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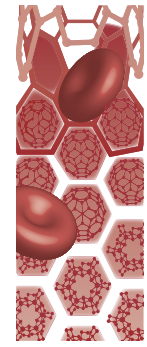

Gemcitabine delivered by fucoidan/chitosan nanoparticles presents increased toxicity over human breast cancer cells

Catarina Oliveira[1,2], Nuno M Neves[1,2,3], Rui L Reis[1,2,3], Albino Martins[1,2] & Tiago H Silva*[,1,2]

[1]3B's Research Group – Biomaterials, Biodegradables & Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering & Regenerative Medicine, AvePark, Parque de Ciência e Tecnologia, 4805-017 Barco, Guimarães, Portugal

[2]ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal

[3]The Discoveries Centre for Regenerative & Precision Medicine, Headquarters at University of Minho, Avepark, 4805-017 Barco, Guimarães, Portugal

*Author for correspondence: tiago.silva@dep.uminho.pt

Aim: To produce marine-origin nanoparticles (NPs) aiming to develop more effective and tolerated therapies for breast cancer. **Materials & methods:** NPs based in two marine-origin polymers (fucoidan and chitosan) were prepared by polyelectrolyte complexation, for the delivery of an antitumor drug model (gemcitabine [Gem]). **Results:** Final formulation resulted in stable NPs around 115–140 nm in size and with a polydispersity index less than 0.2. Gem was encapsulated at a maximum entrapment efficiency of 35–42%. Drug-release studies demonstrated that around 84% of Gem is released within 4 h. Cytotoxicity results of Gem-loaded NPs showed increased toxicity (around 25%) when compared with free Gem. **Conclusion:** The drug-loaded NPs present increased toxicity over human breast cancer cells without increasing toxic effects over endothelial cells.

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Keywords: breast cancer cells • chitosan • drug delivery system • endothelial cells • fucoidan • gemcitabine • nanoparticles • polyelectrolyte complexation

Cancer is one of the leading causes of death worldwide and is characterized by the uncontrolled and fast growth of cells. In 2012, there were around 14 million new cases of cancer and around 8 million resulted in death. By 2035, it is expected to be around 24 million new cases per year [1]. Breast cancer is the most common cancer in females and the second deadliest [2]. Surgery, radiation and systemic therapies (chemotherapy, hormonal and target) are some of the current breast cancer treatment modalities available, depending on the extent and type of cancer [3]. Some of these treatments may present some limitations since, besides affecting the tumor, they also affect the surrounding healthy environment, and may present some side effects [3,4]. For these reasons, the use of natural substances is an interesting alternative due to their low toxicity and biological and chemical similarities to native tissue compositions.

Among natural substances, marine organisms are valuable sources of materials with intriguing properties and characteristics [5]. Fucoidan (Fu) is a naturally derived sulfated polysaccharide extracted from brown seaweed and has been extensively investigated because of its various biological properties such as anticoagulant, antiviral, antiangiogenic, anti-inflammatory, immunomodulating and, most recently, antitumor behavior [6,7]. Looking specifically at its antitumoral behavior, we recently reported that different Fu extracts present different biological responses over human breast cancer and noncancer cell lines with chemical structure apparently playing an important role [8]. Some Fu extracts presented the desired antitumoral behavior, whereas other presented toxicity for both cancer and noncancer cells or where nontoxic for both types of cells, depending on their chemical features.

In this work, we propose the use of nontoxic Fu to develop a natural marine-origin drug delivery system (DDS) for the release of anticancer drugs. Indeed, Fu have been included in nanosystems for diagnostic, drug delivery and regenerative medicine [9,10] and have been combined with chitosan (CH), a marine-derived polycation, to prepare

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nanoparticles (NPs) by polyelectrolyte complexation for the delivery of growth factor drugs and other biological compounds [10–13].

NPs have been developed as DDS for the delivery of drugs or biomolecules. NPs-based DDS presents attractive properties since they can pass through the smallest capillary vessels and penetrate cells/tissue gaps to arrive at the target site [14,15]. They may also improve macromolecule stability, protecting them from enzymatic degradation and controling their release profile. However, they may present some limitations like drug loss during circulation and limited drug-loading capacity.

Gemcitabine (Gem), an antitumor drug, has been applied in the treatment of different cancers such as lung, ovary and pancreatic [16–18]. It has been also used in the treatment of breast cancer, as a single therapy or in combination with other chemotherapeutic agents [19,20]. Gem is a nucleoside analog that inhibits DNA synthesis, by its incorporation into DNA, followed by inhibition of cell proliferation. Gem presents a short biological half-life, being rapidly metabolized. This limits its biological effectiveness, since higher doses are required to reach a therapeutic concentration, increasing the risk of side effects [21,22]. In this sense, there is the unmet therapeutic need to try to enhance Gem biological response by incorporating it into a DDS, which has been previously reported [23,24].

To the best of our knowledge, there are no reports in the literature regarding the development of Fu/CH NPs for the delivery of Gem. In this sense, we propose the combination of Fu (negatively charged) with CH by polyelectrolyte complexation to produce NPs for controlled release of Gem as potential breast cancer therapy. Regarding NP production, there are different parameters that need to be optimized such as polymers concentration and ratio, polymeric solution pH and ionic strength. Therefore, these different parameters were tested and combined to obtain the NPs with smaller size and polydispersity index (PDI). NPs stability is another important aspect that needed to be evaluated. Keeping in mind the tumor microenvironment, NPs toxicity was evaluated over cancer and noncancer cells. The release profile of Gem was analyzed and loaded-NPs tested over endothelial and breast cancer cells as an *in vitro* assessment of antitumor efficiency through cytotoxicity.

Experimental section

Materials

Fu from *Fucus vesiculosus* (molecular weight [MW] by gel permeation chromatography [GPC] 45–75 kDa) was purchased from Marinova (batch number: DPFVF2015505), whereas CH 95/20 (MW by GPC 40–150 kDa) was purchased from Heppe Medical Chitosan GmbH (batch number: 212-261115-04, Halle, Germany). 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were both purchased from Sigma-Aldrich (MO, USA). Centrifugal concentrators Vivaspin 20 300 KD (Sartorius; Stonehouse, UK) were purchased from Fisher Scientific. 2′-Deoxy-2′,2′-difluorocytidine (Gem – $C_9H_{11}F_2N_3O_4$) was purchased from Carbosynth (Berkshire, UK). Human umbilical vein endothelial cells (EA.hy926) and breast cancer cells (MDA-MB-231) were both purchased from ATCC (VA, USA).

Methods

Preparation of NPs

Marine-origin polymeric NPs were prepared by polyelectrolyte complexation. Optimization was performed to study the effect of different parameters over size and PDI. From the results obtained, for each parameter, we chose the production condition that combined the smaller size and PDI values. Different parameters were evaluated, such as concentration of both polymers (ranging from 0.25 to 1 mg/ml); the ratio of polymers solution (CH:Fu – 1:2, 1:1, 2:1); CH and Fu solutions pH; ionic strength (ultrapure water [UPW], osmotized water and 0.9% NaCl) and cross-linking agent concentrations: 600/240, 300/120 and 150/60 mM (EDC/NHS). Each parameter was evaluated separately while all the others were kept constant.

After all the optimization steps, NP parameters were maintained for all further assays. Briefly, CH was dissolved in 1% v/v acetic acid solution at 0.5 mg/ml concentration, whereas the polyanion (Fu) was prepared in UPW to reach a final concentration of 0.5 mg/ml. CH and Fu solutions were adjusted with 1 M HCl and 1 M NaOH, depending on the desired pH. CH solution was adjusted to pH 5, whereas Fu solution was measured and the pH adjusted if its values were not as expected (pH 9). 3 nl of Fu solution (0.5 mg/ml) was added, using a precision syringe pump, to 3 ml of CH solution (0.5 mg/ml) leading to a final theoretical ratio (w/w) of 1:1 and a spontaneous formation of NPs occured. Right after NP production, 300 mM EDC + 120 mM NHS were added to the NP solution. NPs were stirred for 2 h at 600 rpm. After that, NPs were collected using 300 kDa vivaspin

filters for 15 min at 4000 rpm. NPs were washed twice with UPW. Glucose was added in all centrifugation steps to avoid NP aggregation. Finally, NPs were filtered with 0.22 μm filters.

Characterization of NPs

Dynamic light scattering

NP size and PDI were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Samples were diluted in UPW and the size analysis lasted 180 s and performed at 25°C, in triplicate. The zeta potential measurements were performed by a dip cell in automatic mode.

NP tracking analysis

NP tracking analysis (NTA) experiments were performed using a NanoSight NS500 instrument (Malvern Panalytical, Salisbury, UK). This system includes a charge-coupled device camera that allows visualization and tracking of Brownian motion of laser-illuminated particles in suspension. Video images were analyzed using NTA analytical software version 2.3. The measurements were made at room temperature and each video sequence was captured over 60 s.

Scanning electron microscopy & scanning transmission electron microscopy

Measurement of particles' size and morphology observation were obtained using SEM (Scanning electron microscopy; Auriga, Carl Zeiss). A drop of NPs solution was placed in the stub for SEM analysis and left to dry overnight. They were then vacuum-coated with a gold–platinum mixture. Scanning transmission electron microscopy (STEM) analysis was also performed with the same equipment.

NPs physiological & storage stability

To mimic and assess the stability of NPs at physiological conditions, the NPs were diluted in 0.9% NaCl and kept at 37°C. The size and PDI of NPs were measured by DLS every day, until day 7.

To evaluate NPs storage stability after production, NPs were diluted in UPW and kept at 4°C. NPs size and PDI were measured by DLS every week for up to 2 months.

NPs cytotoxicity

Cell culture & cell viability

The cytotoxicity of the optimized NPs was evaluated over human umbilical vein endothelial (EA.hy926, cell line) and breast cancer (MDA-MB-231, cell line) cells. Increasing concentration of NPs were used until toxic effects were observed. The CellTiter 96 AQueous One Solution cell proliferation assay (Promega; WI, USA) was performed at 24, 48 and 72 h. At these time points, the culture medium was removed and the testing samples were rinsed with sterile phosphate-buffered saline (PBS). A mixture of culture medium (without Fetal Bovine Serum and phenol red) and MTS reagent (5:1 ratio) was added to each well and left to incubate for 3 h, at 37°C, in a humidified 5% CO_2 atmosphere. Thereafter, the absorbance of the MTS reaction medium from each sample was read in triplicate at 490 nm in a microplate reader (Synergy HT, Bio-TEK). All experiments were performed in triplicate. This experiment was repeated with Gem-loaded NPs (NPs_Gem), the amount of free Gem being the same as the amount loaded into the developed NPs.

Drug encapsulation & release studies

Drug entrapment efficiency

Gem concentrations, ranging from 0.2 to 3.2 mg/ml, were encapsulated into the NPs' optimized formulation. Gem was added to CH solution and NPs were produced as described in the section titled *Preparation of NPs*, maintaining the same production conditions (CH:Fu:Gem ratio 1:1:0.3).

Entrapment efficiency (EE) was calculated by measuring the initial concentration and nonencapsulated Gem, according to the following formula:

$$\%EE = \frac{(Initial\ concentration - non-entrapped\ drug)}{initial\ concentration} \times 100$$

The samples were analyzed by UV spectroscopy (Shimadzu) at the maximum absorbance of 268 nm. All the experiments were performed in triplicate.

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Gem-release studies

The release kinetics of Gem was measured by placing 1.5 ml of NPs_Gem into dialysis membranes, and this system into 50 ml of 0.9% NaCl at 37°C under 60 rpm stirring. At predetermined time periods (0, 5, 15, 30, 45 min; 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 48 and 72 h), an aliquot of 2 ml of the release solution was taken and immediately measured at the previously defined absorbance. The experiments were performed in triplicate.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism Software. Differences between the different conditions of the cellular assays were analyzed using nonparametric test (Kruskal–Wallis test), and $p < 0.05$ was considered significant. Data are presented as mean ± standard deviations.

Results

Optimization of NPs production parameters

To produce marine-origin polymeric NPs with constant sizes, several processing parameters were tested, namely polymers concentration and ratios, pH of the solution, ionic strength and influence of the cross-linker. Experimental results point out that a decrease in polymers concentration leads to the formation of smaller NPs (Figure 1A). With the same trend, the PDI tends to decrease for concentrations down to 0.5 mg/ml. However, for the lowest concentration, the PDI slightly increased indicating that at this polymers concentration the NPs are less homogenous. In this sense, and by the combination of these two important properties, the working concentration was set as 0.5 mg/ml for both CH and Fu. When Fu is in excess (CH:Fu ratio 1:2) in the final formulation, the NPs present a size around 500 nm and are highly polydispersive (PDI > 0.3; Figure 1B). For the 2:1 ratio, the NPs present a higher PDI than 1:1. Therefore, this last ratio was used for all further experiments. The influence of pH solution was evaluated for each polymer separately, maintaining the pH of the other polymer solution (Figure 1C & D). For the CH solution, pH 5 was chosen, whereas for Fu the testing condition that presented smaller size and PDI values was the one with pH 9 (pH of Fu solution). Ionic strength was also evaluated by dissolving the polymers in different solvents with increasing ionic strength: UPW, osmotized water and 0.9% NaCl. UPW was the condition where the NPs presented the lower PDI and size values (Figure 1E). Taking together, the optimal production parameters were set as: 0.5 mg/ml of both CH and Fu, 1:1 ratio, pH 5 (CH) and pH 9 (Fu) and UPW as the polymers solvent. After setting these parameters, the concentration of a cross-linking agent was evaluated. Different concentrations of EDC/NHS were tested, being the 300 mM of EDC and 120 mM of NHS, the condition that presented the best outcomes in terms of size and PDI (Figure 1F).

NPs characterization

NPs stability

After optimizing all the production parameters, two different conditions were chosen to continue the characterization of the NPs: one without cross-linking (no EDC/NHS) and the other cross-linked with EDC/NHS (300 mM EDC + 120 mM NHS). These two conditions were used to perform stability assays, both at physiological conditions (37°C, 0.9% NaCl; Figure 2A) and for storage conditions (4°C, UPW; Figure 2B). At physiological conditions, both size and PDI increase along the 7 days of the study, presenting values for PDI above 0.3 from day 4 on, concerning noncross-linked NPs. The size of cross-linked NPs was stable along the 7 days, whereas the PDI presents some slightly variations in some time points, these values being below 0.2. For the storage condition, both types of NPs present no significant variations in terms of size and PDI for up to 2 months.

NP properties

Based on stability results, cross-linked NPs were further characterized. NPs present a size of 140 nm, a PDI = 0.172 and a zeta potential of 29 mV by DLS (Table 1). NPs were also evaluated by NTA, presenting a size around 116 nm and a final concentration of 7.81×10^{10}/ml.

Nanoparticle morphology and size in dry state were also observed by SEM and STEM (Figure 3A & B). NPs presented a round shape and a size smaller than 100 nm in both microscopic analyses.

NP cytotoxicity

After optimizing all the parameters, the cytotoxicity of the developed NPs was evaluated over human endothelial (Figure 4A) and breast cancer (Figure 4B) cells. Different concentrations of NPs were tested and, for concentrations

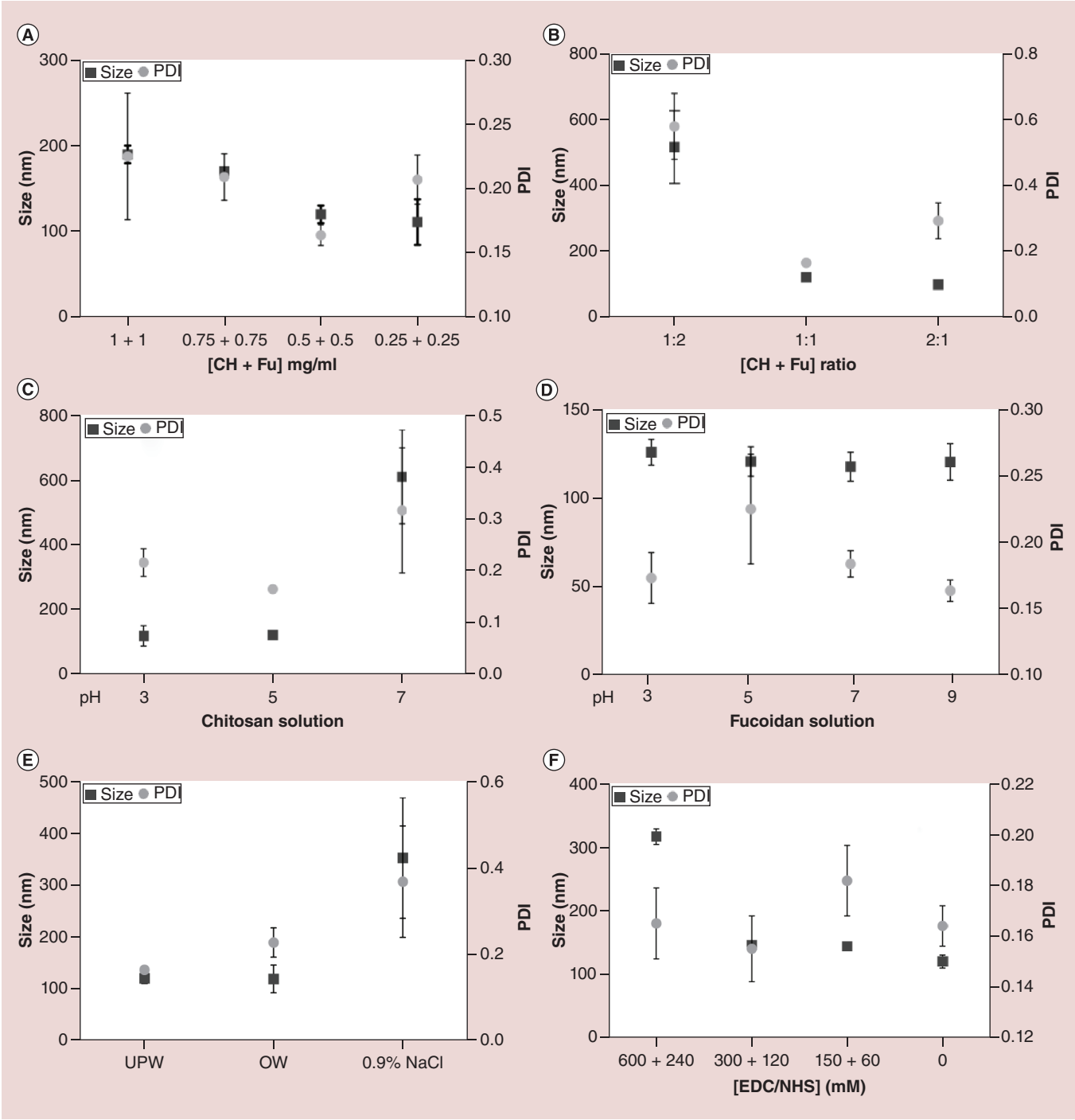

Figure 1. Nanoparticles' size and polydispersity index at different production parameters. (A & B) Evaluation of CH and Fu concentration and ratio (CH + Fu), respectively. **(C & D)** Evaluation of the influence of CH and Fu solutions' pH, respectively. **(E)** Influence of ionic strength and **(F)** Evaluation of different cross-linker (EDC/NHS) concentrations.
CH: Chitosan; EDC: 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; Fu: Fucoidan; PDI: Polydispersity index; NHS: *N*-hydroxysuccinimide.

above 8×10^9 NPs/ml they start to be toxic for both cell types, despite some significant differences in previous concentrations and time points. By SEM analysis, it was possible to observe the presence of the NPs at the surface of both cell types.

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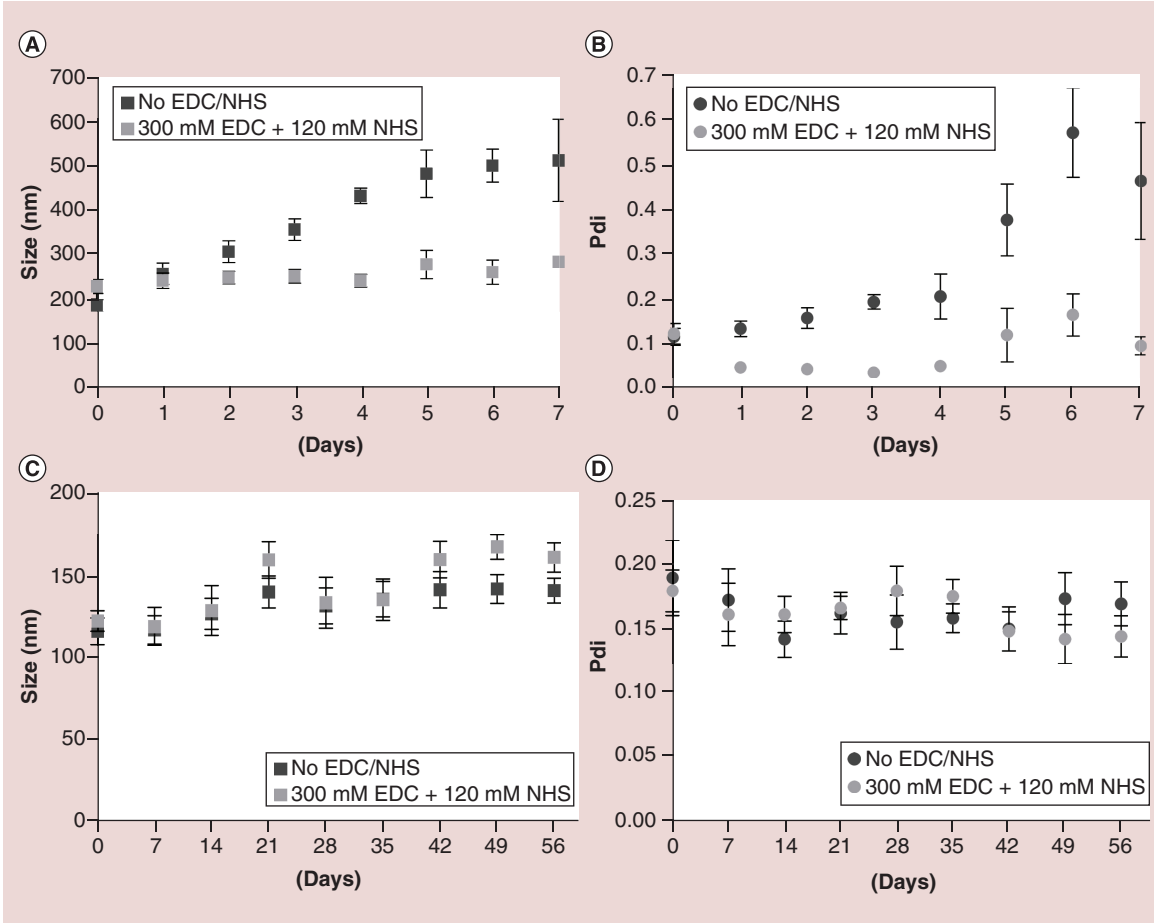

Figure 2. Nanoparticle stability. Size and polydispersity index of NPs at physiological (37°C and 0.9% NaCl; **A & B**) and storage conditions (4°C and UPW; **C & D**). **(A & C)** Regard size and **(B & D)** Regard PDI.
EDC: 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; NHS: *N*-hydroxysuccinimide; PDI: Polydispersity index; UPW: Ultrapure water.

Table 1. Nanoparticles characterization by dynamic light scattering and nanoparticle tracking analysis.

Technique	Property	Value
DLS	Size (nm)	141 ± 17
	PDI	0.172 ± 0.020
	Zeta potential (mV)	28.9 ± 3.5
NTA	Size (nm)	116 ± 17
	Concentration (particles/ml)	$7.8 \times 10^{10} \pm 1.2 \times 10^{10}$

DLS: Dynamic light scattering; NTA: Nanoparticle tracking analysis; PDI: Polydispersity index.

Drug entrapment efficiency & release studies

Gem entrapment efficiency

The drug entrapment efficiency was assessed by testing different Gem concentrations. Concentrations of 0.8 and 1.6 mg/ml present similar values and a maximum EE between 36 and 42%, respectively. At higher concentrations, the system saturates and the EE decreases to values around 30%.

In this sense, 0.8 mg/ml was the chosen concentration to evaluate the Gem-release profile, as well as the cytotoxic effects of NPs_Gem over human endothelial and breast cancer cells. NPs_Gem presented a size around 134 nm and a PDI of 0.186 (Figure 5B), similar to the values observed for the nonloaded NPs (Table 1).

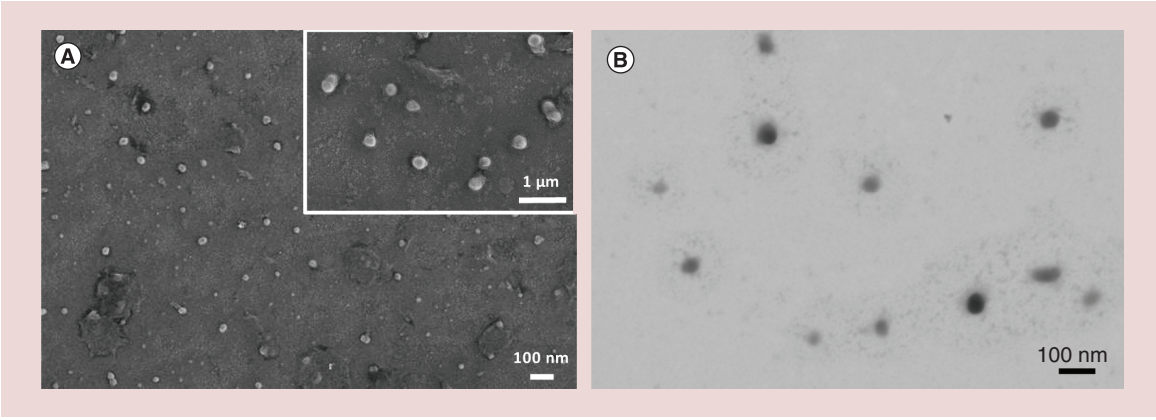

Figure 3. Nanoparticles morphology. Observation by scanning electron microscopy **(SEM; A)** and scanning transmission electron microscopy **(STEM; B)**.

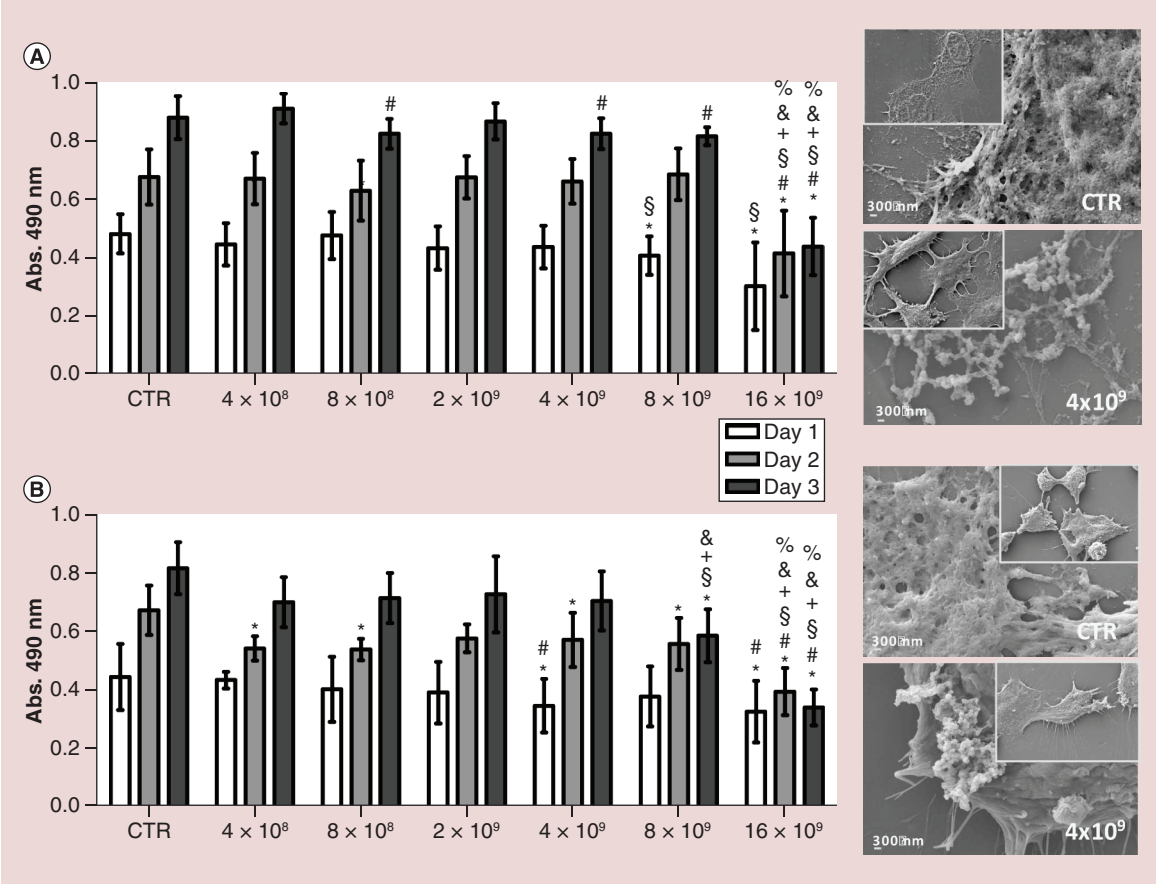

Figure 4. Cytotoxicity of developed NPs. Influence of NPs concentrations over human endothelial **(A)** and breast cancer **(B)** cells at different time points (1, 2 and 3 days). Data were considered statistically significant at $p < 0.05$. *Indicates significant differences compared with CTR; Compared with 4×10^8; §Compared with 8×10^8; +Compared with 2×10^9; &Compared with 4×10^9 and %Compared with 8×10^9. SEM observation of control and 4×10^9 NPs/ml culture conditions at day 3.
CTR: Control; NP: Nanoparticle; SEM: Scanning electron microscopy.

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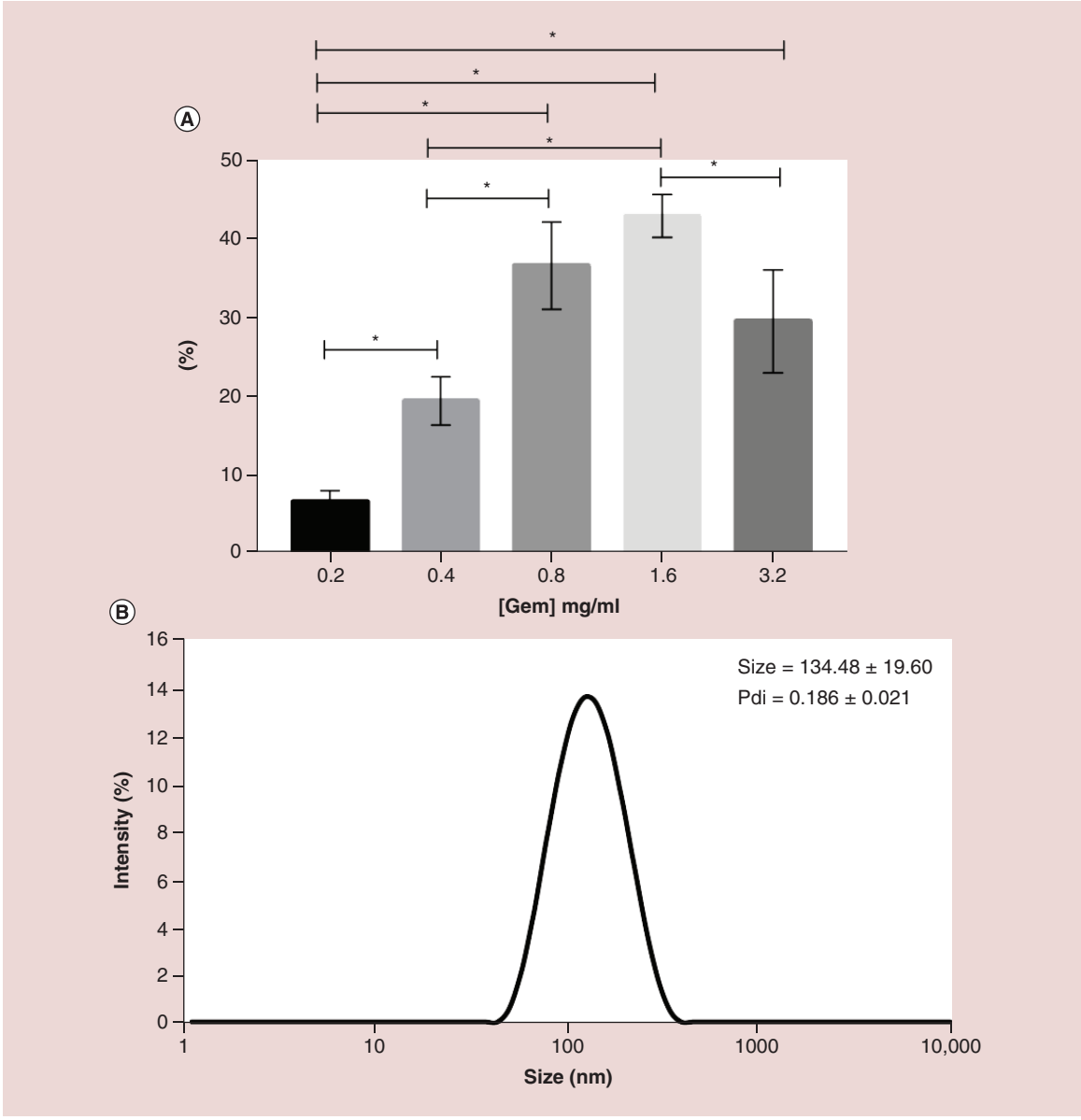

Figure 5. Gem-loaded NPs. Entrapment efficiency **(A)** and size distribution representation **(B)**.
Gem: Gemcitabine; NP: Nanoparticle.

Gem-release profile

The release of Gem from marine-origin polymeric NPs was evaluated at 37°C and 0.9% NaCl to mimic physiological conditions. Figure 6 shows that the developed system releases around 84% of the drug in a sustained way during 4 h, then maintaining a steady-state profile for up to 72 h.

Cytotoxic effects of drug-loaded NPs over cancer & noncancer cells

NPs at 4×10^9 NPs/ml do not present statistical significant differences when compared with the control condition for both endothelial and breast cancer cells, in all time points evaluated (Figure 7), confirming the results already reported in Figure 4. However, NPs_Gem are cytotoxic for breast cancer cells since the first day, but being also cytotoxic at days 2 and 3 for endothelial cells. Free Gem also presents cytotoxicity to endothelial cells with similar values to the ones observed with NPs_Gem. Concerning breast cancer cells, NPs_Gem present increased toxicity at

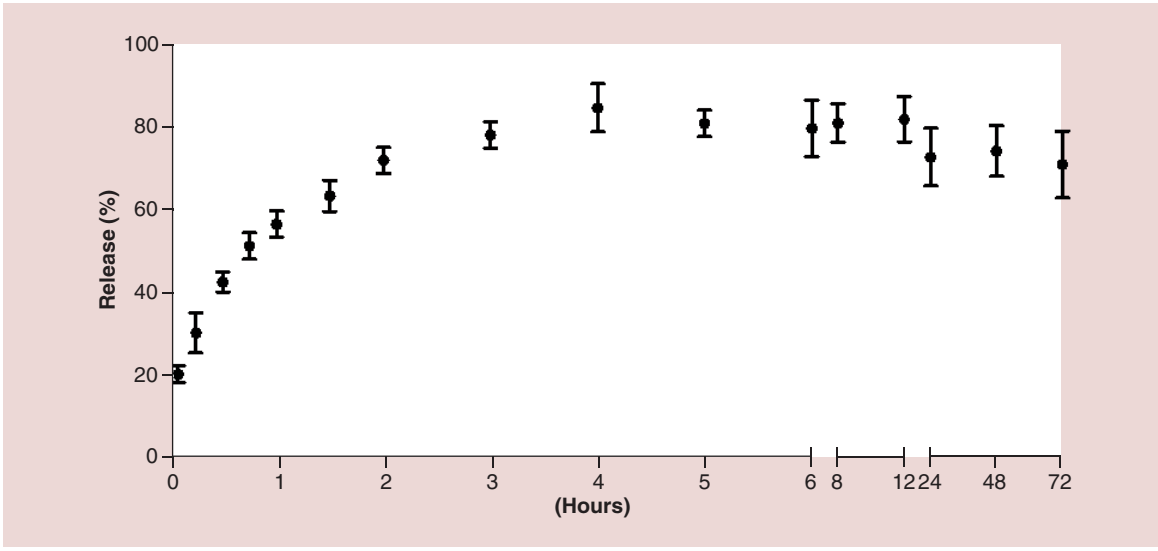

Figure 6. Gemcitabine-release profile from marine-origin polymeric nanoparticles (0.9% NaCl, 37°C, 60 rpm).

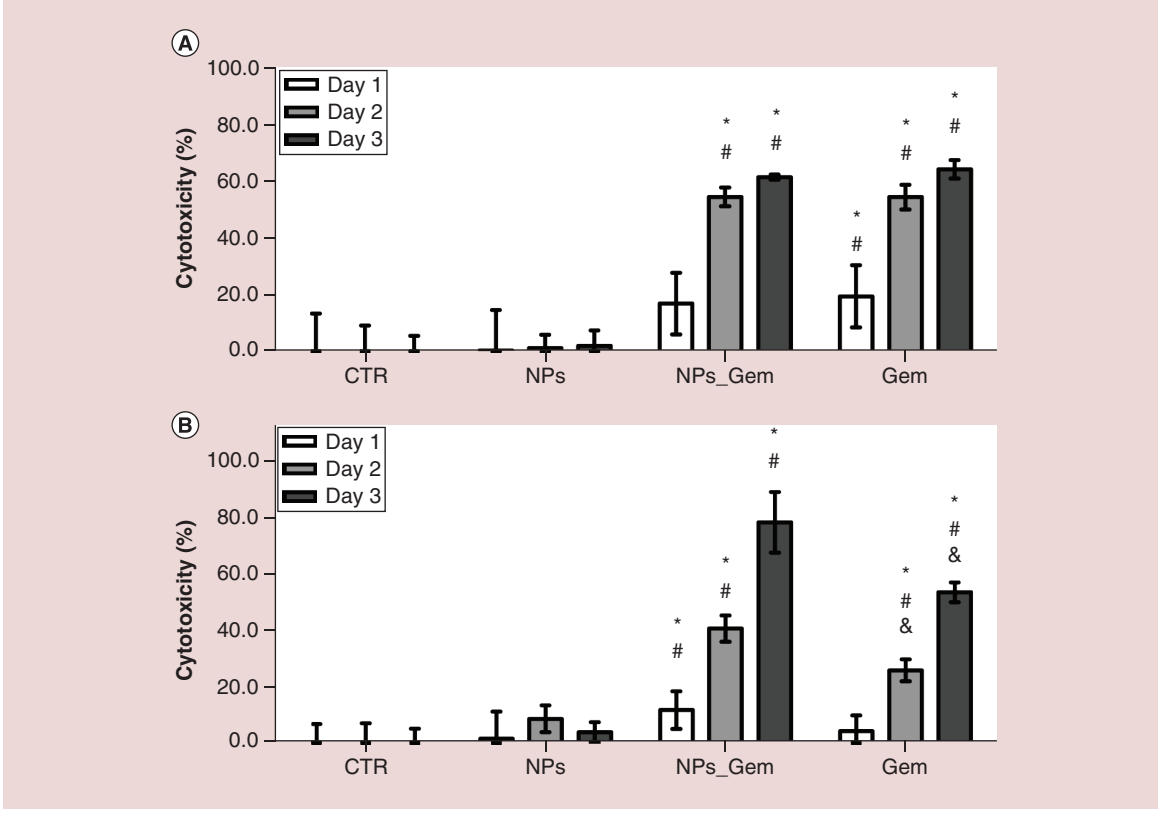

Figure 7. Cytotoxicity of developed Gem-loaded NPs. Influence over human endothelial **(A)** and breast cancer **(B)** cells at different time points (1, 2 and 3 days). Data were considered statistically significant at $p < 0.05$. *Indicates significant differences compared with CTR; #Compared with NPs; &Compared with NPs_Gem.
CTR: Control; NP: Nanoparticle; NP_Gem: Gemcitabine-loaded NP.

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day 2 (15%) and at day 3 (25%) when comparing with free Gem. Furthermore, NP_Gem presents higher toxicity over breast cancer than endothelial cells at day 3 (17%).

Discussion

Polyelectrolyte complexation has been widely reported due to its cost–effectiveness and easy production of NPs. It takes advantage of the interactions between two oppositely charged polymers and a spontaneous formation of the NPs occurs [25]. There are different parameters described to affect NPs size and PDI, namely the polymer concentration and ratio, polymer solution pH and ionic strength [26,27]. Our results showed that by decreasing polymers concentration, it was possible to obtain similar NPs.

However, higher PDI values are observed for both the high and low concentrations, showing obtainance of NPs with a nonhomogenous size distribution. Thus, it seems that at higher concentrations there would be high entropy and at lower concentrations reduced interactions between oppositely charged polymers. The influence of polymers ratio was also evaluated over NPs' size and PDI. When higher amounts of Fu are present (CH:Fu ratio 1:2), the NPs present an increased size and PDI, showing that probably there is the formation of polymeric aggregates instead of NPs. Indeed, the amino groups of CH have a pKa of approximately 6.5, which leads to a protonation in acidic conditions. In solutions with pH >6.5, most amino groups would be deprotonated and CH tends to precipitate [28]. Being deprotonated, the amine groups will be less available to form a complex with Fu, resulting in a less controlled process, with the participation of other types of interactions gaining relevance, which will result in higher variability and consecutively high values of size and PDI, outside the range to be considered as NPs. CH at acidic pHs present protonated amines being as these chemical groups are more prone to form polyelectrolyte complexes with a negatively charged polymer (Fu), with ionic attractive interactions ruling the process, leading to the formation of the desired NPs. Despite similar size values for the three other pHs, pH 5 was the one that presented more homogeneous NPs' size. Fu solution pH seems not to influence the development of NPs size, although a low PDI value is observed when the pH is not adjusted (pH 9; Figure 1C & D). Ionic strength was analyzed by dissolving the polymers in different solvents, UPW being the one that presents the better outcomes in terms of size and PDI. As expected, 0.9% NaCl tends to form bigger and nonhomogenous NPs due to the presence of higher amounts of ions, which would have a charge shielding effect over the protonated groups in polyelectrolytes and thus decreasing the relevance of ionic interactions in the assembly process. Fu/CH NPs may become unstable at physiological conditions due to the deprotonation of CH amine groups. Trying to enhance NPs stability, a cross-linking agent was tested: EDC/NHS. EDC is often used to cross-link amines (NH_2) to carboxylate groups, whose activity is enhanced by NHS, through activation of the carboxylate group [29]. In the present case, the rationale is to cross-link the amine groups of CH with the carboxylic group of the uronic acids that are present in Fu structure [8]. Different concentrations of EDC/NHS were evaluated and the 300 mM EDC + 120 mM NHS presented the smaller PDI. Despite 150 mM EDC + 60 mM NHS present similar results, these concentrations presented higher PDI, reason why a higher concentration was chosen to continue the study. Higher concentrations (600 mM EDC + 240 mM NHS) seem to start precipitating and affecting NPs' size, increasing it to around 300 nm. NPs final formulation was set as: 0.5 mg/ml polymers concentration, 1:1 ratio, pH 5 (CH) and pH 9 (Fu) and UPW as the polymers solvent.

After this optimization process and setting all the parameters, two conditions were chosen to conduct the stability assays: one without cross-linking and the other cross-linked. NPs stability at physiological and storage conditions was assessed. Physiological conditions were mimetized by diluting the produced NPs in 0.9% NaCl, which have an osmolarity similar to circulating plasma 285–295 mOsm/l [30]. Both NPs increase their size to around 200 nm, when in contact with this isotonic solution, due to an increase in the ionic strength with associated charge shielding but also higher water uptake (Donnan effect) [31]. Cross-linked NPs present a quite stable profile along the 7 days, whereas from day 3 on, the noncross-linked NPs start to increase their size, indicating that they probably start to disintegrate at physiological osmolarity. We proceed the characterization of cross-linked NPs that present a size around 140/115 nm (DLS/NTA), a PDI of 0.172, which indicates that these NPs are quite homogeneous and a zeta potential of +29 mV indicating that CH is at the surface of NPs. By NTA, the NPs presented a smaller size due to the fact that NTA is a more precise technique than DLS [32]. By SEM and STEM observation, we were able to confirm the round morphology of the NPs, with a size smaller than 100 nm. The difference between DLS/NTA measurements and SEM/STEM is due to the fact that in the first characterization, NPs are measured in wet state, whereas for SEM/STEM, NPs are analyzed in a dry state.

After all the optimization steps, the cytotoxicity of the developed marine-origin polymeric NPs was evaluated over human endothelial and breast cancer cells at different concentrations. NPs start to become more toxic to both cell types at the highest concentrations (8×10^9 and 16×10^9 NPs/ml) tested. From SEM observation, we were able to observe NPs at the surface of both cell types. From these results, 4×10^9 NPs/ml concentration (nontoxic concentration) was selected to conduct the further experiments.

Breast cancer is sensitive to several cytotoxic drugs that may be used as single or combined therapies. In the past years, new cytotoxic agents have been developed and approved to be used in cancer treatments. Gem has shown antitumoral activity and good toxicity profiles to be used as single-agent therapy for advanced breast cancer. One of the drawbacks of using Gem by intravenous or oral administration is related with its rapid inactivation by enzymatic delamination, low bioavailability and short half-life. These problems require an increase in dosage that may lead to unwanted side effects [19–22]. The use of NPs may overcome some of these limitations since they will be able to protect the drug from enzymatic degradation, diminishing the above-mentioned efficiency problems [33].

The loading of Gem on the marine-origin polymeric NPs was evaluated by testing different Gem concentrations. In Figure 5A, it is possible to observe that, by increasing the concentration of the drug, there is an increase in the EE until it reaches a maximum concentration of 1.6 mg/ml (EE = 42%). At 3.2 mg/ml, the system saturates and the EE decreases to around 30%. NPs_Gem were characterized in terms of size and PDI, showing no significant differences. The release profile was assessed at 37°C and 0.9% NaCl to mimic physiological conditions. From the stability studies, it was observed that the size of the NPs increased, due to the ionic strength of the solution that weakens the electrostatic interactions, leading to the release of Gem from the NPs [10]. Around 84% of Gem was released in the first 4 h in a sustained way, until reaching a steady state.

To assess the antitumoral activity of the developed DDS, it is important to have in mind the tumor microenvironment that comprises not only cancer cells but also noncancer cells (e.g., endothelial, fibroblasts). Indeed, as NPs_Gem are intended to be injected intravenously, the first barrier they will face is the blood vessels wall and thus endothelial cells. Different Fu-based NPs have been developed as anticancer strategies. As example, Fu–cistaplin NPs have been developed and evaluated for colon cancer [34], whereas Fu encapsulated into liposomes showed anticancer activity against osteosarcoma [35]. In another attempt, doxorubicin was incorporated into a protamine/Fu system and these loaded NPs improved inhibitory effects against a breast cancer cell line [36]. However, as far as we know there are no studies in the literature that report the use of Fu to develop NPs for the delivery of Gem. On the other hand, CH-based NPs have been studied for the delivery of antitumoral agents, namely for the delivery of Gem. For example, the loading of Gem into CH magnetic NPs increased Gem efficacy when compared with free Gem [37]. In other study, CH-based NPs were prepared by layer-by-layer technique for the incorporation of Gem and platinum aiming at lung cancer treatments [38]. Other studies reported the use of CH-based NPs for the delivery of other antitumor drugs: PEG-layered CH NPs enhanced the efficacy of ormeloxifene in breast cancer [39] and platinum–gold NPs with CH and doxorubicin presented cell-specific cytotoxicity [40].

In this sense, the combination of these two marine-origin polymers to develop NPs for the delivery of Gem is a novel process with potential positive outcome. We evaluated the Gem loaded-NPs toxicity over human endothelial and breast cancer cell lines. Regarding endothelial cells, both NPs_Gem and free Gem present similar cytotoxicity values. However, concerning breast cancer cells, NPs_Gem increased toxic effects of around 15 and 25% at days 2 and 3, respectively, when compared with free Gem. At day 3, when comparing breast cancer and endothelial cells, it was observed an increased cytotoxicity of around 17% over breast cancer cells. The increased cytotoxicity of the NPs_Gem when compared with free Gem may be attributed to the cellular trafficking pathway, suggesting a more effective uptake of the drug by breast cancer cells. The encapsulation of cisplatin into CH lipid NPs was reported, demonstrating increased apoptosis in cancer cells when compared with free cisplatin [41]. A recent paper reported that CH NPs loaded with curcumin reduced the drug uptake in normal cells, while the same was internalized into cancer cells [42]. Another study reported the internalization of CH-coated NPs loaded with doxorubicin into breast cancer cells cytoplasm and nucleus. The authors stated that the uptake of these NPs may be attributed to the positive charge of their surface. That positively charged CH NPs can have charge-based interaction with the cell membrane and may enter cells either by direct penetration or by endocytosis [43]. Thus, although not presenting a protective effect against the toxicity over endothelial cells, the Fu/CH NPs exhibit a synergistic effect with Gem resulting in a higher toxicity over breast cancer cells. Moreover, these results confirm that, by encapsulating Gem into an NP, the drug is protected from enzymatic degradation, increasing its half-life and biological response.

Conclusion

Marine-origin polymeric NPs were successfully produced by polyelectrolyte complexation, presenting increased stability at physiological conditions when cross-linked with EDC/NHS. Gem was encapsulated in the developed NP system. This DDS increased toxicity over human breast cancer cells, when compared with free Gem, without increasing their toxic effect over endothelial cells. Taken together, the herein-developed DDS is an interesting strategy to be further explored on breast cancer therapies.

Summary points

Nanoparticle production & characterization

- Nanoparticles (NPs) were successfully produced by polyelectrolyte complexation, presenting a size around 115–140 nm.
- NPs present a polydispersity index less than 0.2, demonstrating NPs' size homogeneity.
- Cross-linked NPs presented increased stability at physiological conditions than noncross-linked NPs.

Gemcitabine entrapment efficiency & release profile

- The maximum entrapment efficiency of the system is around 42% for 1.6 mg/ml (above this concentration, the system saturates).
- The system releases around 84% of the drug in the first 4 h.

Drug-loaded NPs cytotoxicity

- Drug-loaded NPs presented increased toxicity (around 25% at day 3) over human breast cancer cells when compared with free gemcitabine.
- Drug-loaded NPs did not present increased toxicity over endothelial cells when compared with free gemcitabine.
- Drug-loaded NPs present higher toxicity over breast cancer cells (around 17%) when compared with endothelial cells.

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