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Enhanced performance of gelatin 5-fluorouracil-containing nanoparticles against squamous cell carcinoma in simulated chronic wounds conditions

Albert Ferriol [a], Maria del Carmen Morán [a,b,*]

[a] Departament de Bioquímica i Fisiologia-Secció de Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Avda. Joan XXIII 27-31, 08028 Barcelona, Spain
[b] Institut de Nanociència i Nanotecnologia-IN2UB, Universitat de Barcelona, Avda. Joan XXIII 27-31, 08028, Barcelona, Spain

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ABSTRACT

Chronic wounds are considered a silent epidemic affecting a significant fraction of the World population. Their treatment supposes a large fraction of the public spending on the health of developed countries. In chronic wounds secondary to burns, trauma, UV light radiation, and diabetes, among others, the development of squamous cell carcinoma (SCC) has been reported. If detected early, 95% of SCC are most comfortable to be treated and cured; however, 5% of advanced SCC is more dangerous and challenging to treat.

It has been reported that the pH value within the wound-milieu influences indirectly and directly all biochemical reactions taking place in this process of healing. Differences in pH values between normal skin and chronic cutaneous wounds could be considered in designing and developing stimuli-responsive nanomaterials. In this work, the anticancer drug 5-fluorouracil (5-FU) inclusion on gelatin-based NPs for SCC treatment has been projected. The present work goal is to prepare and characterize physicochemical and biological properties of new therapeutic-containing NPs for the sustainable delivery of 5-FU under simulated chronic wound conditions. *In vitro* experiments have been performed to assess the biocompatible character of these gelatin-based NPs in terms of their hemolytic and cytotoxicity properties. Due to hyperglycemia impact on both the chronicity of the wounds and chemotherapy efficacy, cellular responses have been determined under euglycemic and hyperglycemic conditions. *In vitro* cytotoxicity studies have reported good selective toxicity against the A431 cell line, demonstrating that gelatin-based NPs are promising dual-responsive delivery systems to SCC targeting under simulated chronic wound conditions.

1. Introduction

Wound healing is an essential physiological process whose purpose is to restore the skin protective barrier and full functionality. Wound healing is a complex process in which many cell types and mediators interact in an exact temporal sequence. Events in wound repair can be divided into four stages: hemostasis, inflammatory phase, proliferative phase, and the posterior stage, named remodeling phase [1].

Chronic wounds do not show normal healing processes and timelines. They seem to be detained in one or more phases of wound healing, and, often they remain too long in the inflammatory stage. When healing processes exceeded three months, they evolve to chronic wounds [2]. Chronic wounds are considered a silent epidemic affecting a significant fraction of the World population. It is estimated that around 2% of the World population would develop a chronic wound during their lifetime [3]. Their treatment supposes a large fraction of public spending on the health of developed countries [4]. Chronicity mainly depends on systemic factors, such as deficient malnutrition, infection, diabetes mellitus, or immune depression. In addition, local factors such as venous or arterial insufficiency and local-pressure effects are the most common pathophysiological causes of impaired wound healing [5]. Chronic wounds are encountered in every specialty, and the occurrence of non-healing wounds will continue to rise as the population ages [6].

During cutaneous wound healing, the pH value indirectly influences all biochemical reactions taking place in the wound-milieu. Under normal circumstances, the milieu varies between a pH of 4–6, which is a critical aspect of the skin's barrier function. This acidic milieu also seems to be essential for the resistance against external chemicals [7].

* Corresponding author at: Departament de Bioquímica i Fisiologia-Secció de Fisiologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona, Avda. Joan XXIII 27-31, 08028 Barcelona, Spain.
E-mail address: mcmoranb@ub.edu (M.C. Morán).

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Acute and chronic wounds reported pH environments in the range of 7.15–8.9, being characteristics for healing and non-healing wounds. Most alkaline pH values have been associated with healing rates lower than wounds in which the pH is closer to neutral [8].

Chronic wounds may degenerate into malignancy. In chronic wounds secondary to burns, trauma, UV light radiation, and other forms of radiation, chronic immunosuppression, and diabetes, among others, the development of squamous cell carcinoma (SCC) has been reported [9]. Together with basal cell carcinoma (BCC), SCC is by far the most common skin cancer, and they are more frequent than any other form of cancer. However, these types of cancer rarely metastasize [10]. About 95% of the SCC of the skin is timely detected and then they are easy to treat and cure. However, the other 5% of SCC cases can spread extensively or aggressively; or have resisted multiple treatments and repeatedly recurred; this case is considered advanced. It is estimated that advanced SCC could promote 15,000 deaths each year in the U.S. Twice as many people die from SCC than from melanoma [11–13]. SCC treatment includes several types of surgery as well as radiation, photodynamic therapy, and topical medications. The latter option comprises the application of 5-fluorouracil or ingenol mebutatea, among others, to treat cancerous and precancerous cells. Therapy with imiquimod activates the immune system to attack cancerous cells [13,14].

In recent years, there has been an increasing effort in developing stimuli-responsive nanomaterials with the hope that they will be established into effective drug delivery vehicles. The success of these drug delivery systems is their capability to respond to endogenous or exogenous stimuli to induce local responses and targeting the drug delivery. Often, their efficiency is correlated with their capability to nanomaterial-facilitated accumulation and/or cellular internalization. Among the different stimuli, the response to small molecules like proteins, nucleic acids, or peptides and local environmental factors, such as pH, temperature, or redox state, has been considered [15].

Differences in pH values between normal skin and chronic cutaneous wounds could be considered in designing and developing stimuli-responsive nanomaterials. Recent studies in our lab have highlighted the properties of gelatin. This protein possesses interesting gelification properties and the strong dependence of ionization with pH [16], which allow us to design nanoparticles (NPs) for the effective intracellular delivery of active biomacromolecules [17–19]. Concerning the applicability of gelatin-based NPs on the chronic wounds environments, it would start by considering the isoelectric point (pI) of gelatin type B (between pH 4.8 and 5.2) and gelatin type A (ranged between 7 and 9 linked to the Bloom value) at pH of the normal skin (pH 5.5) [20].

These differences could be used to form NPs at the skin pH by the interaction of oppositely charged compounds, keeping their protective structure. In the chronic wound surroundings, where the wound-milieu pH would increase until pH 7.15–9.0, gelatin A becomes positively charged. Due to electrostatic repulsion within gelatin B, the protection ceases, and the gelatin-based NP is disrupted, releasing the therapeutical compound. Among the putative drugs to be incorporated, the hydrophilic character of 5-fluorouracil (5-FU) makes this compound an excellent candidate to be encapsulated into gelatin-based nanocarriers [19]. The principal mode of action of 5-FU is as a thymidylate synthase (TS) inhibitor [21]. Unfortunately, the treatment with 5-FU frequently reports primary and secondary resistance [22,23], enforcing the development of a multidisciplinary approach to the efficient delivery of therapeutics to tumor cells.

Besides, cancer treatment can benefit ligands expressed on the tumor cell surface to induce selectivity. It is reported that several cell surface-proteins, over-expressed by tumor cells, contribute to cell invasion. Among them, the role of $\alpha_v\beta_3$ integrin in tumor cell proliferation is well documented [24]. The $\alpha_v\beta_3$ integrin is highly expressed in cells of different tumors, such as glioma (U87MG), epidermoid (A431), and prostate cancers (PC3), among others [25]. Among the suitable structures for tumor targeting, the integrins are recognized to mediate cell adhesion to extracellular matrix and other cells. Interestingly, naturally occurring gelatin contains arginine, glycine, and aspartate – RGD sequence, which is recognized to bind to the integrin-type cell receptors [26].

Thus, gelatin-based NPs may provide an opportunity to increase the therapeutic effect of 5-FU on SCC under chronic wound conditions using a dual strategy. Firstly, to targeting the therapeutic drug to the SCC cells minimizing the harmful effects on the normal skin cells by the action of RGD-motif, and secondly, by releasing the cargo using the charge reversal approach selectively on the surroundings of the chronic wound.

This work aims to prepare and characterize physicochemical and biological properties of 5-FU-containing NPs for sustainable delivery under chronic wound conditions. The physicochemical characterization (particle size, polydispersity index, and degree of 5-FU entrapment) has been evaluated as a function of both gelatin B and 5-FU concentrations and gelatin A gel strength (either low or high blooms values) at a representative pH of the normal skin. *In vitro* experiments have been performed to determine the biocompatible characterization of these gelatin-based NPs in terms of their hemolytic and cytotoxicity properties at the mentioned representative pHs. Due to hyperglycemia impact on both the chronicity of the wounds and chemotherapy efficacy, the potential selectivity towards the squamous cell carcinoma cell line (A431) and the non-tumoral cell lines of mouse dermal fibroblast (3T3) and human epidermal keratinocyte (HaCaT) have been determined at different pHs and under euglycemic and hyperglycemic conditions.

2. Materials and methods

2.1. Materials

Gelatin from bovine skin, gelatin type B, with gel strength 75 bloom value (GB75), gelatin from porcine skin, gelatin type A with gel strength values ranging between 100 (GA100), 175 (GA175),and 300 bloom values (GA300), and 5- Fluorouracil (5-FU) were purchased from Sigma and used as received.

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), L-glutamine solution (200 mM), penicillin-streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin), phosphate-buffered saline (PBS), and trypsin-EDTA solution (170,000 U/L trypsin and 0.2 g/L EDTA), were purchased from Lonza (Verviers, Belgium). 2,5-Diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), Neutral Red Uptake (NRU) and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The 75 cm^2 flasks and 96-well cell culture plates were obtained from TPP (Trasadingen, Switzerland). All other reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation of gelatins and 5-FU solutions

Solutions of gelatin B were prepared by dissolution in PBS pH 5.5 buffer. The concentration of the gelatin ranged from 5 to 40 mg/mL. 5-FU was dissolved in PBS pH 5.5 buffer. A stock solution with a concentration of 10 mg/mL was prepared and then diluted to obtain solutions with concentrations of 2.5 and 5 mg/mL. Solutions of gelatin A were also prepared by dissolution in PBS pH 5.5 buffer. The concentration of the gelatin was fixed at 0.1 mg/mL.

2.2.2. Preparation of gelatin B (5-FU) binary mixtures

5-FU was incorporated into the gelatin network when solutions of gelatin B and 5-FU, previously prepared in PBS pH 5.5, were mixed at a ratio equal to 50% (*v*/v). Consequently, the final concentration of GB and 5-FU were fixed to 2.5–20 mg/mL and 1.25–5 mg/mL, respectively. Then, the obtained mixtures were allowed in the refrigerator overnight to promote gel formation.

2.2.3. Preparation of gelatin B (5-FU)-gelatin A NPs

Mixed gelatin (5-FU) systems (200 μL) were added dropwise into the

gelatin A dissolution (2 mL) under gentle stirring. Under optimal conditions, droplets from the mixed gelatin (5-FU) systems broke under magnetically stirring and instantaneously evolved into discrete particles in contact with the gelatin A solution. The interaction between oppositely charged compounds (gelatin B and gelatin A) forms particles containing 5-FU in the nanometric range. For comparative purposes, particles in the absence of 5-FU were also prepared.

2.2.4. Physicochemical characterization of the gelatin B (5-FU)-gelatin A NPs

2.2.4.1. Size and polydispersity. The size of the obtained NPs was determined by dynamic light scattering (DLS) at 25 °C with Zetasizer Nano ZS90. The size distribution was assessed from the polydispersity index (pdI) that ranges from 0.0 for an entirely monodisperse sample to 1.0 for a polydisperse sample. The interpretation of data was performed considering the size distribution by the intensity of scattered light. Three different measures were performed for each condition. Every single measure consists of 10 sub- measures of 10 s.

2.2.4.2. Loading efficiency. The degree of 5-FU entrapment is expressed through the loading efficiency (LE) values. LE was determined with the equation:

$$\mathrm{LE}\ (\%) = \frac{[\mathrm{Encapsulated\ 5-FU}]}{[\mathrm{Total\ 5-FU}]} \times 100 \quad (1)$$

where

$$[\mathrm{Encapsulated\ 5-FU}] = [\mathrm{Total\ 5-FU}] - [\mathrm{free\ 5-FU}] \quad (2)$$

by spectrophotometry at 267 nm using a nanophotometer (NanoPhotometerTM, Implen). The putative interference due to the presence of NPs was avoided when NPs dispersions were previously centrifuged at 10.000 rpm for 30 min. The quantity of free 5-FU in the solution was determined by interpolation from a calibration curve.

The effect of pH increase from 5.5 to 7.4 and 9.0 (mimicking chronic wound increase) on LE values was also determined. After successive alkalization to pH 5.0 by addition of 0.5 M NaOH solution and incubation for 1 h, the NPs were centrifuged at 10,000 rpm for 30 min. Aliquots of supernatant were subsequently removed for quantification of the free 5-FU. The effect of pH increase on the release efficiency (RE) was evaluated following these equations:

$$\mathrm{RE(pH\ 7.4)(\%)} = \frac{\mathrm{LE\ (5.5)} - \mathrm{LE(7.4)}}{\mathrm{LE\ (5.5)}} \times 100 \quad (3)$$

$$\mathrm{RE(pH\ 9.0)(\%)} = \frac{\mathrm{LE\ (5.5)} - \mathrm{LE(9.0)}}{\mathrm{LE\ (5.5)}} \times 100 \quad (4)$$

2.2.5. In vitro assay with human erythrocytes

2.2.5.1. Obtention and extraction of the erythrocytes. Samples of human blood were obtained from the Banc de Sang i Teixits de Barcelona (Blood and Tissue Bank) from the Catalan Department of Health. Blood was deposited in tubes with anticoagulant EDTA-K3. The blood samples were centrifuged at 3000 rpm at 4 °C for 10 min (Megafuge 2.0 R. Heraeus Instruments). The resulting supernatant containing plasma was extracted with a Pasteur pipette. Then, the blood samples were washed with PBS pH 7.4. This procedure was repeated three times to remove residual leukocytes, and platelets, and concentrate the erythrocytes. Following the last wash, the erythrocytes suspension was diluted (1:1) in PBS pH 7.4, to obtain suitable erythrocytes suspension (cell density of 8×10^9 cell/mL).

2.2.5.2. Hemolysis assay. The hemolysis assay determines the capability of the NPs systems to induce lysis activity of the erythrocytes membrane. Different NPs volumes (25, 50, and 100 μL) were placed in polystyrene tubes, and an aliquot of 25 μL of erythrocytes suspensions was added to each tube. The final volume was 1 mL. The tubes were incubated at room temperature by shaking conditions. After that, the tubes were centrifuged at 10.000 rpm for 5 min. The degree of hemolysis was determined by comparing absorbance at 540 nm (Shimadzu UV-160A) of the supernatants with those of the control samples hemolysed with distilled water. Negative control was obtained by incubating an aliquot of 25 μL of the erythrocyte suspension with PBS pH 7.4, respectively.

2.2.5.3. Agglutination assay. The agglutination assay determines erythrocytes putative aggregation when they were incubated in presence of NPs systems. After the incubation in the hemolysis assay, 100 μL of the erythrocytes suspension were fixed with 100 μL of formaldehyde 4% and kept at 5 °C. After a while, the preparation was diluted by the addition of 200 μL of PBS pH 7.4. A small volume of this preparation (10 μL) was put on a slide and fixed by a coverslip. The sample was studied with an optical microscope with phase contrast (Olympus BX41) using the 40× objective. The images were digitalized using a camera (Olympus digital camera XC50) and analyzed by an image processor (Cell B analysis).

2.2.6. Cell cultures

The murine Swiss albino fibroblast (3T3), the immortal human keratinocyte (HaCaT), and the squamous cell carcinoma (A431) were obtained from Celltec UB. Cells were grown in DMEM medium (4.5 g/L glucose) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C, 5% CO_2. Cells were routinely cultured in 75 cm^2 culture flasks and were trypsinized using trypsin-EDTA when the cells reached approximately 80% confluence.

2.2.7. Cell viability assays

3T3 and HaCaT cells (1×10^5 cells/mL) and A431 cells (5×10^4 cells/mL) were grown at the defined densities into the central 60 wells of a 96-well plate. Cells were incubated for 24 h under 5% CO_2 at 37 °C. The spent medium was then removed, and cells were incubated for 24 h with the corresponding NPs systems, previously diluted 50% (v/v) in DMEM medium supplemented with 5% FBS (100 μL). The influence of pH representative to chronic wound conditions was simulated by incubating the NPs dispersions previously treated with suitable volumes of NaOH 0.5 M to achieve pH 7.4 and 9.0.

2.2.7.1. NRU assay. The accumulation of the neutral red dye in the lysosomes of viable, undamaged cells constitutes the basis of the neutral red uptake (NRU) assay. After the cells were incubated for 24 h with the corresponding systems, the medium was removed, and the NPs were incubated for 3 h with the NR dye (Sigma-Aldrich) solution (50 μg/mL) dissolved in the medium without FBS and phenol red (Lonza). Cells were then washed with sterile PBS, following by the addition of 100 μL of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water to extract the dye. Plates were then placed in a microtitre-plate shaker for 5 min at room temperature to help the total dissolution. Then, the absorbance of the resulting solutions was measured at 550 nm using a Bio-Rad 550 microplate reader. Each treatment effect was calculated as the percentage of tetrazolium salt reduction by viable cells against the control cells (cells without any treatment).

2.2.7.2. MTT assay. In this assay, living cells reduce the yellow tetrazolium salt, 2,5-Diphenyl-3, -(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT) to insoluble purple formazan crystals. After the cells were incubated for 24 h with the corresponding systems, the medium was removed, and 100 μL of MTT in PBS (5 mg/mL) diluted 1:10 in culture medium without phenol red and absence of FBS was added to the cells. The plates were incubated for 3 h, after which the medium was

removed. After that, 100 μL of DMSO (Sigma–Aldrich) were added to each well to dissolve the purple formazan crystals. Agitation, determination of the absorbance of the extracted solution, and each treatment effect were measured at the same conditions as in 2.2.7.1.

2.2.7.3. Selectivity towards cancer cells. The corresponding half-maximal inhibitory concentration (IC_{50}) values for the different formulations at the different pHs were determined from the fitting of concentration-dependent viabilities curves. The corresponding selectivity indexes towards cancer cells were calculated as the following ratio:

$$SI = \frac{IC50\ (non - tumoral\ cell\ line)}{IC50\ (tumoral\ cell\ line)} \quad (5)$$

where either 3T3 fibroblasts and HaCaT keratinocytes were closely used to represent skin model cell lines under normal conditions.

2.2.8. Statistical analyses

Experiments were performed three times on independent occasions unless otherwise stated. Results are expressed as means ± standard deviation. One-way analysis of variance (ANOVA) was used to determine statistical differences between data sets, following the Scheffé post hoc tests for multiple comparisons. IBM SPSS Statistics software was used to execute statistical analyses. Differences were considered statistically significant at $p < 0.001$. Significant differences were illustrated in the figures with an asterisk or other superscript symbols.

3. Results and discussion

3.1. Preparation of gelatin B (5-FU)-gelatin A NPs

The preparation of the gelatin B (5-FU)-gelatin A NPs involves a two-step protocol. The first step consists of the incorporation of the therapeutical drug into the network of the gelatin. One of the most prominent features of gelatin is the capacity to form gels. The formation of these gels is strongly influenced by the concentration of gelatin B and the incubation temperature. The incorporation of 5-FU into the gelatin network was facilitated when dissolutions of gelatin type B and 5-FU were mixed at a ratio equal to 50% (**v**/v). 5-FU concentration was established to 10 mg/mL due to its solubility limitations in an aqueous solution. The incubation of these binary mixtures at low temperature (approximately 10 °C) promoted gel formation for final gelatin type B concentrations ranged between 5 and 20 mg/mL. For binary mixtures prepared at lower gelatin concentration (2.5 mg/mL), gel formation could not be appreciated.

Once the therapeutical drug is incorporated into the gelatin network, the formation of the gelatin-based NPs could be achieved by a dropwise addition method, where drops of the highly viscous binary mixture are added to an oppositely charged gelatin A solution with vigorous stirring. This approach promotes the formation of the NPs by associated phase separation between oppositely charged gelatins. The continuous diffusion of gelatin A throughout the particles promotes the formation of the shell portion stabilizing the NPs with time. Results of NPs formation at two different gelatin A concentrations (0.1 and 1.0 mg/mL) demonstrated few differences in their physicochemical properties. However, NPs prepared at the highest gelatin A concentration have promoted cytotoxicity on fibroblasts and keratinocytes under cell culture conditions (results not shown).

In this series of experiments, three variables were considered: i) gelatin concentration of gelatin B (ranging from 2.5 to 20 mg/mL), (ii) 5-FU concentration (ranging from 1.25 to 5 mg/mL) and (iii) gel strength of gelatin A (either 100, 175 and 300 gel bloom) at 0.1 mg/mL. For comparative purposes, NPs in the absence of 5-FU were also prepared.

The impact of gelatin B concentration was firstly analyzed. Results have demonstrated that the increase of gelatin B concentration systematically reduces the size and improves the 5-FU encapsulation on the obtained NPs (Fig. 1A). No influence on the polydispersity values as a function of the gelatin concentration was found.

Once the optimal gelatin B concentration was established, the effect of the imposed compositions was analyzed separately. Fig. 1 depicts the effect of 5-FU concentration and gelatin A gel strength on the physicochemical parameters of the obtained NPs. Concerning the size, maximum values were obtained by decreasing gelatin A gel strength and increasing the content of 5-FU on the formulations, respectively. Regarding the polydispersity values, these values decreased as the content of 5-FU was increased. The degree of encapsulation of 5-FU on the NPs was evaluated through the loading efficiency (LE) parameter. LE results suggested that the degree of encapsulation of 5-FU entrapment could be modulated by varying 5-FU concentration. In an almost independent way of gelatin A gel strength, LE values became minimum for intermediate 5-FU concentration (2.5 mg/mL) and maximum for the lowest (1.25 mg/mL) and highest (5.0 mg/mL) 5-FU concentration on the formulations.

The increased pH simulating the chronic wounds pH was demonstrated to have a limited effect on the 5-FU release from the NPs (Fig. 1E). The release efficiency (RE) values seem to be a function of both pH and imposed composition. At pH 7.4, RE values followed the trend NPs GA100 > NPs GA175 > NPs GA300, suggesting the effect of pI on the efficiency of 5-FU release under the assayed conditions. The maximum RE values were around 12%. Low release efficiency was found by increasing pH until 9.0.

3.2. Hemocompatibility studies with gelatin B(5-FU)-gelatin A NPs

The treatment of chronic wounds makes it necessary to confirm the hemocompatible character of the proposed NPs. Following the ISO 10993-4 concerning the biological evaluation of medical devices and their interactions with blood, an *in vitro* hemocompatibility assay was carried out. [27]. Under the assayed conditions, the determination of the degree of hemolysis produced by NPs by incubation with red blood cells (RBC) suspension was performed. The effect of NPs on the hemolytic response was evaluated as a function of the ratio (*v*/v) between RBC and NPs dispersion, with values equal to 1:1, 1:2, and 1:4. Fig. 2 A shows representative results as a function of the imposed composition. The degree of hemolysis fluctuates slightly with both the concentration of 5-FU and the RBC/NPs ratio, with values lower than 2% for NPs systems prepared with both GA175 and GA300, achieving 2.5% for discrete conditions in the case of NPs prepared with GA100. Taking into account the criteria for which NPs are classified under non-hemolytic (<2%), slightly hemolytic (with values 2–5%) and hemolytic (values >5%) [28], it could be concluded that the proposed NPs showed a non-hemolytic character.

The interaction of NPs with RBC can cause significant erythrocyte deformation and/or aggregation with damaging consequences, despite the NPs having little hemolytic activity [29]. Changes in the morphology and the distribution of the erythrocytes by incubation of different NPs systems were analyzed by optical microscopy, and representative images are shown in Fig. 2B. From the obtained results, it can be deduced that even at the highest concentration of both gelatin B and 5-FU at the maximum RBC/NPs ratio (1:4), any agglutination phenomena are produced, independently of the gel strength of the gelatin A. The absence of erythrocyte deformation is consistent with the isotonicity [30] of the NPs formulations, whose osmolarity values ranged from 306 mOsm/L to 311 mOsm/L.

3.3. Cell viability studies with gelatin B(5-FU)-gelatin A NPs

The assessment of the viability of the proposed cell lines (3T3, HaCaT and A431) by interaction with the obtained NPs includes evaluating the effect of pH 5.5 on these biological assays. Close control of pH conditions is crucial for optimal culture conditions. pH values in the range 7.2–7.4

A

Gelatin B concentration (mg/mL)	Size (nm)	pdi	LE (%)
2.5	300-1000	0.5-0.8	0-50
5	65-300	0.5-0.8	0-25
10	50-200	0.5-0.8	0-25
20	60-130	0.5-0.8	30-60

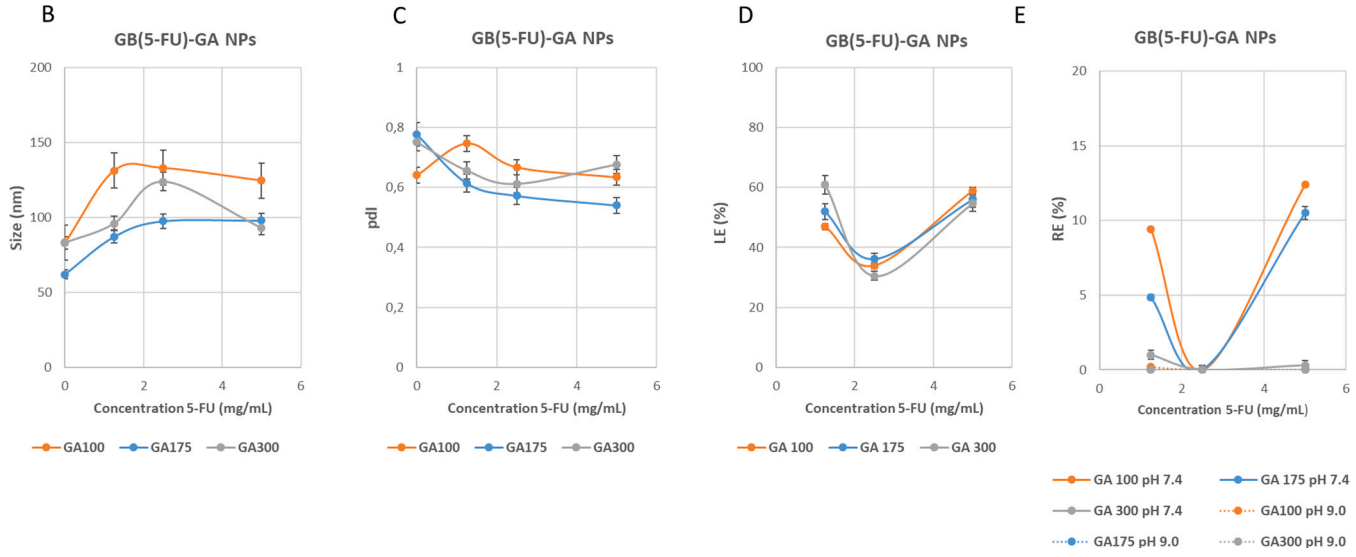

Fig. 1. Effect of gelatin B concentration on the physicochemical properties of NPs (A). Representative results on the particle size (B), polydispersity (C), LE values (D), and RE values as a function of pH (E) for gelatin B concentration equal to 20 mg/mL. Results are expressed as means ± standard deviation.

are preferred for most cells. However, fibroblast-like cells seem to prefer slightly alkaline conditions (pH 7.4–7.7), while continuous transformed cell lines have a preference for more acid pH conditions (7.0–7.4) [31]. The NRU and MTT endpoints were used to evaluate cell viability.

Through the NRU method, cell viabilities ranging between 60 and 80% were obtained for the three cell lines (Fig. SM1). Although these results demonstrated good biocompatible properties, this endpoint demonstrated poor selectivity to discriminate between NPs (either in the absence of presence of 5-FU) or 5-FU in solution. No significant differences were found between either NPs prepared in the absence or presence of 5-FU, 5-FU containing NPs, and 5-FU in solution, independently of the cell line. Due to the NRU endpoint characteristics [32], the obtained results suggested that the plasma membrane and/or lysosomal compartments of these cells remained undamaged after the incubation with the different formulations.

When cell viability assays were performed using the MTT method, results were more sensitive to the imposed compositions (Fig. SM2). The MTT endpoint demonstrates that the proposed gelatin-based NPs are biocompatible drug delivery systems at pH 5.5. Cell viability values ranging between 80 and 170% as a function of the gelatin A gel strength A and cell line, were found. Nevertheless, by incorporating the antitumoral drug, an increase in the cytotoxic responses with values ranged between 35 and 72% was observed. Besides, these studies demonstrated that 5-FU in solution at identical concentrations to those included in the NPs could not induce cytotoxicity on the tested cells, in agreement with previous results in our lab [19]. Considering that the MTT assay is a measurement of cell metabolic activity within the mitochondrial compartment [33], the obtained results suggested that 5-FU in solution involves a low interaction with the mitochondrial compartment. However, the decrease in cell viability by the inclusion of 5-FU on gelatin-based NPs strongly suggests that uptake and internalization are mediated by the NPs system [19,34,35].

Further insights into the efficiency of 5-FU NPs were obtained by evaluating the cell response at both the pH on the normal skin and two representative pH values corresponding to chronic wounds (7.4 and 9.0). Besides, the effect of glucose concentration was also evaluated. For this purpose, both low-glucose DMEM type (1 g/L glucose) and high-glucose DMEM type (4.5 g/L glucose) were used. Concentrations of glucose close to 1 g/L (5.5 mM) are considered euglycemic conditions under *in vitro* conditions, whereas diabetic conditions can be simulated for glucose concentrations up to 10 mM. Cell viability studies demonstrated that the NPs could effectively deliver 5-FU into the cells under both glucose conditions (Fig. 3).

Experiments performed under euglycemic conditions demonstrated how the loss of cell viability only occurs for NPs containing 5-FU, with values ranged between 20 and 70% depending on the cell line, pH, and gelatin A gel strength. When the effect of 5-FU concentration was evaluated, results demonstrated that NPs with an initial 5-FU concentration equal to 1.25 and 2.5 mg/mL promoted similar cell response, in agreement with an identical amount of 5-FU encapsulated (taking into account the LE values systems (Fig. 1D)). However, the increase of 5-FU content for NPs prepared with 5 mg/mL did not induce any additional decrease in cell viability.

For NPs prepared in the absence of 5-FU, cell viability reaches 100% or, in some cases, can induce proliferation. The degree of proliferation improves by increasing either the pH or the gelatin A gel strength, depending on the cell line. The effect of pH on cell proliferation can be correlated with the optimal culture conditions concerning pH, close to 7.4 [31] for the selected cell lines. Regarding the effect of the gelatin gel strength, it is reported that proliferative capability in both 2D and 3D cultures is mainly affected by gel strength induced by gel concentration changes [36]. In this work, differences in gel strength are inherent to

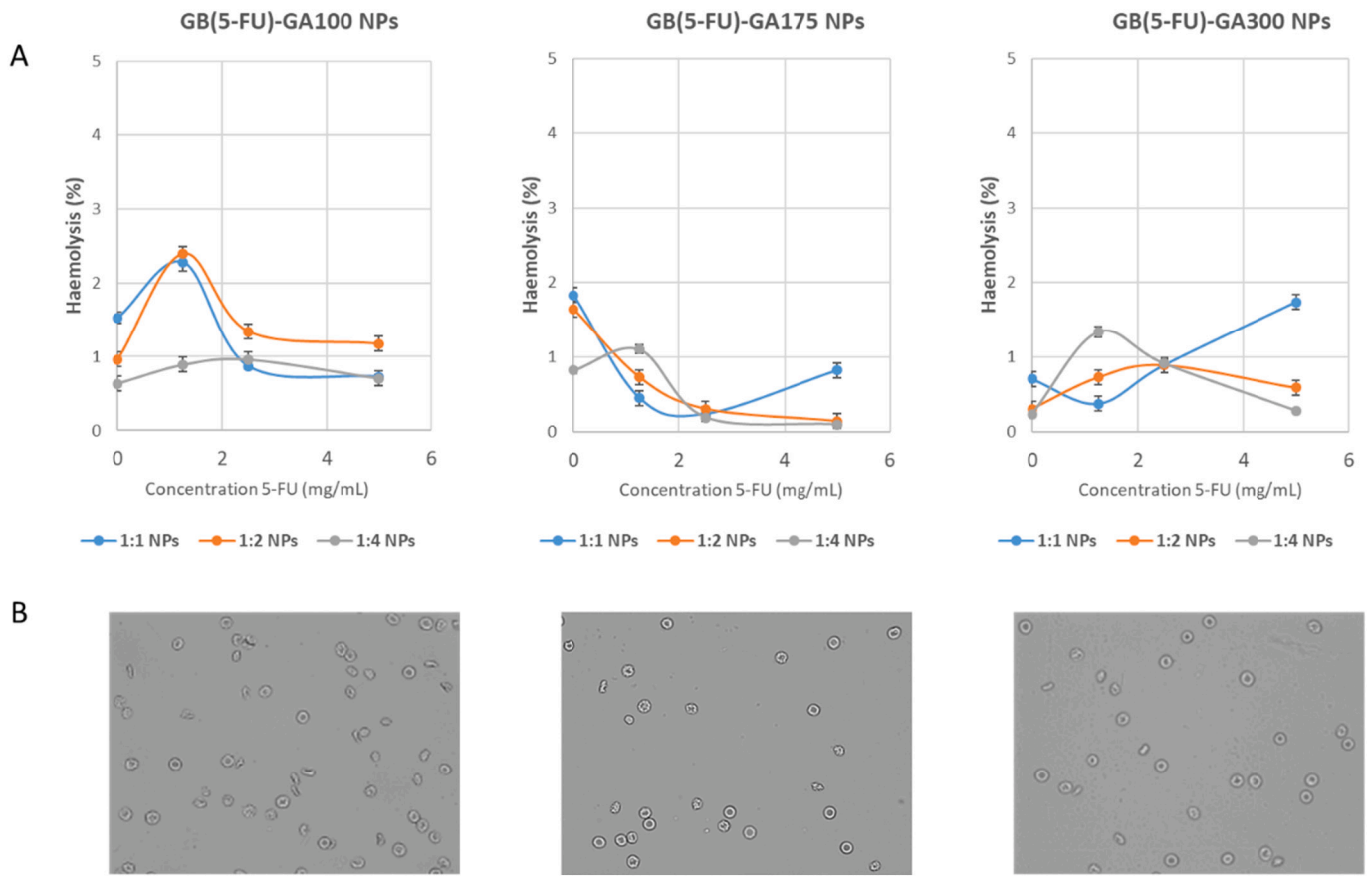

Fig. 2. Representative results of the percentage of hemolysis induced by the NPs systems as a function of the RBC/NPs (v/v) (1:1, 1:2 and 1:4) prepared with gelatin A with different gel strength values (A). Representative images of erythrocytes after incubation with 5FU NPs (5-FU 5 mg/mL) at RBC/NPs (v/v) (1:4) for the three different gel strength values of gelatin A (B).

gelatin A, at equal concentration conditions.

When experiments were performed under hyperglycemic conditions, similar results were obtained (Fig. 3B). Interestingly, the incubation of cells with blank NPs increases the proliferation rate by comparing those data observed under euglycemic conditions. Besides, by increasing glucose concentration, the incubation of cells with 5-FU NPs promotes the increased deleterious effect of the antitumoral drug due to pH and the imposed compositions.

The effect of glucose on cell culture is not an easy issue. The literature demonstrated that high-glucose media (4.5 g/L glucose) is the optimal cell culture condition for both fibroblasts and epithelial cells. Alternatively, the consequence of growing cells under conditions that are nearly diabetic is that the glycation and glycoxidation processes modify cells and cell products. Therefore, by increasing glucose concentration up to 25 mM glucose, cells will secrete relatively high amounts of lactic acid, which may impair proliferation and metabolism relatively rapidly. Some cell culture protocols described the role of high-glucose DMEM on the controlled grown of some tumor cells with fast growth speed and difficult attachment [31,37].

The corresponding concentration required to inhibit cell growth by 50% (IC_{50}) in terms of the initial 5-FU concentration on the NPs was determined by fitting the concentration-dependent viabilities curves. Fig. 4 summarizes the obtained results as a function of pH and gelatin A gel strength under both glucose conditions. Results demonstrate that the cellular response is highly dependent on both the glucose concentration and the cell line. When considering the fibroblasts cell line, the euglycemic conditions promote low sensitivity to the deleterious effect of NPs, independently of the pH and gelatin A gel strength. In all cases, the corresponding IC_{50} values result to be higher than the highest 5-FU concentration (IC_{50} values >5 mg/mL). However, by increasing the glucose concentration on the culture media, 3T3 fibroblasts showed high sensitivity to the cytotoxic effect of 5-FU containing NPs, with IC_{50} ranged between 1.2 and 1.3 mg/mL, in an almost independent manner of the pH and gelatin A gel strength.

The response of the HaCaT keratinocyte cell line promotes opposite results as a function of the glucose concentration. Results obtained under hyperglycemic conditions became an excellent example of our hypothesis to the controlled encapsulation and release of 5-FU as a function of gelatin A gel strength. As previously discussed, the pI of gelatin A depends on its gel strength value: pI values close to 7 must be considered to the low gel strength, whereas pI values could move close to 9 for higher gelatin gel strength [20]. Cell viability responses induced by NPs prepared with gelatin A at the lowest gel strength values (GA 100) followed the proposed hypothesis. The highest IC_{50} values were found for all systems at the normal skin pH value (pH 5.5), with lower IC_{50} values with significant differences for pH values close to those of chronic wounds (pH 7.4 and 9.0). Changes in IC_{50} values must be correlated with the controlled release of 5-FU because of the vehicle destabilization as a function of pH. NPs prepared with gelatin A with 175 and 300 blooms did not show any differential behavior as a function of pH, showing identical and highest IC_{50} values. These results could be correlated with the inability to reach their pI values. Consequently, gelatin A remains as a positively charged compound, and no destabilization and release occur. Results obtained under euglycemic conditions did not show any predictable trend, with the lowest IC_{50} values for experiments conducted at the normal skin pH.

Differences in cell behavior of these non-tumoral cell lines upon incubation with the NPs systems might be correlated with differences in

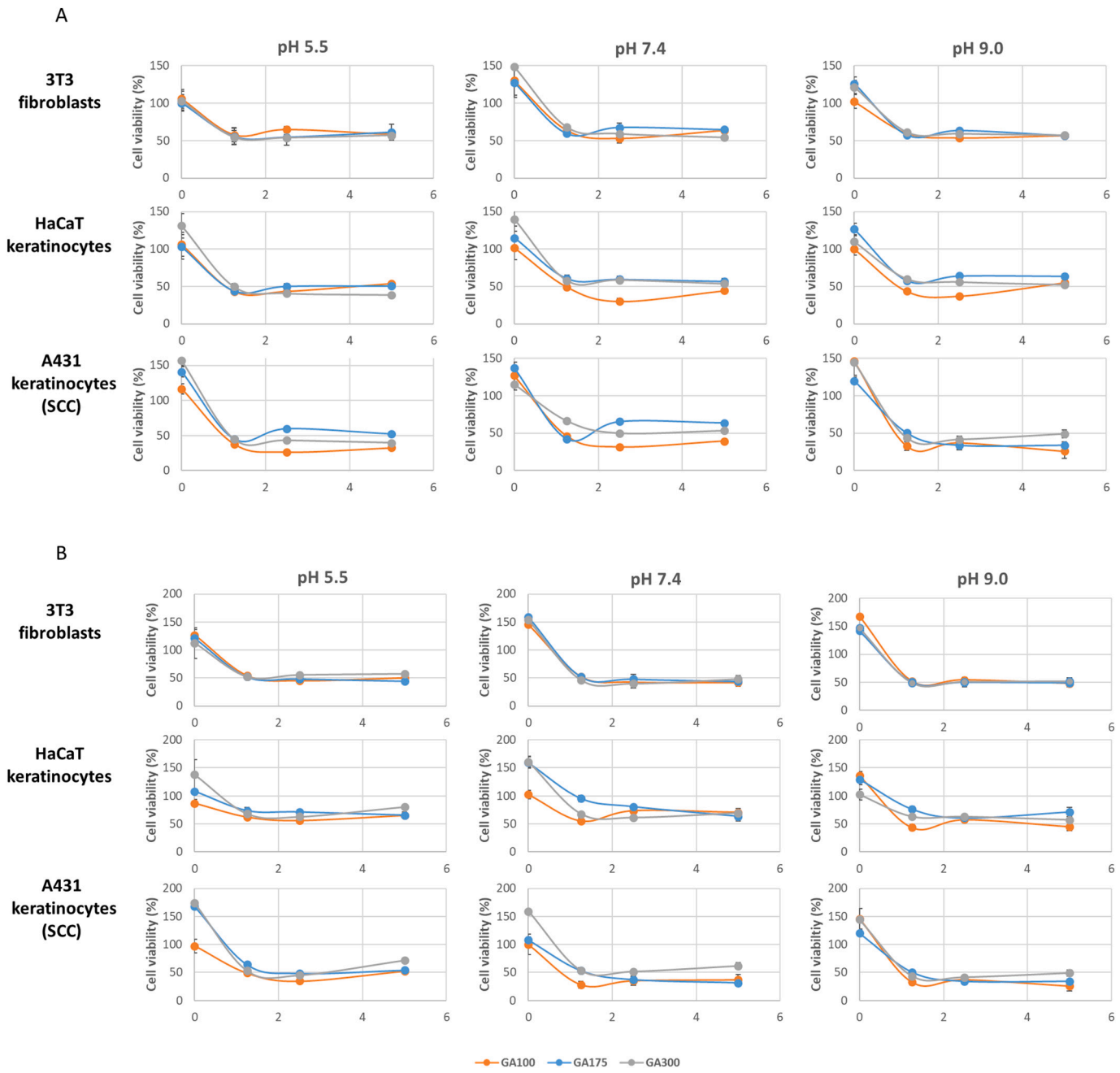

Fig. 3. Representative cell viability derived from the cell viability studies under simulated euglycemic (A) and hyperglycemic conditions (B) incubated with gelatin-based NPs including 5-FU (5-FU NPs), during 24 h, determined by MTT method. Results are expressed as the average of three independent experiments ± standard deviation.

cell characteristics. Changes in morphologic and physiologic properties between 3T3 and HaCaT cell lines, especially the differing ability to resist oxidative stress, could explain differences in sensitivity. Hence, whereas keratinocytes constitute an example of representative epidermal skin cells, fibroblasts are located in the dermal skin layer. For this reason, 3T3 cells are generally most sensitive than HaCaT cells to the deleterious effect of incubated substances [38], as can be found in this work under the standard cell culture conditions of these two cell lines (4.5 g/L glucose).

Interestingly, the squamous cancer cell line (A431) reported the lowest IC_{50} values, with values ranged between 0.6 and 1.7 mg/mL, and 0.7–2.4 mg/mL, at high and low glucose conditions, respectively, with significant differences between the gel strength of gelatin A and pH values. The role of glucose concentration on chemotherapy has reported discrepant results. In most studies, hyperglycemia decreases the anti-proliferative effect of chemotherapy but chemotherapy effect seemed to be potentiated, in some studies.

The diminution of chemotherapy efficacy seems to differ between cell lines instead of the type of chemotherapy [39]. Although the IC_{50} values seem to depend on glucose concentration, the obtained results showed significant differences with those values obtained in both non-tumoral cell lines (3T3 and HaCaT). The increase of efficiency on the A431 cell line could be correlated with tumor cell capability to recognize the RGD motif on gelatin as a strategy for tumor targeting. Although tumoral cell lines overexpressed integrins, previous results in our lab have demonstrated to be intensely dependent on cell line type [19]. The obtained results demonstrated that our NPs take advantage of this therapeutical approach to target A431 cells.

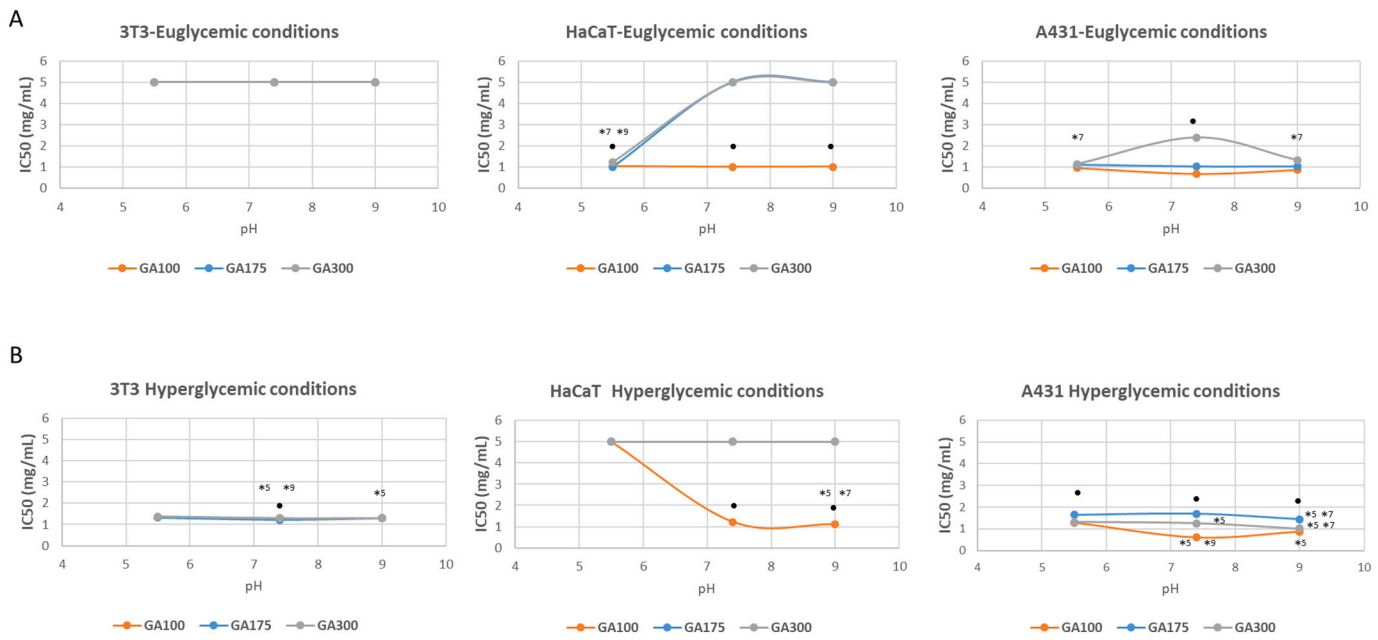

Fig. 4. Half maximal inhibitory concentration (IC_{50}) values derived from the cell viability studies under simulated euglycemic (A) and hyperglycemic conditions (B) incubated with gelatin-based NPs including 5-FU (5-FU NPs), during 24 h, determined by MTT method. Results are expressed as the average of three independent experiments ± standard deviation. $^{\bullet}p < 0.001$ indicates significant differences between gelatin A gel strength at the same pH values, and $^{*}p < 0.001$ indicates significant differences between treatments for the same gelatin A gel strength at different pH values (indicated as *5,* 7 or*9). In all cases, IC_{50} values equal to 5 corresponds to systems for which the concentration required to inhibit cell growth by 50% compared with control cells was not achieved even at the highest 5-FU concentration (5 mg/mL).

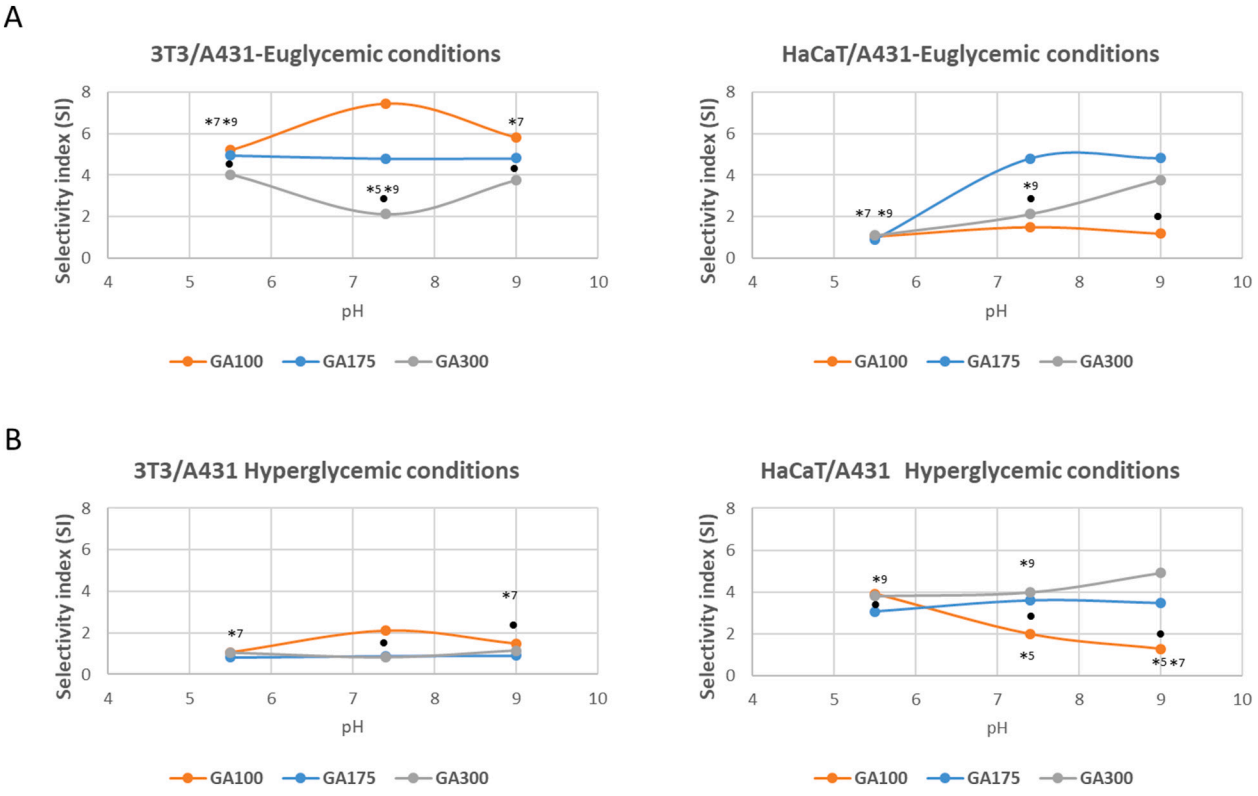

Fig. 5. Selectivity indexes (SI) derived from the cell viability studies of cells incubated with gelatin-based NPs including 5-FU (5-FU NPs), during 24 h, determined by MTT method. Results are expressed as the average of three independent experiments ± standard deviation. $^{\bullet}p < 0.001$ indicates significant differences between gelatin A gel strength at the same pH values, and $^{*}p < 0.001$ indicates significant differences between treatments for the same gelatin A gel strength at different pH values (indicated as *5,* 7, or*9).

The hypothetical ideal treatment of cancer is by administering a drug by selective toxicity. Selective toxicity can be defined as the property of toxic substances to harm or kill particular species of cells (*i.e.* cancer cells) without producing any harmful effect to normal cells [40]. In this work, once the IC_{50} values were determined, the corresponding selectivity indexes (SI) between different cell lines were determined (Fig. 5). The selective toxicity becomes better as the selectivity indexes increase.

The SI values suggested that the degree of selectivity strongly depends on the concentration of glucose on the cell culture media. Accordingly, in the case of the euglycemic conditions, it can be deduced that all the NPs present an excellent selectivity for the tumoral cells (A431), especially in comparison with the 3T3 cells. Significant differences ($p < 0.001$) between gelatin A gel strength values were found in all cases. The SI maximum was found with NPs prepared with GA100 at pH 7 ($SI > 7.5$). SI values ranged between 2 and 4, for NPs systems prepared with GA300 and GA175, respectively. Compared with the HaCaT cell line, the SI maximum was found with NPs prepared with GA175 at either pH 7 or pH 9 ($SI = 5$).

Otherwise, under hyperglycemic conditions, the NPs systems offered low selectivity for the tumoral cells (A431) compared with 3T3 fibroblasts. SI values are maximum for the NPs prepared with GA100 at pH 7 ($SI = 2$), with significant differences between gel strength and pH. Interestingly, the stimulated diabetic conditions promote notable selectivity for the target cells (SCC cells) compared to cells with similar characteristics (HaCaT keratinocytes), which would coexist in the same layer of the skin under physiological conditions. In all cases, significant differences between the gelatin A gel strength and pH were found. The SI maximum corresponds to NPs prepared with GA300 at pH 9 ($SI = 5$). In all cases, significant differences ($p < 0.001$) between SI values under euglycemic and hyperglycemic conditions were found.

From the obtained results, it can be deduced that gelatin type A gel strength is a controlling parameter on the mode of action of the proposed NPs. As it can be derived from the SI values, NPs prepared with gelatin with different bloom values allow the modulation of the selectivity between representative skin cells, both non-tumoral and tumoral, under the imposed conditions of both pH and glucose concentrations.

3. Conclusions

The interaction of oppositely charged gelatin type A and gelatin type B at the pH of the normal skin has been used to prepare NPs including 5-FU. NPs with optimal values concerning size (60–130 nm), pdI (0.5–0.8) and LE (30–60%) values have been obtained at the highest gelatin B concentration (20 mg/mL).

The biological evaluation of NPs by interaction with erythrocytes has demonstrated that the gelatin-based NPs are hemocompatible independently of the RBC/NPs ratio, gelatin A gel strength, or concentration of 5-FU on the NPs. The degree of hemolysis (<2%) allows to confirm that NPs are non-hemolytic as requires the ISO 10993-4. Additionally, even at the maximum RBC/NPs ratio (1:4) (*v*/v), no agglutination phenomenon has been produced.

Representative cell lines from both epidermis and dermis layers and non-tumoral and tumoral characteristics have been used to assess the *in vitro* cell viability induced by the NPs systems. Unlike 5-FU in solution, 5-FU-containing NPs can reduce selectively the viability of the tumoral cells, depends on glucose concentration, pH, and gelatin A gel strength.

Non-tumoral cell lines (3T3 and HaCaT) have shown to be highly sensitive to glucose concentration, promoting some unexpected results as a function of pH and gelatin A gel strength. Notably, cells associated with SCC (A431 cell line) have shown the lowest IC_{50}, independently of glucose conditions. Together with the dependence of pI of gelatin A with gel strength, the presence of the RGD-motif on the gelatin structure confers excellent properties to the targeted delivery of 5-FU to A431 cells.

Under the euglycemic conditions, the NPs showed good selectivity for the cancer cells (A431), especially compared to the 3T3 cells, with SI values that can reach 7.5. By increasing the glucose concentration to a diabetic condition analogous, these NPs act with selectivity, reaching the target cells (A431) with SI values up to 5 compared to the HaCaT cells.

The obtained results have demonstrated how the presence of RGD-motif on gelatin and the differences in ionization properties of this protein at the pH of chronic wounds confers to the proposed gelatin-based NPs promising properties as delivery systems to the targeting of squamous cancer cells with selective toxicity.

CRediT authorship contribution statement

M.C.M. conceived of the presented idea. A. F. and M.C.M. developed the gelatin-based NPs, performed the cell viability studies, and analyzed the experiments. M.C.M. wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors have approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.msec.2021.112073.

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Maria del Carmen Morán (BS-Chemistry-1996; MS-Chemistry-1997; PhD-Chemistry-2002 UB) is Associate Professor at the Department of Biochemistry and Physiology, Faculty of Pharmacy and Food Science at the University of Barcelona (UB). She did a PhD at the Department of Surfactant Technology-Spanish Council for Scientific Research (IIQAB-CSIC) and then a postdoctoral research at the Department of Chemistry at the University of Coimbra-Portugal (2006–2010) by means of UE founding. Through a Ramon y Cajal contract, she incorporated at the Department of Physiology-UB (2010-). Her scientific background is now being applied to more biologically oriented questions to prepare efficient therapeutic delivery systems and their *in **vitro*** characterization towards selected cell lines under the bulk and substrates conditions.

Albert Ferriol (BS-Biotechnology-2018, University of Lleida) has coursed his master's degree in the field of Nanoscience and Nanotechnology at the University of Barcelona (UB) (2018–2019). His Master's thesis project has been developed at the Department of Biochemistry and Physiology, Faculty of Pharmacy and Food Science (UB). He was involved in preparing and optimizing drug delivery systems and their cytotoxicity and potential selectivity towards selected non-tumoral and tumoral cell lines. He is currently a Jr. Scientist in an R&D company. His research is focused on the biofunctionalization of nanostructures and their biological characterization.