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Research paper

β-Casein nanoparticle-based oral drug delivery system for potential treatment of gastric carcinoma: Stability, target-activated release and cytotoxicity

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ABSTRACT

We studied a potential drug delivery system comprising the hydrophobic anticancer drug paclitaxel entrapped within β-casein (β-CN) nanoparticles and its cytotoxicity to human gastric carcinoma cells. Paclitaxel was entrapped by stirring its dimethyl sulfoxide (DMSO) solution into PBS containing β-CN. Cryo-TEM analysis revealed drug nanocrystals, the growth of which was blocked by β-CN. Entrapment efficiency was nearly 100%, and the nanovehicles formed were colloidally stable. Following encapsulation and simulated digestion with pepsin (2 hours at pH = 2, 37 °C), paclitaxel retained its cytotoxic activity to human N-87 gastric cancer cells; the IC_{50} value (32.5 ± 6.2 nM) was similar to that of non-encapsulated paclitaxel (25.4 ± 2.6 nM). Without prior simulated gastric digestion, β-CN-paclitaxel nanoparticles were non-cytotoxic, suggesting the lack of untoward toxicity to bucal and esophageal epithelia. We conclude that β-CN shows promise to be useful for target-activated oral delivery of hydrophobic chemotherapeutics in the treatment of gastric carcinoma, one of the leading causes of cancer mortality worldwide.

1. Introduction

Gastric cancer is a major cause of cancer mortality worldwide [1]. Many of the current chemotherapeutic drugs for the treatment of multiple human malignancies are administered intravenously (IV). IV administration of chemotherapeutics is a major source of discomfort and stress to patients, and high costs due to multiple hospitalizations required to complete the multiple IV sessions of chemotherapeutic regimens [2]. The availability of suitable and effective oral therapeutic agents would make a significant contribution to patients' quality of life, may significantly reduce costs, and may prove more effective than current treatment modalities.

Beta-casein (β-CN), one of the four main caseins in bovine milk, comprises 17% proline residues, leading to an open tertiary structure, which is easily accessible to gastric proteases. Hence, we have proposed [3–5] that it may serve as an oral delivery vehicle for releasing chemotherapeutic cargo in the stomach for the treatment of gastric carcinoma. Another important feature of β-CN making it highly suitable for this task is its pronounced amphiphilic structure [6,7], which enables it to self-assemble in aqueous solution, thereby forming stable micellar structures [8,9]. Beta-CN micelles contain 15–60 β-CN molecules, and they have radius of gyration (Rg) values ranging between 7.3 and 13.5 nm. The critical micellization concentration (CMC) ranges between 0.05 and 0.2% w/v, depending on temperature, pH, solvent composition, and ionic strength [10]. We have recently shown [3–5] that β-CN nanoparticles can entrap and deliver hydrophobic chemotherapeutics such as mitoxantrone, vinblastine, irinotecan, docetaxel, and paclitaxel. Paclitaxel (Taxol) commonly used for the treatment of gastric cancer [11–13] was chosen for further development of β–CN nanovehicles for target-activated release in the stomach with the aim of future treatment of gastric carcinoma.

Oral delivery of paclitaxel was investigated under different preclinical and clinical stages [12,14–18]. The reported delivery systems include: (a) Polyoxyethylated castor oil (Cremophor EL)

Abbreviations: β-CN, beta casein; C_{infl}, drug concentration at the inflection point of the cytotoxicity curve; CMC, critical micellization concentration; CPMB, counts per minute of β emission; DMSO, dimethyl sulfoxide; GIT, gastrointestinal tract; IC_{50}, drug concentration (nM) required to inhibit cell growth by 50%; IV, intravenous; *k*, cytotoxicity-mechanism related parameter of the cytotoxicity model; P_0, percent of live cells at zero drug concentration; P_∞, percent of live cells at "infinite" drug concentration, i.e., percent of drug resistant cells; PBS, phosphate-buffered saline; Rg, radius of gyration; SDS-PAGE, sodium dodecyl sulfate – polyacrylamide gel electrophoresis; XTT, 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide.

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and 50% (w/v) ethanol (Paxoral™); this delivery system is commonly used for IV administration, but it causes hypersensitivity reactions, acute nausea, and vomiting [12,14,16–18], and (b) polysorbate 80, which showed better absorption of orally given paclitaxel [15] compared to Cremophor EL. These delivery systems are synthetic surfactants and organic solvents aimed at solubilizing the drug in aqueous solution. In contrast, β-CN is a natural milk protein, whose structure is analogous to a di-block copolymer [19]. As such, β-CN micelles are expected to be extremely more stable than those of low molecular weight surfactant micelles, and the kinetics of premature release of entrapped hydrophobic molecules are expected to be several orders of magnitude slower compared to release from low molecular weight surfactants [20], which is a major advantage of using β-CN for this task, compared to the use of small surfactants. Additional advantages of β-CN over other oral delivery systems mentioned above are as follows: First, unlike β-CN, these systems cannot protect upper gastrointestinal tract (GIT) regions such as mouth and esophagus (on the contrary, Cremophor EL and other surfactants and solvents are notorious for damaging blood vessels and tissues they come in contact with around injection sites [21]); Second, such synthetic drug delivery vehicles including Cremophor EL are absorbed via the small intestine into the blood and may inflict undesirable side effects, including severe anaphylactoid hypersensitivity reactions, hyperlipid emia, abnormal lipoprotein patterns, aggregation of erythrocytes, and peripheral neuropathy [21], whereas β-CN is a component of natural GRAS (generally recognized as safe) food material. The target-activated release enabled by the natural properties of β-CN would allow the use of lower doses, consequently minimizing drug side effects and eliminating the need for undesirable artificial solubilizing agents. Complementary nano, micro, or macrocoatings made of other biopolymers should enable a programmed degradation pattern and release the drug cargo at the desired site further down the GIT. Moreover, one may increase the therapeutic index of the chemotherapeutic regimen by loading a synergistic combination of drugs within these nanoparticles. The major goal of the current research was to examine the performance of a β-CN-based delivery system comprising the hydrophobic anticancer drug paclitaxel and to determine its cytotoxicity to human gastric carcinoma cells with and without prior simulated gastric digestion.

2. Materials and methods

2.1. Drugs and chemicals

Paclitaxel (T7191, purity >97%, cytotoxic drug), β-CN from bovine milk (C6905, 90% purity), and pepsin from porcine gastric mucosa (P6887, 3260 unit/ mg protein, 0.92 mg protein/mg solids) were purchased from Sigma-Aldrich Ltd., Rehovot, Israel. [^{3}H] Paclitaxel [2,6-bemzamido-3H(N)] (MT-1646) (250 micro Curie, $5.55 * 10^8$ DPM) was purchased from Moravek Biochemicals, Brea, CA, USA. UltimaGold™ was obtained from Perkin Elmer, Waltham, MAS, USA. Cell proliferation kit (based on 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide (XTT)) was purchased from Biological Industries Ltd., Beth-Haemek, Israel.

2.2. Paclitaxel encapsulation in β-CN

A 10 mM stock solution of paclitaxel in dimethyl sulfoxide (DMSO) was freshly prepared prior to use. A stock solution of [^{3}H]paclitaxel was prepared by mixing [^{3}H]paclitaxel and unlabeled paclitaxel at $1{:}10^6$ molar ratio. A stock solution of β-CN was prepared by dissolving 1 mg/ml or 5 mg/ml β-CN in 0.1 M phosphate-buffered saline (PBS) at pH 7.0, ionic strength 0.1 M. PBS contained 80 mM NaCl, 5.65 mM Na_2HPO_4, and 3.05 mM NaH_2PO_4. The entrapment of paclitaxel in β-CN nanoparticles at 6:1 drug/ β-CN molar ratio (250 μM paclitaxel), representing the optimal paclitaxel/β-CN molar ratio for maximal capacity of paclitaxel-β-CN nanoparticles according to our previous study [3–5], was performed by adding [^{3}H]paclitaxel (final radiolabeled fraction of 1 ppm) in DMSO to 1 mg/ml β-CN stock solution in PBS while continuously stirring. The volume percentage of DMSO in PBS was 2.5%. The samples were equilibrated for ~16 h at room temperature (20–24 °C).

Cytotoxicity of paclitaxel in undigested β-CN nanoparticles was studied in serum-free medium (SFM), as described below in the "Cytotoxicity Assay" section. To obtain a final β-CN concentration of 1 mg/ml (above β-CN CMC[4]) and not to exceed 20% PBS, the entrapment of paclitaxel in β-CN nanoparticles was performed by adding 10 mM stock solution of paclitaxel in DMSO to 5 mg/ml β-CN stock solution in PBS while continuously stirring, to obtain different paclitaxel concentrations (0.5–5000 nM). The samples were equilibrated for ~16 h at room temperature (20–24 °C). Prior to the cytotoxicity experiment in SFM, the samples were diluted 5-fold and equilibrated for ~16 h at room temperature (20–24 °C).

2.3. Stabilization of paclitaxel by β-CN in an aqueous solution

Drug stability in different solvents as a function of time (for two weeks) was studied by dissolving 250 μM paclitaxel (1 ppm labeled) solution in: PBS, DMSO, and in 1 mg/ml β-CN in PBS (6:1 paclitaxel/β-CN molar ratio). The concentration of the dissolved drug as a function of time was determined by measuring [^{3}H] labeled paclitaxel using a liquid scintillation analyzer (Tri-Carb® liquid scintillation analyzer 2900TR, PerkinElmer® Shelton, CT, USA). At each time point, 200 μl of the solution was sampled and added to 3.5 ml UltimaGold™ and measured with a liquid scintillation analyzer. The concentration of paclitaxel was determined using a calibration curve of counts per minute of β emission (CPMB) vs. disintegrations per min (DPM), which were converted to the concentration of paclitaxel in the solution (tritiated and non-tritiated). The calibration curve was prepared by adding different volumes of [^{3}H] paclitaxel stock solution to DMSO to obtain different concentrations of [^{3}H] paclitaxel; then, 200 μl of the labeled drug in DMSO was added to 3.5 ml UltimaGold™ and measured using the liquid scintillation analyzer.

2.4. Microscopy

2.4.1. Light microscopy

Light microscopy image of pure 250 μM paclitaxel in PBS and 2.5% DMSO was compared to the image of the same concentration of paclitaxel entrapped in β-CN nanoparticles (6:1 drug/β-CN molar ratio). Light microscope images were taken at x100 magnification using Nikon Eclipse Ti-S microscope (Nikon Instruments Inc., Melville, NY).

2.4.2. Cryogenic transmission electron microscopy (cryo-TEM)

Cryo-TEM was used for the imaging of entrapped paclitaxel in β-CN nanoparticles at 6:1 molar ratio in PBS and 2.5% DMSO. An image of pure β-CN in the same buffer solution was taken for comparison. Vitrified specimens for cryo-TEM were prepared in a controlled environment vitrification system (CEVS) at 25 ± 0.01 °C and 95–99% relative humidity, to ensure fixed temperature and to avoid water loss from the solution during sample preparation. The specimens were prepared as thin liquid films (10–500 nm thick), on perforated polymer/carbon films and quenched into liquid ethane at its freezing point (−183 °C). This process is designed to prevent water crystallization during thermal fixation. In this manner, component segregation and rearrangement are mostly prevented, and the original fluid nanostructure is preserved. The

technique and apparatus were described in detail previously [22–24]. The vitrified samples were then stored under liquid nitrogen (−196 °C), transferred to an Oxford CT 3500 cooling holder via its "work station," and observed in a Philips CM 120 microscope at about −175 °C. Images were recorded at a 120 kV acceleration voltage, in a low-dose mode, to minimize electron-beam radiation damage. We used a Gatan Multi Scan 791 cooled CCD camera, with the Digital Micrograph 3.1 software package, to acquire the images. Images were recorded under focus of about 2 μm to enhance phase contrast.

2.5. Simulated gastric digestion

Gastric digestion was performed by simulating gastric conditions: pH = 2, 37 °C with continuous shaking for two hours using the gastric protease pepsin [25]. To determine the optimal β-CN/ pepsin w/w ratio, a stock solution of 1 mg/ml pepsin in 10 mM HCl was freshly prepared. The pH of a 1 mg/ml β-CN solution in PBS was adjusted from 7.0 to 2.2 using 1 M HCl [26]. The stock solution of pepsin was added to 1 mg/ml β-CN in a PBS solution at different β-CN/pepsin w/w ratios (from 5000:1 to 5:1) and incubated for 2 h, at 37 °C, while shaking continuously. The proteolytic reaction was terminated by increasing the pH to 7.0 with 1 M NaOH[26]. Degradation of β-CN as a function of β-CN is as follows: pepsin w/w ratio was studied using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The controls were β-CN in PBS at pH 7 (without any pH adjustments) in the absence of pepsin, and β-CN in PBS at pH 2 (after pH reduction) in the absence of pepsin.

To find the optimal simulated digestion time when β-CN is fully degraded (to maximally release the drug), and to assure the entrapped drug does not adversely affect protein digestibility, degradation of pure 1 mg/ml β-CN and of paclitaxel-β-CN nanoparticles (6:1 paclitaxel/β-CN molar ratio) as a function of simulated digestion time (up to 2 h) were compared using SDS-PAGE. A complementary method was used to follow the release of the drug with the time of digestion by observing the fraction of the drug which passes an ultrafiltration membrane. This was measured by centrifugal ultrafiltration using Microcon® centrifugal filter devices with a molecular weight cutoff (MWCO) of 10 kDa (Millipore, Billerica, MA 01821, USA). A sample of 300 μl of β-CN-drug nanoparticle solution, taken at different times during the simulated digestion, was centrifuged for 20 min at 13,000*g*, following which the sample reservoir above the membrane was practically dry. The amount of drug in the permeate was determined by liquid scintillation counting. To estimate the amount of paclitaxel that did not pass the membrane, the sample reservoir was placed upside down in a vial containing 300 μl PBS, the membrane was wet by PBS by shaking it a few times followed by spinning for 3 min at 1000*g*, and the amount of paclitaxel washed from the membrane was determined by scintillation counting. Prior to each centrifugal ultrafiltration, the total concentration of paclitaxel in the sample was also determined by liquid scintillation counting as described above. About 70% of total added paclitaxel was recovered from the centrifugal filter devices. The possibility that some of the released drug aggregated and thus did not pass the membrane, as well as the fact that about 30% of the drug remained in the membrane, precluded accurate determination of the fraction of drug released. Still, plotting the fraction of paclitaxel passing the membrane (as percent of the total recovered drug) vs. digestion time, allowed to determine the time for maximal release, which is the time required to achieve a constant maximal fraction of the drug in the permeate.

Simulated digestion for the cytotoxicity experiments: The pH of 1 mg/ml pure β-CN in PBS solution and of paclitaxel-β-CN nanoparticles at 6:1 paclitaxel/ β-CN molar ratio in PBS solution was adjusted from 7.0 to 2.2 by 1 M HCl. Pepsin dissolved in 10 mM HCl was added to the solutions at 50:1 β-CN/ pepsin w/w ratios (final pepsin concentration 0.02 mg/ml) and incubated for 2 h, at 37 °C, while continuously shaking. Aliquots of 100 μl were drawn at different times during the incubation, and the reaction in these aliquots was stopped by increasing the pH to 7.0 with 1 M NaOH. The controls were as follows: 0.02 mg/ml pure pepsin after a 2 hr digestion, and 1 mg/ml β-CN without pepsin and without decreasing the pH. Paclitaxel-loaded nanocapsules solution of 1 mg/ml β-CN at 6:1 paclitaxel/ β-CN molar ratio was digested at optimal simulated gastric digestion conditions of 50:1 β-CN/pepsin w/w ratio, at 37 °C, while continuously shaking for 20 min and was prepared for cytotoxicity assay on N-87 cells. Untreated paclitaxel in PBS precipitated as a function of time (Fig. 1). Hence, following 20 min of simulated digestion, the drug was centrifuged for 20 min at 13000*g*. The supernatant was removed and the sediment was dissolved in DMSO and left for ∼16 h to equilibrate at room temperature (20–24 °C). The concentration of paclitaxel was determined using liquid scintillation counting.

2.6. Tissue culture

Human gastric cancer N-87 cells were kindly provided by Prof. Yosef Yarden (Dept. of Biological Regulation, Weizmann Institute of Science, Rehovot, Israel). N-87 cells were maintained in RPMI-1640 medium (Invitrogen™ – GIBCO® Carlsbad, California), containing 10% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin (Biological Industries, Beth-Haemek, Israel), and incubated under humidified atmosphere containing 5% CO_2. For experiments using serum-free medium (SFM) growth, N-87 cells were gradually adapted to grow in SFM; the complete growth medium was diluted by 25% in serum-free RPMI-1640 each trypsinization and incubated under humidified atmosphere containing 5% CO_2. After a month of growth, the adapted cells grew in absolutely serum-free RPMI-1640 medium.

2.7. Cytotoxicity assay

The cytotoxic activity of paclitaxel entrapped in β-CN nanoparticles with and without prior simulated gastric digestion at the above optimal conditions was compared to the cytotoxic activity of untreated paclitaxel. The final paclitaxel concentration range in the serum of all systems was 0.1–10,000 nM. The preparation of undigested paclitaxel–β-CN nanoparticles is described in the "paclitaxel encapsulation in β-CN" section. To measure paclitaxel cytotoxicity following simulated digestion, paclitaxel in DMSO solution at a known concentration, as explained in "Simulated gastric digestion" section, was diluted in full serum RPMI-1640 medium to obtain final concentrations of 0.1–10,000 nM. The cytotoxic activity of paclitaxel following simulated digestion was compared to that of untreated paclitaxel in complete growth medium, and

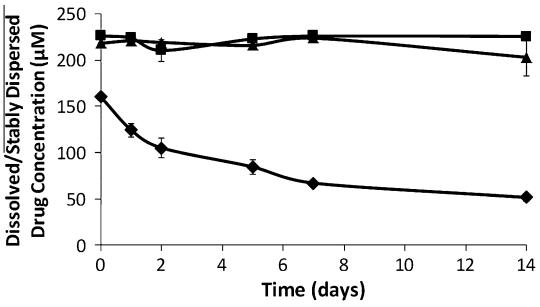

Fig. 1. Stability of paclitaxel over time in different solvents: DMSO (■), PBS (♦), and encapsulated in β-CN in PBS (▲).

the cytotoxic activity of paclitaxel-β-CN nanoparticles without simulated digestion was compared to that of untreated paclitaxel in SFM. The latter was used in this experiment both because human serum albumin competes with β-CN for paclitaxel binding, and because our primary goal was to develop an oral delivery system, hence, unlike in systemic delivery, serum albumin is not expected to come in contact with the nanoparticles. Still, in experiments, where binding competition posed no interference, a complete growth medium was used, as the cells can grow more rapidly in it. Untreated paclitaxel was prepared by diluting 10 mM stock solution of paclitaxel in DMSO in complete growth medium and in SFM thereby obtaining final concentrations of 0.1–1000 nM.

Cytotoxic activity was measured using an XTT-based colorimetric cell proliferation kit. Exponentially growing cells were seeded at 5×10^3 cells/well in 96-well plates (90 μl of growth medium/well). After an overnight incubation, cells were exposed to different drug concentrations (10 μl drug solution/well) for 72 h. Cellular viability was determined by adding the XTT reagent and an activation reagent at 50:1 v/v ratio (total volume 50 μl/well) and incubating for 2 h at 37 °C. Following solubilization of the dye, absorbance was determined by a microplate reader (ASYS Hitech GmbH, Austria). Values presented are means of at least three independent experiments, each performed in triplicate. Cells grown in a drug-free medium or with added 0.1 mg/ml β-CN, 0.1 mg/ml digested β-CN, or 10% PBS stock solution served as controls.

2.8. Statistical analysis

The encapsulation of paclitaxel in β-CN was performed in duplicate for all experiments. The calibration curve and the drug dissolution in DMSO and PBS for the experiments on stabilization of paclitaxel by β-CN were made in duplicate, and an average value and a standard error (standard deviation divided by square root of the number of replicates) were calculated at each point.

About 10 similar images were collected by light microscope and cryo-TEM from three different samples for each experiment, and one representative image is presented.

To determine the optimal pepsin/β-CN mass ratio for simulated gastric digestion, the digestion of 1 mg/ml β-CN by different concentration of pepsin was performed in duplicate. A scan of one representative SDS-PAGE gel is presented. The digestion of pure β-CN and paclitaxel-loaded β-CN at optimal pepsin concentration was also performed in duplicate, and one representative SDS-PAGE gel is presented for each experiment. The paclitaxel release experiment as a function of digestion time was performed in duplicate. An average value and a standard error were calculated at each point.

For each cytotoxicity assay, three independent experiments were performed (i.e., preparation of pure paclitaxel, pure β-CN and paclitaxel entrapment in β-CN solutions, simulated gastric digestion of pure β-CN and β-CN-paclitaxel nanoparticles, and three independent cell lines growing for 3 weeks separately). Each of these three independent experiments was performed in triplicate. The presented values are averages of these three experiments, and standard error is presented by error bars for each point.

To analyze drug cytotoxicity data and compare the free drug, the encapsulated drug and the drug released by simulated digestion, we have developed the following model equation (Eq. 1):

$$P = P_0 + \frac{P_\infty - P_0}{1 + \left(\frac{C_{infl}}{C}\right)^k} \quad (1)$$

Here, P is the percent of surviving cells at each drug concentration, P_0 is the percent at zero drug concentration (ideally 100%, however, as this parameter was obtained from the model fit, small deviations from 100% were possible), P_∞ is the percent of drug-tolerant cells, C_{infl} is the drug concentration (nM) at the inflection point, C is the drug concentration (nM), and k is a cytotoxic-mechanism related curve-steepness parameter. The values of the parameters were obtained through non-linear model-fitting by least square minimization, using Microsoft Excel™ Solver. The IC_{50} was calculated numerically from the obtained model equation. Drug concentrations necessary to inhibit cell growth by 50% (IC_{50}) compared to untreated controls were determined by fitting the data to the model equation (Eq. 1). The statistical analysis of variance of the encapsulated vs. the unencapsulated paclitaxel was made using a two-sample T-test, assuming unequal variances, using Microsoft Excel™.

3. Results

3.1. Stabilization of paclitaxel in aqueous solution by β-CN

The stability of paclitaxel in β-CN nanocapsules was compared to its stability in PBS and also in DMSO which is a good solvent for paclitaxel. The β-CN nanocapsules system was prepared by dissolving paclitaxel in DMSO, followed by its encapsulation in 1 mg/ml β-CN in PBS (at a molar ratio of 6:1 paclitaxel/ β-CN). Each system contained 250 μM paclitaxel ([^{3}H] labeled at 1 ppm). The three different systems were studied as a function of time for 2 weeks. The concentration of the dissolved or stably dispersed drug as a function of time is presented in Fig. 1.

This concentration was determined by liquid scintillation counting by sampling the liquid from the top of the vial following vortexing. It is evident from Fig. 1 that the concentration of stably dispersed paclitaxel encapsulated in β-CN was constant during the two weeks period of the experiment and was practically equal to the concentration of paclitaxel dissolved in DMSO (about 220 μM). However, when paclitaxel was added predissolved in DMSO into PBS in the absence of β-CN, it formed an unstable dispersion, and the concentration of dispersed paclitaxel decreased as a function of time to 50 μM during the two weeks of observation due to precipitation.

To gain further insight into drug encapsulation and stability in aqueous solution, a light microscopy image of pure paclitaxel in PBS containing 2.5% DMSO (Fig. 2a) was compared to an image of paclitaxel entrapped in β-CN nanoparticles at 6:1 drug/β-CN molar ratio in the same aqueous solution, (Fig. 2b).

It is evident that in the absence of β-CN, paclitaxel forms large droplets of about 15–20 μm after its addition to PBS (predissolved in the DMSO). A closer look reveals needle-shaped crystals protruding radially from the droplet surface, meaning that the drug tends to crystallize under these conditions. However, when paclitaxel was added to a β-CN-containing solution, small sub-micron dots can be observed.

A closer observation, at a much higher magnification, was performed by Cryo-TEM as presented in Fig. 2c and d.

Fig. 2c surprisingly reveals a few paclitaxel nanocrystals, which are clustered together (the crystal cluster was ~250 nm long and 150 nm wide). This image revealed that the drug crystallizes even in the presence of β-CN, but apparently the protein prevents crystal growth. The grainy surface and the grainy contour of the crystal suggest it is covered with β-CN molecules that are adsorbed to its surface. This way β-CN encapsulates the drug crystals and keeps them nanometric in size. Moreover, β-CN micelles are also discernible as round gray aggregates, mainly seen in the bottom part of Fig. 2c above the carbon grid and around the paclitaxel crystal cluster. Comparing β-CN micelles in this figure to empty β-CN micelles depicted in Fig. 2d, the empty β-CN micelles in Fig. 2d have diameters of about 15–20 nm, in agreement with other studies [10] and our previous dynamic light scattering results [4]. The diameter of

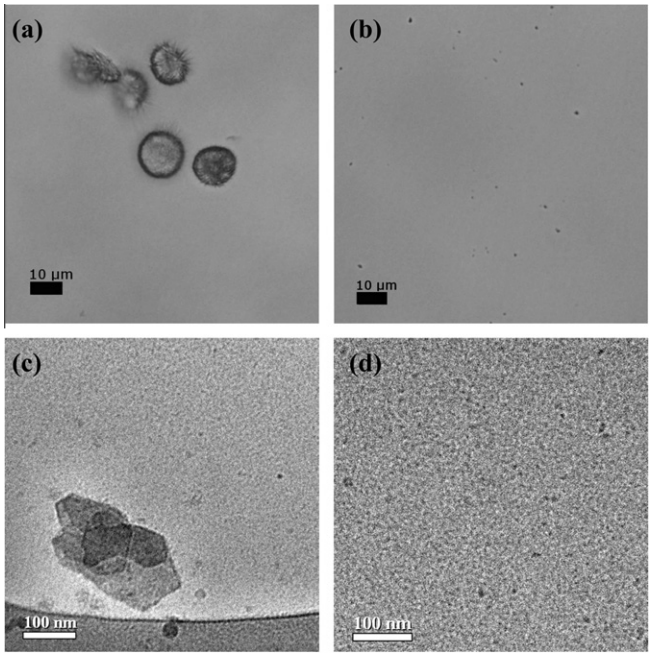

Fig. 2. Top: Light microscope images of (a) pure paclitaxel in PBS containing 2.5% DMSO and (b) paclitaxel-β-CN nanoparticles in PBS containing 2.5% DMSO. Bottom: cryo-TEM images of (c) paclitaxel-β-CN nanoparticles in PBS containing 2.5% DMSO (same system as in b) and (d) pure β-CN in PBS containing 2.5% DMSO.

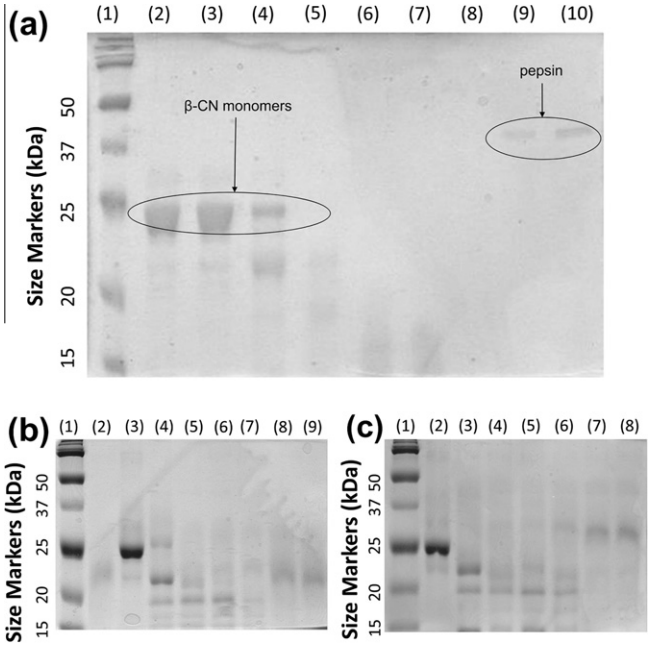

Fig. 3. Top: (a) SDS-PAGE analysis of simulated digestion products of β-CN as a function of pepsin concentration at the following conditions (1 mg/ml β-CN, pH = 2, for 2 hr, at 37 °C, continuously shaking) as a function of β-CN/pepsin w/w ratio. (1) MW markers, (2) no pepsin at pH 7, (3) no pepsin at pH 2, (4) 5000:1 at pH 2, (5) 1000:1 at pH 2, (6) 500:1 at pH 2, (7) 100:1 at pH 2, (8) 50:1 at pH 2, (9) 10:1 at pH 2, (10) 5:1 at pH 2. Bottom: SDS-PAGE analysis of simulated digestion products of β-CN as a function of time; simulated digestion conditions (β-CN/pepsin 50:1 w/w ratio, pH = 2, at 37 °C, continuously shaking) as a function of digestion time. (b) 1 mg/ml pure β-CN: (1) size markers, (2) control: 0.02 mg/ml pure pepsin after 120 min digestion (3) 0 min, (4) 1 min, (5) 5 min, (6) 10 min, (7) 20 min, (8) 60 min, (9) 120 min. **(c)** 250 μM paclitaxel encapsulated in 1 mg/ml β-CN nanoparticles (6:1 paclitaxel/β-CN molar ratio): (1) size markers, (2) 0 min, (3) 1 min, (4) 5 min, (5) 10 min, (6) 20 min, (7) 60 min, (8) 120 min.

some of the micelles in the presence of paclitaxel in Fig. 2c is higher (about 30–40 nm), and blotted areas can be seen in their cores, which may indicate entrapped paclitaxel. The β-CN micelles in Fig. 2d appear darker than those in Fig. 2c, because the carbon grid at the bottom of Fig. 2d (which is absent in Fig. 2c), created a sharper contrast, thus the rest of the image became lighter, compared to Fig. 2d.

3.2. Simulated gastric digestion and target-activated paclitaxel release

To determine the optimal β-CN/pepsin w/w ratio, β-CN proteolysis was studied as a function of β-CN/pepsin w/w ratio. Pepsin was added to β-CN in a PBS solution at various β-CN/pepsin w/w ratios (from 5000:1 to 5:1) and then incubated for 2 h, at 37 °C, and pH 2, while continuously shaking. Fig. 3a depicts the pattern of β-CN breakdown products as a function of β-CN/pepsin (w/w) ratio. The controls were β-CN in PBS at pH 7.0 and pH 2, both in the absence of pepsin.

No significant β-CN degradation was observed in the absence of pepsin at the acidic pH (pH 2), compared to untreated β-CN at pH 7.0. Expectedly, the concentration of intact β-CN molecules diminished with increasing pepsin concentration (decreasing β-CN/pepsin ratio) while short β-CN peptides appeared. As pepsin concentration was increased, peptides became shorter, and at the highest pepsin concentrations studied, no β-CN peptide bands (above 15 kDa) were discernible. At a 50:1 β-CN/pepsin molar ratio, neither β-CN monomers nor short peptides were observed. Hence, the optimal β-CN/pepsin w/w ratio was found to be 50:1. It is noteworthy that at 10:1 and 5:1 β-CN/pepsin ratio 0.1 mg/ml and 0.2 mg/ml pepsin, respectively, a band of intact pepsin was observed (the molecular mass of pepsin is 35 kDa), which remained due to the relatively high initial enzyme concentration, while at lower enzyme concentrations it was all self-digested.

To test whether or not the presence of paclitaxel affects the enzymatic digestion of β-CN and to estimate the time for maximal release of the drug, degradation of pure β-CN was compared to the degradation of β-CN in β-CN-paclitaxel nanoparticles (6:1 paclitaxel/β-CN molar ratio) as a function of simulated digestion time (up to 2 h) using SDS-PAGE. Results are presented in Fig. 3b and c.

It is evident that the presence of paclitaxel had no significant effect on the progress of digestion with time. In both Fig. 3b and c, after 5 min of digestion, no β-CN monomers could be observed. Up to ~10–20 min of digestion peptides were still visible, while at 60 min of digestion, no β-CN peptides were observed, in agreement with previous studies of Schmelzera and coworkers [26], who studied the peptic digestion of β-casein. A control of 2 h self-digestion of 0.02 mg/ml pure pepsin was also performed, wherein a band with a MW of 20–25 kDa was observed (Fig. 3b lane 2). A band of 20–25 kDa was also discernible at different times of digestion, the MW of which decreased as a function of time, but it became more intense at 60 and 120 min. After 2 h of digestion, this band appeared with the same MW as pure pepsin digestion after 2 h (Fig. 3b lane 9 vs. lane 2, respectively), but it was more intense in the presence of β-CN (lane 9). This band might be a combination of auto-degradation peptides of pepsin and some residual intact or slightly digested β-CN monomers.

The time of maximal drug release during simulated gastric digestion was further studied by a complementary observation focused on the drug, rather than the protein. As described in the materials and methods section, paclitaxel concentration was determined in the permeate obtained by centrifugal ultrafiltration at different times during simulated gastric digestion of paclitaxel-loaded β-CN nanoparticles. The fraction of paclitaxel found in the permeate with simulated digestion time is presented in Fig. 4; the fraction of paclitaxel in the permeate increased during the first 10–20 min of digestion, within which, apparently the maximal

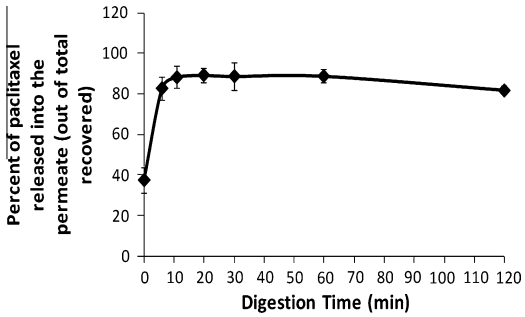

Fig. 4. Fraction (%) of paclitaxel released into the permeate (out of the total paclitaxel recovered from the ultrafiltration centrifugal filter devices), as a function of simulated gastric digestion duration.

amount of paclitaxel was released from β-CN-paclitaxel nanoparticles, and no further paclitaxel release was observed at longer digestion times.

3.3. Paclitaxel cytotoxicity

The cytotoxic activity of paclitaxel after encapsulation (1 mg/ml β-CN at 6:1 paclitaxel/β-CN molar ratio) and simulated gastric digestion at the optimal conditions (50:1 β-CN/pepsin w/w ratio, at 37 °C, pH 2, while shaking continuously for 20 min) was compared to that of untreated paclitaxel on N-87 gastric cancer cells in complete RPMI-1640 growth medium. Surviving fraction versus the concentration of untreated paclitaxel as well as paclitaxel after encapsulation and simulated digestion is presented in Fig. 5.

The data show that encapsulation and simulated digestion did not diminish the cytotoxic potential of paclitaxel, as the IC_{50} values were not significantly different ($p = 0.215$) from those of untreated paclitaxel. The summary of the model parameters (Eq. 1) obtained is presented in Table 1.

The cytotoxicity of undigested β-CN-paclitaxel nanoparticles was also determined in SFM (as described in the above section 2.7 "Cytotoxicity assay"). The cytotoxic activity of the undigested paclitaxel-β-CN nanoparticles was compared to that of untreated paclitaxel, both tested in SFM. As shown in Fig. 5, undigested β-CN-paclitaxel nanoparticles had no cytotoxic effect on N87 cells grown in SFM, whereas untreated paclitaxel showed cytotoxicity

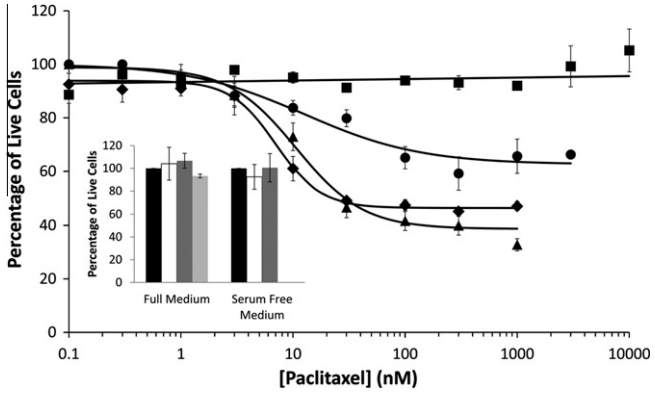

Fig. 5. Percentage of live cells as a function of paclitaxel concentration: paclitaxel – β-CN nanoparticles following simulated digestion in full RPMI 1640 medium ▲ vs. undigested paclitaxel – β-CN nanoparticles in serum-free RPMI 1640 medium ■, and vs. untreated paclitaxel in full RPMI 1640 medium ♦ and in serum-free RPMI 1640 medium ● (lines represent the model fit (Eq. 1)). *Inset*: Percentage of live cells in the different control solutions: 1 mg/ml β-CN ▩, 0.1 mg/ml digested β-CN ▩, 20% PBS □, and in medium only ■ (full RPMI 1640 medium-left, and serum-free RPMI 1640 medium-right).

on these cells. The cytotoxicity of the untreated paclitaxel in SFM was lower than that of the untreated paclitaxel in complete growth medium probably because of some paclitaxel precipitation in SFM which lacks serum proteins, while in serum-containing growth medium, it is presumably partly solubilized by bovine serum albumin, which is known to bind various molecules and drugs including paclitaxel [27].

The inset in Fig. 5 presents the controls: cells cultured in growth medium only, cells in growth medium containing 20% added PBS as well as cells in a medium containing 1 mg/ml pure undigested or 0.1 mg/ml digested β-CN. No significant cytotoxic effect was observed with these controls.

4. Discussion

4.1. Paclitaxel stabilization by β-CN in an aqueous solution

Based on the paclitaxel stability experiments in different solvents (Fig. 1), paclitaxel is unstable in PBS and it aggregates and precipitates with time, in agreement with previous studies [28,29]. The concentration of the dissolved or dispersed paclitaxel decreased, apparently because it precipitated and partly adsorbed to the test tube, thus even vortexing prior to sampling failed to redissolve or disperse it. However, encapsulation of paclitaxel in β-CN nanoparticles stabilized this taxene drug in PBS and prevented aggregation and precipitation in agreement with previous studies which showed that paclitaxel encapsulation prevents it from aggregation in an aqueous solution [29–31]. These results are in agreement with the findings of a clear solution of paclitaxel encapsulated in 1 mg/ml β-CN at 12:1 paclitaxel/β-CN molar ratio compared to the results of the turbid solution containing visible white aggregates of untreated paclitaxel at higher concentration (504 μM) dissolved in PBS, which were described in our previous paper [3].

If the unencapsulated drug precipitates with time, then any unencapsulated or released drug in the β-CN system in Fig. 1 would have precipitated. Therefore, the fact the graph of paclitaxel in β-CN remained almost identical to that in DMSO, means the entrapment efficiency remained stably very close to 100%. The lack of cytotoxicity of paclitaxel in non-digested β-CN nanoparticles shown in Fig. 5 provides a strong support to this result of excellent entrapment efficiency. This high entrapment efficiency was quite constant (within experimental error) during the two weeks observation period (Fig. 1) without any additional stabilizers or preservatives. Addition of preservatives or heat-sterilization (caseins are fairly heat-stable) may further prolong β-CN stability in PBS.

In agreement with paclitaxel stabilization by β-CN in aqueous solution which was shown above, light microscopy and cryo-TEM analyses demonstrated that the presence of β-CN stabilized paclitaxel in the aqueous solution. Light microscope images show that in the absence of β-CN, paclitaxel formed large droplets ~15–20 μm in diameter, with protruding needle-like crystals. The spherical droplet core is likely to be also crystalline, but its spherical shape indicated a quick liquid-phase separation which had occurred right after DMSO dissipation in the water, following addition of paclitaxel in DMSO solution into the buffer as discussed in our previous study [3]. Later-on, the slower processes of crystallization outside (and most likely also inside) the droplet apparently occurred, leading to the formation of a corona of typical needle-like crystals on the droplet surface. The size and the needle-like shape of the crystals on the droplet surface are in agreement with observations of Castro et al [28] (although the droplets were not observed there, probably due to differences in concentrations and different preparation procedures).

When paclitaxel is added to β-CN solution, as shown in the cryo-TEM analysis (Fig. 2b left), in addition to small paclitaxel

Table 1
Cytotoxicity Model Parameters (Eq. (1)) and IC_{50} Results.

	IC_{50} (nM)	C_{infl} (nM)	k	P_∞ (%)	P_0 (%)
Untreated[a] paclitaxel	22.1 ± 4.7	7.5 ± 1.3	2.61 ± 0.67	46.3 ± 0.7	93.7 ± 1.7
Treated[a] paclitaxel	30.4 ± 7.8	10.7 ± 2.9	1.67 ± 0.32	39.3 ± 3.2	99.3 ± 1.0

[a] Treated paclitaxel had undergone encapsulation in β-CN followed by simulated gastric digestion. Values reported are means of three independent experiments, each performed in triplicates ± standard error.

nanocrystals surrounded by adsorbed β-CN molecules, which stabilize it and prevent it from further crystal growth or aggregation up to micrometric dimensions, molecularly bound and smaller paclitaxel nanocrystals might also be entrapped in the cores of β-CN micelles. The inhibition of paclitaxel aggregation by β-CN encapsulation was proposed in our previous paper [3]. The current study indicates that following the initial aggregation, which is terminated at a smaller aggregate size by the protein, paclitaxel later crystallizes. Paclitaxel crystals in aqueous solution have been observed in other studies [28,32]. Tubulin was shown to facilitate nucleation of paclitaxel, modifying the shape of its resulting crystals [28], and immunoglobulins in blood were shown to suppress paclitaxel crystallization [32]. However, the suppression of paclitaxel crystal size from micro to nanoscale and modification of crystal morphology (from needle to more uniform polygonal structures), via adsorption to β-CN, are apparently reported here for the first time.

4.2. Simulated gastric digestion and target-activated release

Based on our SDS-PAGE results (Fig. 3b and c) which showed that β-CN peptides were still seen up to ~10–20 min of incubation with pepsin and that about the same time was required to reach maximal paclitaxel release (Fig. 4), the simulated digestion time for the subsequent *in vitro* experiments was set at 20 min to ensure the completion of paclitaxel release. Based on our SDS-PAGE analysis of β-CN degradation as a function of time, the presence of the drug did not significantly affect the digestibility of β-CN. This study showed only small differences in band pattern between pure β-CN degradation and that of paclitaxel-loaded β-CN nanoparticles (6:1 paclitaxel/β-CN molar ratio); however, the optimal digestion time was identical: after 5 min of digestion no more intact β-CN molecules were observed, and breakdown peptides were only seen until 10–20 min. The results of paclitaxel release correlated with the results of β-CN degradation; the maximal amount of drug was released after about 10–20 min of simulated digestion. These results reveal an excellent target-activated release effect for possible future treatment of gastric cancers using β-CN nanoparticles, based on the open structure of the β-CN, which evolved, as all caseins, to be easily digestible by gastric proteases.

4.3. Paclitaxel cytotoxicity

The cytotoxic activity of paclitaxel after encapsulation (in 1 mg/ml β-CN at 6:1 paclitaxel/β-CN molar ratio) and following simulated gastric digestion at the optimal conditions (50:1 β-CN/pepsin w/w ratio, at 37 °C and pH 2, while shaking continuously for 20 min) was compared to the cytotoxic activity of untreated paclitaxel on N-87 cells in complete RPMI-1640 growth medium. According to the calculated IC_{50} values, after encapsulation and simulated digestion, paclitaxel did not lose its potent cytotoxic activity, as its IC_{50} value (30.4 ± 7.8 nM) was insignificantly different from that of untreated paclitaxel (22.1 ± 4.7 nM) and in reasonably good agreement with Gong et al., [33] who reported an IC_{50} of 15.8 nM. We have shown that β-CN is rapidly and efficiently digested by gastric proteases such as pepsin, and the model hydrophobic drug paclitaxel was promptly released from β-CN-paclitaxel nanoparticles following β-CN digestion. Moreover, the cytotoxic activity of paclitaxel was fully retained following encapsulation and simulated gastric digestion.

To examine the potential for the protection of the upper regions of the GIT, that is, mouth and esophagus from paclitaxel during ingestion, provided by paclitaxel entrapment in β-CN, the cytotoxicity of paclitaxel-β-CN nanoparticles without prior simulated gastric digestion was examined in SFM RPMI-1640 medium. The association constant of paclitaxel to human serum albumin (HSA) is much higher than that of paclitaxel to β-CN: 1×10^5–2.1×10^6 M^{-1} [27] vs. $(6.3 \pm 1.0) \times 10^3$ M^{-1} [3], respectively. The maximal paclitaxel molar binding ratio of human serum albumin (HSA) and β-CN is similar 6.6 [27] and 7.3 ± 1.2 [3]. If both β-CN and HSA are present in the medium, HSA will favorably compete with β-CN on paclitaxel binding thus facilitating paclitaxel uptake by the cells, thereby causing an increased cytotoxic effect. On the other hand, HSA is not significantly present in the bucal cavity, esophagus, or the lumen of the GIT. Hence, SFM was chosen for these experiments (It is noteworthy that the control of 0.1 mg/ml digested β-CN in SFM – light gray, right side of the inset in Fig. 5 – was unneeded and thus was not performed, as this experiment was aimed at observing pregastric effects). We have shown that N-87 gastric carcinoma cells that were grown in SFM retained complete sensitivity to untreated paclitaxel. Therefore, it is evident that when paclitaxel is entrapped within β-CN nanoparticles, it is not cytotoxic. The proposed drug delivery system is thus expected to protect the bucal cavity and the esophagus from paclitaxel (and maybe other hydrophobic drugs) and will apparently release it efficiently in the stomach.

The abilities of β-CN to protect tissues from paclitaxel before the protein is digested and to achieve "target-activated release" of β-CN in the stomach are unique in comparison with other drug delivery systems proposed for paclitaxel including Cremophor EL[12,14,16–18] and polysorbate 80 [15].

5. Conclusions

We conclude that β-CN displayed a very good entrapment efficiency, capacity, and stabilization properties for paclitaxel in aqueous solution. The finding that undigested β-CN-encapsulated paclitaxel was not cytotoxic to the gastric cancer cell line studied suggests that β-CN may possibly protect upper GIT regions including mouth and esophagus from paclitaxel (and possibly other cytotoxic cargos) and efficiently release it in the stomach, without compromising drug cytotoxicity. β-CN shows promise to serve as a useful nanoscopic vehicle for the aqueous solubilization and stabilization of this antitumor agent in oral delivery preparations and possibly other hydrophobic therapeutic drugs and drug combinations aimed at treating malignant and non-malignant gastric and small intestine disorders.

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