoparticles. The scale bar is 200 nm.

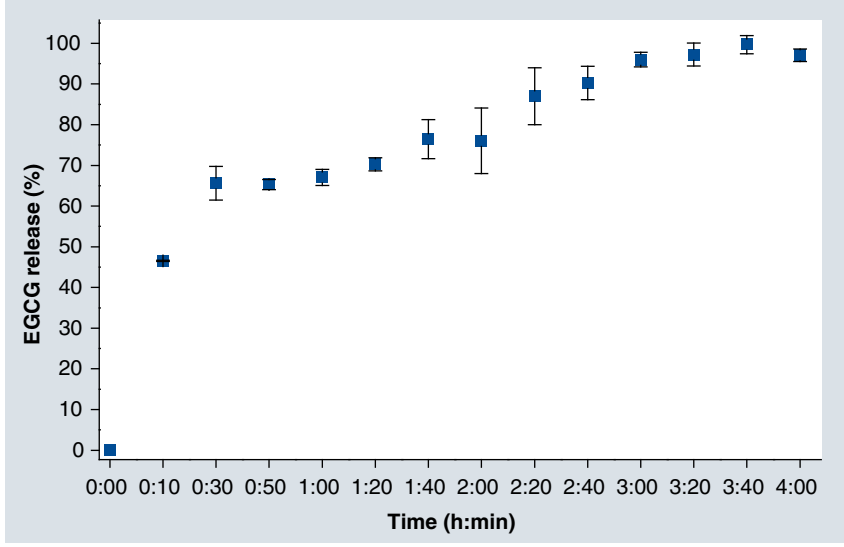

Figure 4. *In vitro* epigallocatechin-3-gallate release profile from nanoparticles.
EGCG: Epigallocatechin-3-gallate.

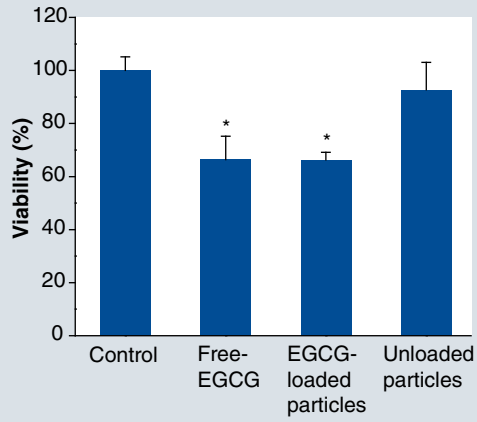

Figure 5. Viability of Du145 cells exposed for 64 h to free EGCG, EGCG/nanoparticles and unloaded nanoparticles, as assessed by 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyl tetrazolium bromide reduction assay.
*Significantly different from control ($p < 0.01$).
EGCG: Epigallocatechin-3-gallate.

and inflammation [31]. The release of EGCG from NPs at pH 7.4 follows a sigmoidal profile. This behavior might be related to the presence of different particle size populations. The dependence of the release rate with particle size is well studied for microspheres [32]. Larger spheres generally release encapsulated compounds more slowly and over longer time periods, with other properties (e.g., polymer molecular weight, initial porosity and drug distribution within the sphere) being equal. The water uptake by larger NPs, resulting in increased solubilization of the drug and swelling of the polymer matrix, may cause an increasing release rate with time.

Encapsulated EGCG, at a concentration of 100 µM, was able to reduce cell viability and induce caspase-3 activation in cultured prostate cancer cells, comparable to the free EGCG [33,34]. A similar conclusion was attained with encapsulated EGCG in polyelectrolyte-coated gelatin NPs of 200 nm, which blocked HGF-induced intracellular signaling in the breast cancer cells as free EGCG [2]. A reduction in the number of the colonies by 10–20%, on applying low concentration (1–2 µM) of EGCG/NPs, is to be regarded as a demonstration of the ability to inhibit cell proliferation by NPs at lower concentration than that for free EGCG. Our results are in agreement with the previous study using PLA–PEG NPs, in which it was shown that low concentrations of encapsulated EGCG (2.7 µM) is able to reduce the number of colonies more efficiently than free EGCG [1]. A possible reason for the efficacy increase at low EGCG concentrations is the efficient delivery by NPs. Moreover, it is expected that NPs increase EGCG stability. It was found that the catechin is more stable in the presence of cells than in cell medium. Such behavior is attributed to stabilizing factors from cells such as proteins and antioxidants [23]. Gum arabic has antioxidant properties [35] and, thus, might augment protection of EGCG molecules, which is important for oral administration [36].

The molecular mechanisms of the biological effects of EGCG are still being studied, but it has been proposed that EGCG could exert its activity by binding to receptors (e.g., EGF-receptor) or intracellularly by inhibiting protein kinases [23]. It was demonstrated that there is a size-dependent cellular uptake of biodegradable particles [37,38]. Particles of 50, 100 and 200 nm were internalized and distributed throughout the cells after 2 h, whereas 500 nm beads were localized largely at the periphery of the cells [37]. It is reasonable to assume that maltodextrin–gum arabic NPs are internalized by the cells. Gum arabic-coated iron oxide NPs with hydrodynamic diameters of approximately 120 nm were shown to have higher affinity to enter tumor cells than starch-coated NPs and were not cytotoxic [39].

At concentrations higher than 5 µM, the inhibitory effect of encapsulated EGCG was retained, but it was less pronounced when compared with free EGCG, which may be attributed to a saturated and limited capability of cellular uptake of NPs, as it has been observed for poly(D,L-lactide-co-glycolide) NPs [38,40]. The decrease in NP uptake at higher concentration is possibly caused by aggregation or reveals competing binding sites. Aggregation, at some

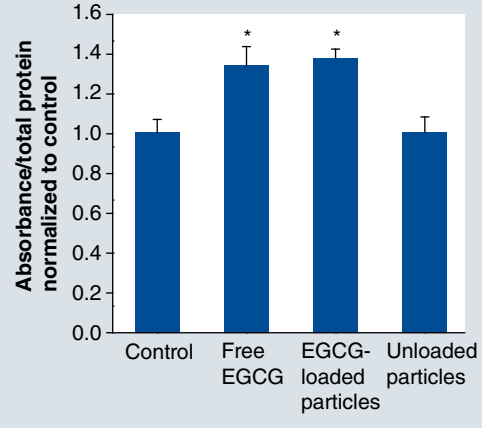

Figure 6. Activation of caspase-3 in Du145 cells exposed for 64 h to free EGCG, EGCG/nanoparticles and unloaded nanoparticles.
*Significantly different from control ($p < 0.01$).
EGCG: Epigallocatechin-3-gallate.

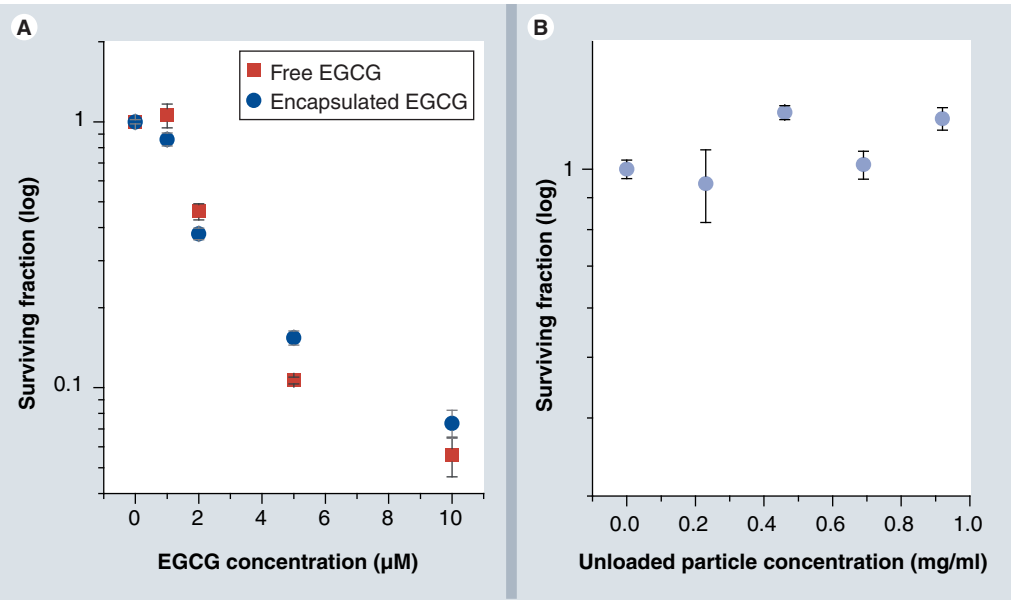

Figure 7. Surviving fraction of DU145 assessed by clonogenic assay. (A) Cells incubated with free and encapsulated EGCG, normalized to initial EGCG concentration. **(B)** Cells incubated with unloaded particles.
EGCG: Epigallocatechin 3-gallate.

extension, might be favorable since it may prevent small particles from escaping a tumor interstitium. The size of liposomes containing EGCG was shown to strongly influence the accumulation of the active molecule in basal cell carcinomas [41]. Large vesicles (>300 nm) were more readily entrapped by the tumor.

Conclusion

This work proposes the application of two components, which are already used in pharmaceutical and food industries (US FDA approved) as carriers for cancer prevention and therapy. The importance of the study relies on the ability of NPs to retain EGCG inhibitory effects of the proliferative activity of prostate cancer cells, high encapsulation efficiency (>80%) and the possibility of scaling up the process. Free EGCG is readily oxidized in the air. The aim of using the maltodextrin–gum arabic matrix was to increase its stability during storage and after administration. Furthermore, the system was not cytotoxic to Du145 prostate cells, suggesting that the polysaccharide NPs can be used as nanochemopreventive tools.

Future perspective

The concept of functional food and beverage as well as nutritional supplements is becoming increasingly important for consumers owing to claimed health benefits. It is now believed that many natural compounds, such as flavonoids, a subclass of polyphenols, exhibit biochemical and pharmacological activities, including modulation of carcinogenic metabolism and inhibition of cell proliferation. However, despite promising preclinical results, applicability of chemopreventive agents to humans has limited success due to inefficient delivery and bioavailability. If novel formulations of flavonoids efficiently reach the target tissue/organ, their medical benefit could increase significantly. We expect that the field of nanochemoprevention will be subject to continuous research in the coming

Executive summary

- Chemoprevention is the most cost-effective strategy for cancer control.
- Nanochemoprevention has recently emerged as a novel approach using encapsulated agents.
- The inhibitory action of epigallocatechin gallate (EGCG), a potential chemopreventive agent, on proliferation of prostate cancer cells was studied.
- We have developed a protective encapsulation system for EGCG based on maltodextrin and gum arabic nanoparticles.
- The results of cell viability and clonogenic potential show that the system was able to retain EGCG anticancer activity, while at the same time providing efficient protection to the molecule.

years owing to recent promising results concerning the efficacy of NPs in improving the performance of clinical/medical prevention/therapies in oncology.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

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- ▪▪ of considerable interest

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