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Pectin-coated chitosan microgels crosslinked on superhydrophobic surfaces for 5-fluorouracil encapsulation

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ABSTRACT

5-Fluorouracil (5-FU)-loaded chitosan microgels for oral and topical chemotherapy were prepared applying a superhydrophobic surface-based encapsulation technology. Drug-loaded chitosan dispersions were cross-linked and then coated with drug-free chitosan or pectin layers at the solid-air interface in a highly efficient and environment-friendly way. The size of the microgels (with diameters of ca. 280 and 557 μm for the chitosan seeds and pectin-coated microgels respectively) was the lowest obtained until now using similar biomimetic methodologies. The microgels were characterized regarding 5-FU release profiles in vitro in aqueous media covering the pH range of the gastrointestinal tract, and cytotoxicity against two cancer cell lines sensitive to 5-FU. Owing to their control of 5-FU release in acidic medium, calcium pectinate-coated microgels can be considered as suitable for oral administration. Growth inhibition of cancer cells by 5-FU was greater when incorporated to chitosan microgels; these being potentially useful for treatment of skin and colorectal tumors.

1. Introduction

Oral chemotherapy is attracting increasing attention since it can provide convenient home-based therapy with lesser collateral effects than intravenous treatments, leading to a better quality of life (Banna et al., 2010). In fact, more than twenty antitumor drugs have been recently approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for oral administration, and most of the new molecules under research are thought to be developed as oral medicines (Krishnaiah & Khan, 2012; Ruddy, Mayer, & Partridge, 2009). As an example, 5-fluorouracil (5-FU) has been shown suitable for systemic oral therapy, without expecting differences in drug efficacy compared to continuous infusion (Miura et al., 2010). In addition to systemic absorption, the oral route also enables site-specific treatment of colorectal cancer, which has been reported as the fourth most frequent cause of cancer death (Pisani, Bray, & Parkin, 2002). Mucoadhesive formulations can provide high local concentrations of antitumor agents and prolonged contact with the tumor tissue, using lower doses and thus causing less damage in healthy tissues (Haupt, Zioni, Gati, Kleinstern, & Rubinstein, 2006; Krishnaiah & Khan, 2012). Chitosan-coated alginate microspheres (Urbanska, Karagiannis, Guajardo, Langer, & Anderson, 2012) and pellets of hyaluronic acid-coupled chitosan nanoparticles (Jain, Jain, Ganesh, Arve, & Beg, 2010) loaded with oxaliplatin, Eudragit S-100 coated hydroxypropyl methylcellulose granules (Ciftci & Groves, 2006) and gelatin-coated chitosan microspheres (Bhat et al., 2012) loaded with 5-FU, and chitosan microspheres with valdecoxib (Thakral, Ray, & Majumdar, 2012) have been recently shown useful for colorectal cancer treatment. Bioactivation of 5-FU due to the cellular enzymatic activity is notably promoted in colorectal tumor tissue compared to normal tissue (Wei, Qing, De-Ying, Bai, & Li-Fang, 2008). Moreover, chitosan can reduce the side effects caused by anti-metabolite drugs such as 5-FU, particularly the injury to the small intestinal mucosa membrane, the incidence of diarrhea and the reduction of the blood leukocyte number, without loss of antitumor activity (Kimura & Okuda, 1999). Combination of chitosan with 5-FU might be also suitable for the topical treatment of precancerous and cancerous lesions of the skin (targeting to atypical keratinocytes) and tumors affecting vaginal-uterine tissues (Lam et al., 2012; McGillis & Fein, 2003). Chitosan may increase the penetration of 5-FU through the epithelial cells and, thus, enhance the effectiveness of the treatments (Sabitha et al., 2013).

The design of microparticles for drug delivery has to face up to two relevant difficulties: (i) small and hardly predictable drug entrapment efficiency and (ii) poor control of the drug release

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rate through the short diffusion pathway (Bhattarai, Gunn, & Zhang, 2010; Sinha et al., 2004). Recently, a bioinspired approach to obtain polymer microgels with 100% encapsulation yield has been developed exploiting the way aqueous droplets adopt spherical forms on superhydrophobic surfaces, followed by a step of hardening/cross-linking of the polymer at the solid-air interface (Lima, Song, Blanco-Fernandez, Alvarez-Lorenzo, & Mano, 2011; Song, Lima, & Mano, 2010). This methodology avoids harsh conditions and organic solvents and may enable a very precise and reproducible dosing in the microgels of narrow therapeutic range, labile drugs and molecularly targeted proteins and peptides used in the cancer treatment.

The aim of this work was to develop chitosan-based microgels for oral administration or skin/mucosal application of 5-FU, implementing a methodology consisting of (i) deposition of droplets of drug-containing chitosan aqueous solutions on superhydrophobic surfaces, (ii) cross-linking of chitosan in glutaraldehyde atmosphere, and (iii) optionally, coating of the microgels by successive deposition and cross-linking of drug-free chitosan or other polysaccharide (e.g. pectin) solutions in order to attain a better control of drug release. Former trials with bioinspired superhydrophobic surfaces involved acrylic-modified polymers and UV polymerization (Lima, Custodio, Alvarez-Lorenzo, & Mano, 2013; Lima et al., 2011). As an advantage, the present work avoids monomeric species and initiators and employs natural polysaccharides (Muzzarelli et al., 2012), being a safer and greener approach. To carry out the work, first superhydrophobic surfaces that mimic the hierarchical micro- and nano-structure and the roughness of the Lotus leaves were obtained on polystyrene in order to attain water contact angles greater than 150° and sliding angle lower than 10° (Neto, Custodio, Song, & Mano, 2011; Song et al., 2010). In addition to chitosan solely microgels, semi-interpenetrating networks (semi-IPN) of chitosan with hyaluronic acid and different varieties of Eudragit® were prepared. Sodium hyaluronate (SH) has been shown able to modulate cell adhesion, growth and migration, wound healing and cancer metastasis (Jain & Jain, 2008; Zeng, Toole, Kinney, Kuo, & Stamenkovic, 1998). Moreover, hyaluronic acid receptors such as CD44 and Rhamm are overexpressed in cancer cells, including colorectal ones (Kobel et al., 2004). Two acrylic copolymers namely Eudragit® E PO (soluble at pH > 5) and NE 30 D (time-dependent dissolution) were included in the microgels to explore the possibilities of tuning 5-FU release rate as a function of pH (Lunter & Daniels, 2012; Paharia et al., 2007). After hardening, some chitosan microgels were coated, also on the superhydrophobic surfaces, with pectin cross-linked with $CaCl_2$ in order to facilitate colon specific release (Liu, Fishman, Kost, & Hicks, 2003; Sriamornsak, 2011). The developed approach may enable a precise regulation of the amount of drug encapsulated and the pectin-coated microgels provide pH-responsive controlled drug release.

2. Materials and methods

2.1. Materials

Polystyrene sheets (60 mm × 60 mm) from square Petri dishes (Bdbioscience, Enzifarma, Portugal) and polystyrene granules of injection molding grade were used for the obtaining of syperhydrophobic surfaces (Song et al., 2010). Chitosan (chitosan 222, average deacetylation degree 76.2%, Mw 359,000 (s.d. 11,570) Da (Barreiro, Coronilla, Concheiro, & Alvarez-Lorenzo, 2005)) was from George S. Daras (Marseille, France); sodium hyaluronate (SH) was from Guinama (Valencia, Spain); and 5-fluorouracil (5-FU) was from Fagron Iberica S.A.U. (Barcelona, Spain). 1H,1H,2H,2H-Perfluorodecyltriethoxysilane (PFDTS, 97%), crystal violet, formic acid, fetal bovine serum (FBS), glutamine, non-essential amino acids (NEAA), pectin from apple (galacturonic acid content 77.5%, methoxy content 7.8%), pectin from citrus fruits (galacturonic acid content 84%, methoxy content 10%) and penicillin–streptomycin solution were from Sigma–Aldrich Co. (St. Louis, MO, USA). Dulbecco' Modified Eagle's Medium (DMEM) with high glucose content was from Gibco (Invitrogen, Spain) and Eagle's Minimum Essential Medium (EMEM) from the American Type Culture Collection (Manassas, VA). Glutaraldehyde solution (25%, v/v), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$) and orthophosphoric acid (85%) were from Merck (Hohenbrunn, Germany). Eudragit® E PO and NE 30 D were provided by Evonik (Germany) and 2-(4-morpholine)ethanesulfonic acid (MES) by Fischer Bioreagents (Leicestershire, UK). Purified water (resistivity > 18 MΩ cm; MilliQ®, Millipore, Spain) was obtained by reverse osmosis. All other reagents were of analytical grade.

2.2. Methods

2.2.1. Preparation and characterization of polystyrene superhydrophobic surfaces

Polystyrene sheets were washed with ethanol in an ultrasonic bath for 15 min and treated with a solution of polystyrene (70 mg/ml) in tetrahydrofuran and ethanol (2:1.3, v/v). Then, the sheets were immersed in ethanol for 1 min and dried under nitrogen flow before argon plasma treatment (30W; Plasma Prep5, Gala Instruments, Germany) for 20 s. Finally, the sheets were immersed in a PFDTS solution (1% in ethanol) for at least 24 h, and then removed and air dried. The roughness of the surfaces before and after treatment and after being used were observed by means of Scanning Electron Microscopy (SEM, FESEM ULTRA Plus instrument, Zeiss, Oberkochen, Germany). Water contact angle was measured at room temperature in static mode using a Phoenix 300 goniometer (SEO, Korea).

2.2.2. Purification of chitosan and Eudragit® E PO

Chitosan was purified following a precipitation procedure previously described (Signini & Campana Filho, 1999). Briefly, chitosan (10 g) was dispersed in acetic acid 2% (v/v) and filtered first through a cellulose membrane of 7–11 μm pore size (ALBET-Hahnemühle, Barcelona, Spain) and then through a nylon membrane of 0.45 μm pore size (Lida Manufacturing Corp., Kenosha, WI, US). The pH of the solution was increased up to 8 with NaOH 2 M and the precipitated chitosan was washed with water until the medium reached pH 7, and then with ethanol:water 80:20 and 90:10 (v/v). The purified chitosan was freeze-dried. Purification of Eudragit® E PO was carried out by dialysis of a solution of the acrylic polymer (5 g) in phosphate buffer pH 2 (50 ml) against phosphate buffer pH 2 for 2 days, and against water for 5 days more using dialysis tubes of 12,400 Da MWCO (Sigma–Aldrich Co., St. Louis, MO, USA). Finally, purified Eudragit® E PO was freeze-dried.

2.2.3. Preparation of chitosan microgels

Dispersions of chitosan, chitosan/SH, and chitosan/Eudragit® in acetic acid 1% (v/v) were prepared at various concentrations (Table 1). 5-FU was added to aliquots of the solutions up to 0.5% (w/v). Droplets (2.5 μl) of the polymer/s solutions containing 5-FU were placed on the superhydrophobic surface of polystyrene sheets using a micropipette. Then, the sheets with the droplets were transferred to a dessicator containing 500 ml of glutaraldehyde solution (25%, v/v in water) at the bottom in order to create a glutaraldehyde atmosphere able to cross-link the microgels. After 1 or 4 h, the microgels were transferred to another dessicator that was connected to vacuum for 4 h for removal of unreacted glutaraldehyde. Chitosan cross-linking was monitored by FTIR analysis (Bruker model IFS-66v, Bruker Optics, Karlsruhe, Germany) of the

Table 1
Formulations of chitosan, chitosan/sodium hyaluronate, chitosan/Eudragit® varieties studied.

Formulation	Uncoated microgels				Coated microgels		
	Chitosan (%, w/v)	SH (%, w/v)	Eudragit® E PO (%, w/v)	Eudragit® NE 30 D (%, w/v)	Layers of chitosan	Layers of apple pectin	Layers of citrus pectin
1	1						
2	1.5						
3	2						
4	1	1					
5	1.5	0.5					
6	1.5		2.5				
7	1			1			
8	1.5				1		
9	1.5				2		
10	1.5				3		
11	1.5					1	
12	1.5						1

microgels; samples of 5 units were taken out from the glutaraldehyde atmosphere at different times, compressed with KBr and the IR spectra recorded in the 400–4000 cm^{-1} interval at 2 cm^{-1} resolution.

2.2.4. Preparation of coated microgels

Droplets of 5 μl of chitosan solution (1.5%, w/v) without drug were poured onto the preformed drug-loaded chitosan (1.5%, w/v) microgels placed on the superhydrophobic surfaces, which were then transferred to the glutaraldehyde atmosphere and processed as described above. Microgels coated with up to three layers of chitosan without drug were obtained by successive application of the droplets and repetition of the cross-linking process. In parallel, chitosan microgels (without chitosan layers) were coated with pectin by deposition of one droplet (5 μl) of pectin solution (2%, w/v) without drug onto each microgel, followed by one droplet of $CaCl_2$ solution (5%, w/v, 3 μl) in order to crosslink the pectin. Immediately after, calcium-pectinate coated microspheres were transferred to the glutaraldehyde atmosphere and processed as previously described.

2.2.5. Microgels characterization

2.2.5.1. Physical characterization. SEM images of microgels were taken under environmental conditions and after gold-coating using an EVO LS15 microscope (Zeiss, Oberkochen, Germany). Differential scanning calorimetry (DSC) scans of placebo and 5-FU-loaded chitosan microgels (5 mg in non-closed aluminum pans) were recorded from 30 to 300 °C at a heating rate of 10 °C/min in a DSC Q100 (TA Instruments, New Castle, DE) fitted with a RCS cooling unit and using nitrogen as the purge gas (50 ml min^{-1}). The size of dried chitosan microgels (uncoated, three layers chitosan-coated and pectin-coated) was evaluated using an Olympus SZ-CTV optical stereomicroscope (Tokyo, Japan) at 5X connected to a JVC TK-S350 video camera (Tokyo, Japan). Twenty microgels of each formulation were sized using the analysis image software analySIS® (Soft Imaging System, Münster, Germany). Digital pictures of chitosan microgels were also taken before and after the release study.

2.2.5.2. Swelling. Non-dried non-coated chitosan (prepared from 1.5% solution; formulation 2), chitosan/SH (prepared from 1.5%/0.5% solution; formulation 5), and chitosan/Eudragit® E PO (prepared from 1.5%/2.5% solution; formulation 6) microgels (samples of 5 units, in triplicate) were placed in contact with water, HCl 1% or phosphate buffer pH 7.4 (1 ml). At regular times, microgels were collected, weighed after removal the excess of swelling media with a soft tissue paper, and returned to the medium. The degree of swelling was estimated as follows:

$$\text{Swelling}\ \ (\%) = 100\left(\frac{W_t - W_0}{W_0}\right) \quad (1)$$

where W_0 and W_t represent the initial weight of the microgel and the that of the swollen specimen at each predetermined time. After 6 h in the medium, the aspect of the microgels was observed using an Olympus SZ-CTV optical stereomicroscope (Tokyo, Japan) at 5× connected to a JVC TK-S350 video camera (Tokyo, Japan).

2.2.5.3. Pectin erosion. Pectin films were prepared with the same solutions of pectin (from citrus fruits and from apple) at 2% used for the coating of chitosan microgels. Pectin solutions (4 ml) were poured on polyethylene dishes (51-mm diameter) and cross-linked with $CaCl_2$ 5% (w/v) (2.4 ml; i.e., the same ratio as that used for the coating). Then, the films were transferred to a dessicator containing glutaraldehyde (25% in water) in the bottom, kept for 4 h, and then transferred to an oven and dried for 24 h at 50 °C. Weighed pieces of the films were placed in vials containing water, HCl 1% or phosphate buffer pH 7.4 (10 ml). The vials were kept at 37 °C under oscillatory movements (30 osc/min) for 1 h and then the films were dried and weighed.

2.2.5.4. 5-FU release. 5-FU-loaded microgels (5 units) were placed in Eppendorf tubes containing 1 ml of water, HCl 1%, or phosphate buffer pH 7.4. At predetermined times, 200 μl of medium were taken and placed in the wells of Costar 96 well UV plates (Corning, NY, USA), and the absorbance was measured at 280 nm in a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany). The sample was replaced with 200 μl of fresh medium. All experiments were carried out in triplicate.

2.2.5.5. Cytotoxicity. HeLa cells (CCL-2, American Type Culture Collection, Manassas, VA) in 100 μl of DMEM-high glucose supplemented with 10% FBS, 2% glutamine, 1% NEAA and 1% penicillin–streptomycin were seeded in a 96-well plate (15,000 cells per well). In parallel, SK-MEL-28 cells (HTB-72, American Type Culture Collection, Manassas, VA) in 100 μl of EMEM supplemented with 10% FBS and 1% penicillin–streptomycin were seeded in another 96-well plate (5000 cells per well). The cells were incubated for 24 h at 37 °C, 5% CO_2 and 90% relative humidity. Then, microgels (3 or 5 units) were placed in some wells and 100 μl more of each medium were added. For the positive controls, the culture medium was removed and 5-FU solutions in 200 μl culture medium were added. Cells in culture medium without microgels nor 5-FU were used as negative controls. The plates were incubated at 37 °C, 5% CO_2 and 90% relative humidity for 6, 24 and 48 h in case of HeLa

cells, and for 24, 48 and 72 h in case of SK-MEL-28 cells. At those times, the microgels and the medium were removed from the wells, and the living cells were fixed with glutaraldehyde (11%, 20 μl) for 15 min under shaking. Then, the wells were washed with 100 μl of water three times, and the cells were stained with 20 μl of a violet crystal solution (0.1% in phosphoric acid 200 mM, formic acid 200 mM, MES 200 mM; pH adjusted to 6 with NaOH 5 M) for 15 min under shaking. The wells were washed with water (100 μl) four times in order to remove the residual crystal violet solution, and then dried for 2 h. Cristal violet was redissolved with an acetic solution (10%, 100 μl) for 15 min under shaking and the absorbance was measured at 595 nm using a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany). All experiments were carried out in triplicate.

3. Results and discussion

3.1. Preparation and characterization of superhydrophobic surfaces

Polystyrene sheets surface was made superhydrophobic following a previously described approach that involves (i) creation of a hierarchical structure on the surface and (ii) treatment with a low surface free energy coating (Lima et al., 2011). Micro and nanostructures were formed through the precipitation of polystyrene on the surface of polystyrene sheets. The aggregation of more polystyrene around the precipitation nuclei decreased the surface tension (Yuan et al., 2007). Finally, plasma treatment of the surfaces generated reactive hydroxyl groups which then reacted with the fluorocarboxylate groups of PFDTS increasing even more the hydrophobicity. SEM images showed the rough topography with micro and nanometric structures (<5 μm) of the modified surfaces (Fig. 1).

When used to prepare successive microgels batches, changes in the structure of the superhydrophobic surfaces could alter the morphology and the homogeneity of the features of the particles. To elucidate the relevance of those changes, the surfaces were analyzed again after microgels preparation. SEM micrographs revealed no changes in the surface structure at any magnification after preparation of 10 batches of chitosan solely microgels (Fig. 1). Furthermore, contact angle measurements confirmed the superhydrophobicity of the modified polystyrene as prepared (157°) and after being used for obtaining chitosan microgels (151°). Thus, the surfaces stood well the production of chitosan or chitosan-SH microgels. By contrast, after preparation of the semi-IPN microgels of chitosan and Eudragit® NE 30D the surfaces became altered at the micro and nanometric scales (Fig. 1 D1 and D2) even after the first batch preparation, and the water contact angle decreased up to 66.5°. This finding is related to the presence of an emulsifier (1.5% of Nonoxynol 100) in the composition of the commercial dispersions of Eudragit® NE 30D. As the superhydrophobic nature of the surfaces depends not only on the roughness but also on their low surface free energy, the surfactant leads to a decrease in the hydrophobicity that limits the reusability of the surfaces. Differently, Eudragit® E PO, provided as a solid powder of poly[butyl methacylate-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate] 1:2:1 (Evonik, 2013), did not damage the superhydrophobic surfaces.

3.2. Preparation of non-coated microgels

The volume of the drops of polymer/drug solution placed on the superhydrophobic surface determined the size of the microgels. Although the topography of the modified surfaces could enable the obtaining of few micrometers spherical droplets, the manual pipeting did not allow going below 2.5 μl droplets (initial radius

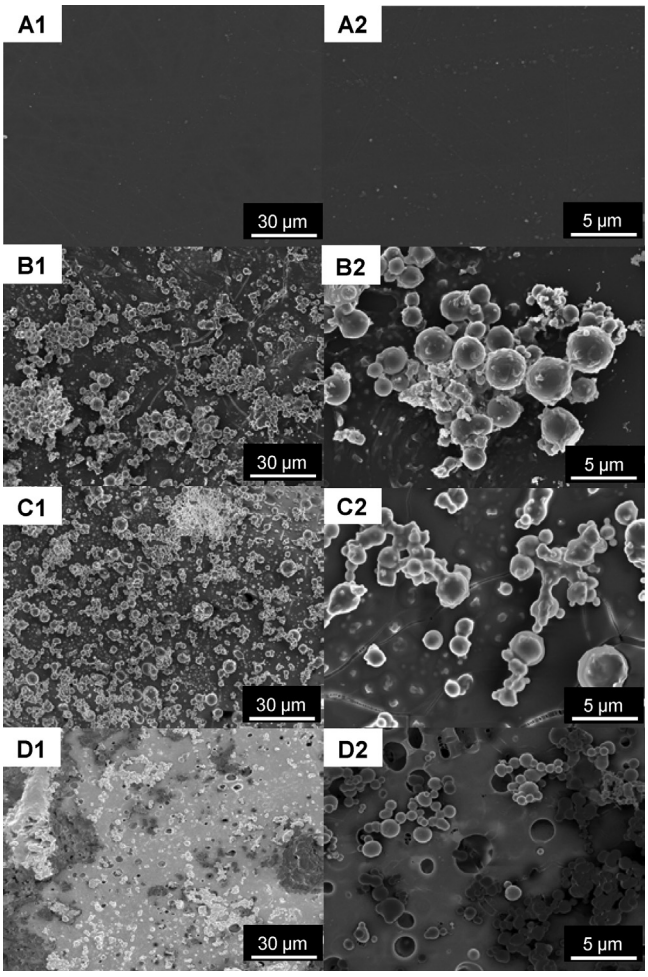

Fig. 1. SEM images of polystyrene surfaces at various magnifications: unmodified sheets (A1 and A2), just modified sheets to turn into superhydrophobic (B1 and B2), and modified sheets after being used to obtain 10 batches of chitosan microgels (C1 and C2) or 1 batch of chitosan/Eudragit® NE 30D microgels (D1 and D2).

840 μm). The droplet components were in situ cross-linked on the superhydrophobic surfaces in glutaraldehyde atmosphere, instead of immersion in diluted glutaraldehyde solution, to avoid premature leakage of the drug. This methodology provided chitosan microgels homogeneously cross-linked, without requiring further steps to remove the excess of glutaraldehyde. Microgels also containing SH or an Eudragit® variety can be considered as semi-IPNs (formulations 4–7 in Table 1) since only chitosan is cross-linked under the conditions of the process. The time required for the cross-linking was monitored by means of FTIR analysis (Fig. 2). Formation of the C=N bond of the imine group of the Shift base was seen as an increase in the absorption of the band at around 1634–1659 cm^{-1}, and thus this band was used as an index of the cross-linking of chitosan with glutaraldehyde (Kildeeva, Perminov, Vladimirov, Novikov, & Mikhailov, 2009). A second important band at 1570 cm^{-1} due to ethylene bonds (C=C) appeared after 3 h of treatment in glutaraldehyde atmosphere (Monteiro & Airoldi, 1999). An increase in the absorption region of the methylene groups (3000–2800 cm^{-1}) was also observed after 3 h of cross-linking, which is characteristic of the reaction of chitosan with glutaraldehyde (Kildeeva et al., 2009). In the 30 min to 4 h time frame, no typical band of the free aldehyde groups at 1720 cm^{-1} was detected. Only after 4.5 h of cross-linking a slight peak appeared at 1720 cm^{-1} due to valence vibrations of free aldehydic groups (Monteiro & Airoldi, 1999).

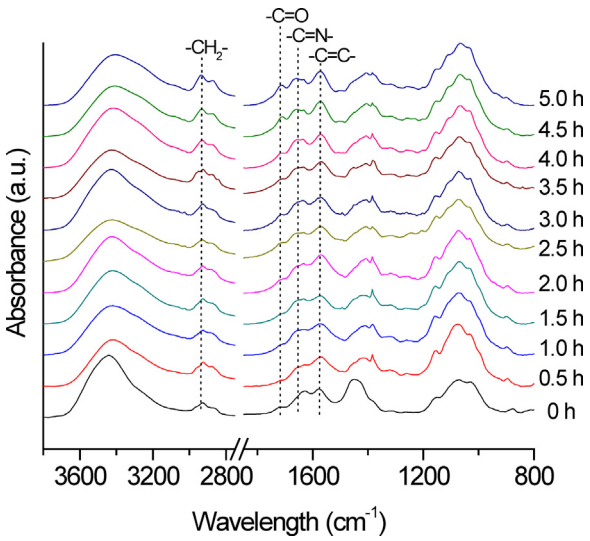

Fig. 2. FTIR spectra of chitosan microgels after different cross-linking time in a glutaraldehyde atmosphere. The bands that underwent the main changes are indicated in the plot.

Therefore, 4 h seems to be an adequate time for the cross-linking of chitosan without risk of incorporating free glutaraldehyde. Nevertheless, for subsequent studies, the microgels were exposed to glutaraldehyde atmosphere for 1 h and 4 h in order to evaluate the effect of this variable on their performance. DSC runs of non-loaded and 5-FU-loaded microgels did not show the characteristic melting peak of 5-FU at 285 °C, indicating that the drug intercalated inside the chitosan microgels at the molecular level.

Digital and SEM images (Fig. 3) confirmed the spherical shape and the typical yellow color of the microgels cross-linked for 4 h. SEM images taken under environmental conditions without staining revealed the smooth surface of chitosan 1.5% (Fig. 3 – 3A) and chitosan 1.5%-Eudragit® E PO 2.5% (Fig. 3 – 6A) microgels. The shape and the homogeneous structure of the particles were kept after drying under vacuum (Fig. 3 – 3B and 3C). By contrast, roughness and small pores were evidenced at the surface of the chitosan-SH microgels (Fig. 3 – 5A), which could be associated to a phase separation due to coacervation between these oppositely charged polymers (Vasiliu, Popa, & Rinaudo, 2005).

3.3. Preparation of coated microgels

The 5-FU-loaded chitosan microgels were subjected to two different coating treatments with drug-free layers of (i) chitosan that was cross-linked in glutaraldehyde atmosphere, or (ii) pectin subsequently cross-linked with $CaCl_2$. Chitosan microgels maintained the spherical shape after deposition of successive chitosan or pectin droplets for coating, and also after drying (Fig. 3 – 10B and 10C). Three different formulations were prepared with single, double or triple layers of chitosan using 1.5% polymer solution. To do that, drops of 5 μl of chitosan solution without drug were poured on vacuum-dried microgels placed on the superhydrophobic surfaces, cross-linked for 4 h in glutaraldehyde atmosphere and dried (formulation 8). The drop volume (5 μl) was sufficiently large for the complete wrapping of the microgels. This process was repeated once and twice more for obtaining doubly- and triply-coated microspheres respectively (formulations 9 and 10). Feret mean diameter of dried uncoated and triply-coated microgels was 280 ± 44 μm and 557 ± 29 μm, respectively. SEM of transversal sections showed a homogeneous continuous structure without defined limits between the core and the layers (Fig. 3 – 10B and 10C), which is explained by the fact that the coating solution is of the same nature as that used to form the seed microgels. Partial diffusion of the coating in the seed microgels can be expected, enabling a good binding; in fact, no delamination of the coated particles was observed during their handling.

Two varieties of pectin were tested for the coating; namely pectin from apple and pectin from citrus fruits with 77.5% and 84% content in galacturonic acid, respectively. Low methoxy-pectins, as the ones used in the present study, require the employment of calcium ions for the cross-linking (Sriamornsak, 2011). Ca^{2+} enhances the susceptibility of this polysaccharide to enzymes in the human large bowel, since bacteria require this ion for their activity (Liu et al., 2003). Once cross-linked with $CaCl_2$, the pectin-coated microgels were hardened in the glutaraldehyde atmosphere, since this treatment has been previously reported to improve the in vivo colon-specific drug release from calcium-pectinate beads (Das & Ng, 2010). In addition to the cross-linking, ionic interactions between the negatively charged pectin and cationic chitosan that may lead to polyelectrolyte complexes can contribute to delay the release of active compounds (Barakat & Almurshedi, 2011; Das, Chaudhury, & Ng, 2011). The homogeneous and clear calcium-pectinate shell enabled the visualization of the yellow chitosan

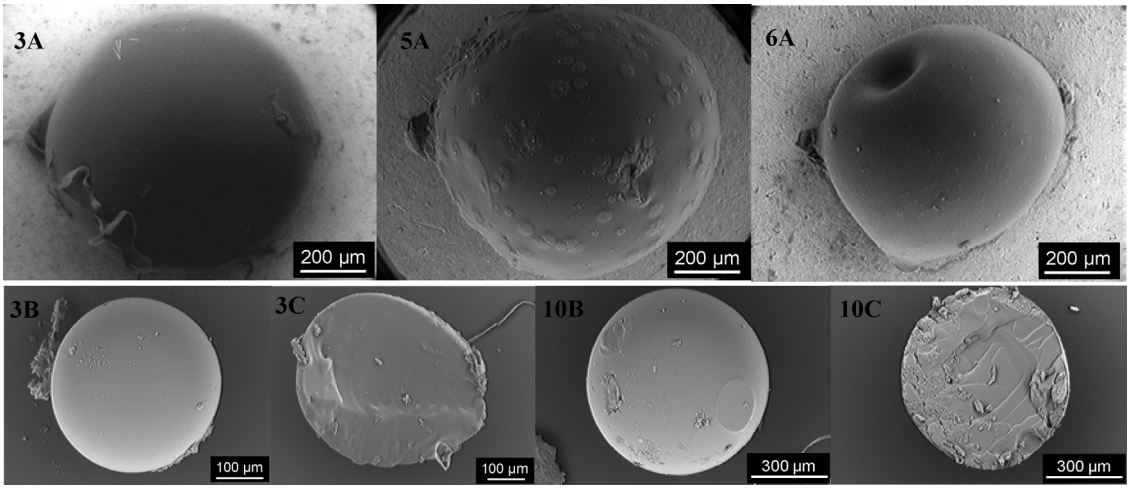

Fig. 3. SEM images under environmental conditions (A), and SEM images of dried microgels (B) and of their transversal sections (C). Numbers correspond to the formulations in Table 1.

core, from which the shell did not detach during handling. Apple pectin-coated and citrus pectin-coated microgels had Feret diameters of 561 ± 44 μm and 524 ± 50 μm.

3.4. Swelling

The changes in weight and diameter of freshly prepared non-coated microgels (i.e., not dried) when placed in water, HCl 1% and phosphate buffer pH 7.4 at room temperature were monitored for 6 h (Fig. 4).

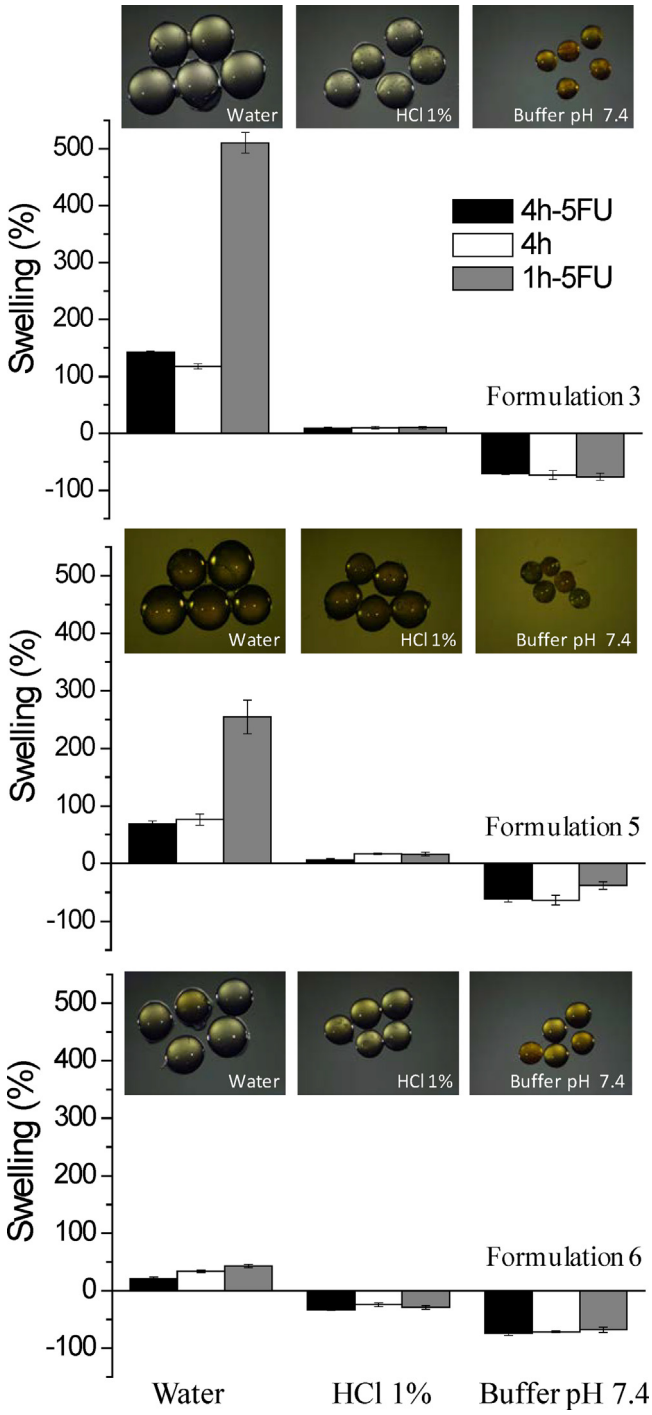

Fig. 4. Changes in swelling degree of chitosan microgels (formulations 3, 5 and 6) after 6 h in water, HCl 1% or phosphate buffer pH 7.4, and the corresponding digital images (all taken at the same magnification level).

In general, the degree of swelling was greater in water than in the other two media, but notable differences were observed depending on the composition of the microgels. Those prepared with chitosan solely and cross-linked for 1 h swelled in water more than any other formulation. The swelling in HCl 1% was smaller. It should be noted that chitosan microgels were prepared from dispersions in acetic acid 1% (v/v) and therefore, the polymer was already protonated. HCl 1% is also a good solvent for chitosan, but its greater ionic strength (compared to water) may be responsible for the partial shrinking (i.e., lower swelling compared to the initial diameter of microgels in acetic acid medium). No erosion was appreciated. It should be noticed that semi-IPNs of chitosan and Eudragit® E PO did not swell, but shrank in acid medium, which is explained by the low solubility in HCl of this Eudragit® variety. Interestingly, all microgels shrank in buffer pH 7.4, as the chitosan chains deprotonized. No significant effect of 5-FU on the swelling pattern was observed. During the whole length of the swelling study, the microgels did not disintegrate and kept their spherical shape (Fig. 4).

3.5. 5-FU release profiles

Drug release profiles from chitosan uncoated microgels (formulations 1–7 as in Table 1) in HCl 1% (pH 0.65), water and phosphate buffer pH 7.4 are shown in Fig. 5. Five microgels, each containing 0.0125 mg of 5-FU (encapsulation efficiency 100%), were immersed in 1 ml of medium (i.e., maximum release would correspond to 0.0625 mg/ml, well below drug solubility ~10–12 mg/ml). Drug release rate from microgels with the lowest proportion of chitosan (formulation 1) was faster in buffer pH 7.4, where the release was completed in 30 min, compared to water or HCl 1% (release completed in 1 h). This trend is quite the opposite to that observed for the swelling; i.e., the greater the swelling, the slower the release was. Changes in drug solubility as well as in the possible interactions with chitosan can explain this finding. In buffer pH 7.4, the solubility of the drug is greater than in acid medium (Singh, Singh, & Singh, 2005) and thus it could be squeezed out the microgels as they shrunk. As expected, microgels cross-linked for 1 h released the drug significantly faster than those cross-linked for 4 h (Table 2), since their mesh size should be quite large because of a smaller cross-linking density and greater degree of swelling (Fig. 4). In case

Table 2
5-Fluorouracil released after 15 min of assay in the different media tested. The time of cross-linking (1 h or 4 h) is indicated at the end of the codes of the microgels formulations. Mean values and, in parenthesis, standard deviations.

Formulation-cross-linking time	Water	HCl 1% pH 0.65	Phosphate buffer pH 7.4
1-1 h	19.29 (3.89)	76.58 (6.58)	61.47 (4.31)
1-4 h	31.43 (7.86)	37.00 (4.00)	46.06 (6.40)
2-1 h	33.27 (8.07)	72.15 (2.06)	67.34 (6.20)
2-4 h	34.81 (4.08)	57.02 (2.76)	59.72 (4.13)
3-1 h	19.49 (4.80)	38.44 (2.49)	43.40 (3.36)
3-4 h	28.84 (2.45)	32.25 (2.08)	32.95 (3.53)
4-1 h	70.67 (7.81)	27.04 (3.92)	83.22 (5.98)
4-4 h	46.74 (8.69)	30.65 (3.54)	35.03 (5.44)
5-1 h	23.02 (8.03)	18.47 (8.37)	76.13 (5.87)
5-4 h	79.93 (6.88)	20.89 (7.58)	77.97 (1.04)
6-1 h	32.04 (5.96)	46.83 (5.95)	35.97 (6.98)
6-4 h	47.71 (6.73)	34.13 (1.96)	34.66 (6.84)
7-1 h	63.46 (3.07)	33.78 (0.99)	81.54 (8.42)
7-4 h	28.85 (7.14)	38.77 (7.85)	85.40 (8.45)
8-4 h	8.98 (0.95)	31.25(5.30)	14.12 (6.72)
9-4 h	9.94 (2.43)	10.84(2.84)	12.89 (6.17)
10-4 h	7.83 (5.23)	13.80(1.74)	8.09 (3.14)
11-4 h	31.78 (24.04)	15.72 (6.42)	58.49 (6.35)
12-4 h	30.16 (15.34)	34.22 (5.47)	50.07 (14.18)

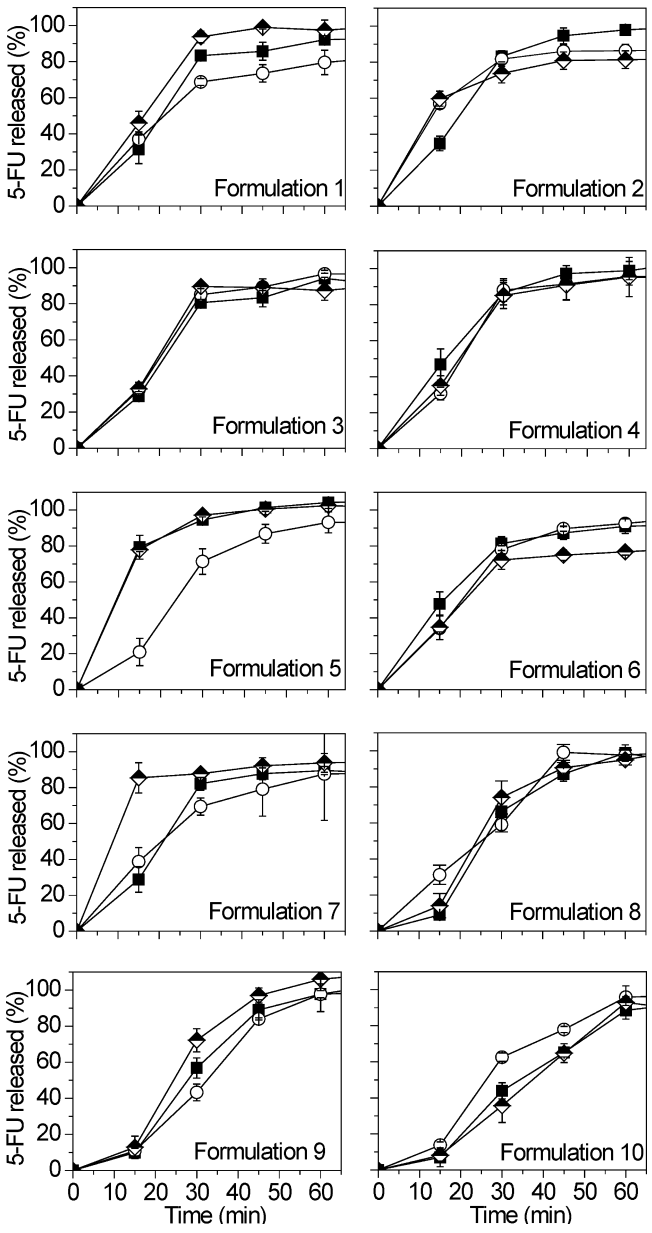

Fig. 5. 5-Fluorouracil release profiles in water (filled squares), HCl 1% (unfilled circles) and phosphate buffer pH 7.4 (semi-filled diamonds) from microgels cross-linked for 4 h. Formulation numbers as in Table 1.

of formulations 2 and 3, drug release profiles were quite similar in the three media.

The semi-IPN microgels based on chitosan and SH showed a similar behavior to those of chitosan solely, and the slower release was again observed in acid medium (formulation 5). The microgels containing the smallest proportion of SH showed a burst in water and in buffer pH 7.4. As the content in SH increased, the drug release rate became slower probably because of polyelectrolyte complex formation by both polymers (formulation 4). Semi-IPN microgels of chitosan and Eudragit® also showed pH-dependent release (formulations 6 and 7). Microgels cross-linked for 4 h sustained the release better in acid medium than in buffer pH 7.4, disregarding the Eudragit® variety. In water a strong effect of the cross-linking time was observed; those microgels prepared with chitosan and Eudragit® NE 30D cross-linked for only 1 h exhibited a very rapid release, which reveals that the chitosan mesh was not sufficiently small to retain the acrylic polymers. Thus, the microgels rapidly expanded in water (Table 2) and the acrylic polymer and 5-FU could abandon the insufficiently formed chitosan network. No differences were observed in the release profiles in water and in HCl 1% (Table 2). The percentage of 5-FU released at 15 min from the microgels in the three media is reported in Table 2. Non-coated chitosan 1.5% microgels (formulation 2) and chitosan/Eudragit® E PO semi-IPNs (formulation 6) provided a slightly more controlled release in any of the media than the other microgels. In view of these results, chitosan 1.5% microgels were chosen for further optimization of the release profiles by means of coating.

Coating with successive cross-linked layers of drug-free chitosan solution led to a delay in the release (Fig. 5, formulations 8–10). Interestingly, coating with only one layer of calcium cross-linked pectin led to remarkably lower 5-FU release rate, particularly at pH acid (Fig. 6, formulations 11 and 12) when pectin from apple was used. This pectin variety has lower methoxy content than pectin from citrus fruits and thus it can be cross-linked to a larger extent with Ca^{2+}, minimizing the entrance of HCl medium and thus rendering a more sustained release. By contrast, once in phosphate buffer at pH 7.4 fast swelling of the coating (disregarding the source of pectin) occurred, followed by a rapid release of the drug (Fig. 6, formulations 11 and 12). Weakness of the ionic cross-linking as well as of the interactions of the pectin layer with the chitosan core is behind the faster release observed as the pH increased (Das et al., 2011). This effect may be desirable for the colorectal cancer treatment in order to attain specific release in the site where the tumor is located. Previous reports on preparation of 5-FU-loaded chitosan microspheres prepared by combining the emulsification technique and a two-step solidification method using both glutaraldehyde and tripolyphosphate as cross-linking agents resulted in encapsulation efficiencies of 30–78% and more sustained release at pH 6 compared to pH 2 (Sun, Gu, Gao, & Gao, 2010). Therefore, coating with pectin of 100% 5-FU encapsulated chitosan microgels

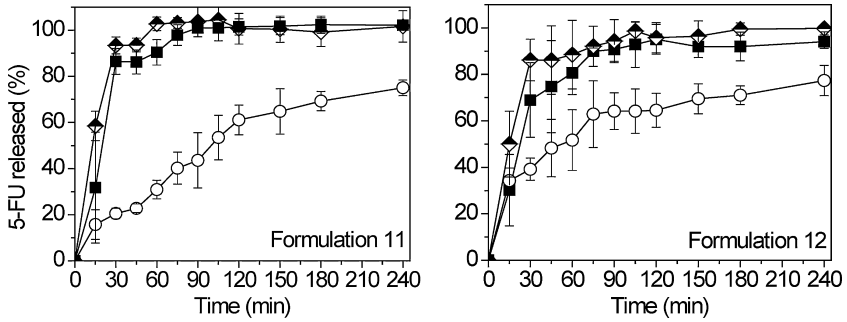

Fig. 6. 5-Fluorouracil release profiles in water (squares), HCl 1% (circles) and in phosphate buffer pH 7.4 (diamonds) from apple pectin-coated (formulation 11) and from citrus pectin-coated microgels (formulation 12).

appears as adequate approach to invert the pH-dependence of the drug release process. Long sustained release profiles of resveratrol previously observed for pectin/chitosan composite particles should be mainly related to the very low solubility of resveratrol (0.01 mg/100 ml), which is 5 orders of magnitude lower than that of 5-FU (Das et al., 2011). In our case, the high hydrophilic character of 5-FU is a challenge for obtaining prolonged release profiles since as the medium can enter in the formulation, the drug easily dissolves and diffuses out of the microgels. Complete prevention of 5-FU release at acid pH may require further optimization of the chitosan cores.

To gain an insight into the behavior of the shell of the microspheres, an erosion study was performed with pectin films. Calcium cross-linked apple-pectinate films eroded 73.0 (s.d. 1.3)%, 82.1 (s.d. 2.7)% and 91.6 (s.d. 2.5)% after their immersion in water, HCl 1% and phosphate buffer pH 7.4 respectively. On the other hand, citrus fruits–calcium pectinate films lost 82.0 (s.d. 5.7)% after immersion in water and completely disappeared in HCl 1% and phosphate buffer pH 7.4. As explained above, the differences in weight lost can be related to the different methoxy content of the pectin varieties: the higher the methoxy content, the lower the cross-linking extent and so the faster the erosion process. When orally administered, the small size of the microgels compared to the interdigestive pyloric canal (around 1–2 mm) may lead to short residence time in the stomach, and as a consequence pectin shell may still partially remain when enter in the gut and only a small fraction of drug is expected to be released in the stomach. Coating-free chitosan seeds may then contact directly with colonic cancer cells, taking advantage of their mucoadhesive properties.

3.6. Cytotoxicity

Since the pectin layer would disappear from the microgels in the gut and maybe not needed for other applications (e.g. dermal), cytotoxicity tests were carried out with non-coated chitosan 1.5% and chitosan 1.5%/Eudragit® E PO semi-IPN and triply-coated chitosan 1.5% microgels (formulations 2, 6 and 10, respectively). Triply coated chitosan microgels were included in the test because they represent the formulation processed for a longer time in glutaraldehyde atmosphere and thus the one that could cause more concerns regarding toxicity. Preliminary tests carried out with non-loaded microgels obtained from as-supplied chitosan and Eudragit® resulted in unacceptable cell toxicity levels. That was the reason of the purification step of chitosan and Eudragit® polymers before microgels preparation. The experiments were carried out with placebo and 5-FU-loaded (0.0125 mg) microgels against two drug-sensitive tumor cell lines, HeLa (epithelial adenocarcinoma of cervix) and SK-MEL-28 (malignant melanoma) (Paolino et al., 2008; Takara et al., 2002).

The microgels were placed in direct contact with the cells previously adhered to the bottom of 96-well plates. This methodology is considered to be more reliable than the indirect-contact method (Saw, Cao, & Ng, 2005). 5-FU solutions in DMEM cell culture medium were used as positive controls for HeLa cells at three concentrations: 2.4 μM was chosen as a concentration close to the IC_{50} (Takara et al., 2002), whereas 1.45 mM and 2.40 mM were selected as the drug concentration that, respectively, 3 or 5 microgels can provide to the cell growth medium after complete release. Growth inhibition of HeLa cells was studied after 6, 24 and 48 h in contact with the drug solutions and the microgels (Fig. 7). 5-FU solutions at the lowest concentration tested led to 53% cell growth inhibition at 48 h, i.e. close to IC_{50} as expected. Cell growth inhibition values of 78.1% and 83.3% were observed for 1.45 and 2.40 mM 5-FU, respectively. Chitosan microgels without 5-FU showed a low toxicity (cell growth inhibition of 28.4% and 30.1% after 48 h for three and five microgels, respectively). By contrast, the cell inhibition effect of

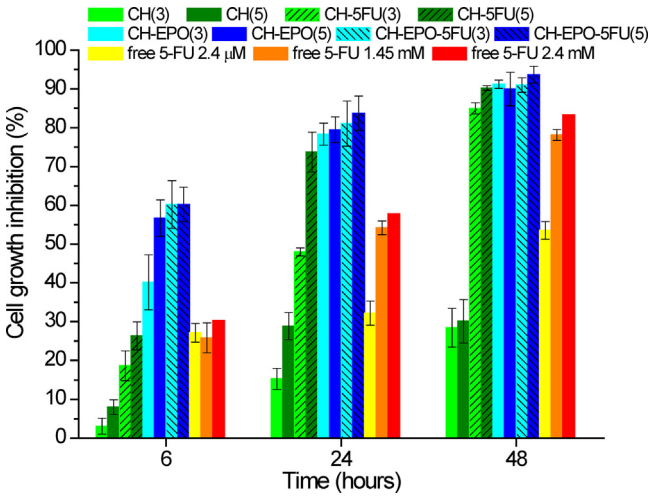

Fig. 7. Growth inhibition of HeLa cells caused by chitosan 1.5% (CH) and chitosan 1.5%-Eudragit® E PO 2.5% (CH-E PO) microgels (3 or 5 microgels per culture) with and without 5-FU 0.5%, and of free drug solutions.

5-FU-loaded chitosan microgels progressively increased as a function of time, being able to cause nearly 90% cell growth inhibition in 48 h. Thus, the 5-FU-loaded chitosan microgels resulted even more cytotoxic than the free drug. This could be associated with promotion of the anticancer effect of 5-FU by the chitosan, as previously observed (Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004). Since this cell line does not have P-glycoprotein in its membrane, removal of the drug by efflux from the inside of the HeLa cells is not expected. Thus, the whole amount of drug encapsulated inside the microgels would be available for causing cytotoxic effects against the cancer cells. On the other hand, chitosan-Eudragit® E PO microgels resulted to be quite toxic by themselves and even more when loaded with 5-FU, leading to more than 50% cell growth inhibition in the first 6 h. After 48 h in direct contact with HeLa cells, both non-loaded and drug-loaded chitosan-Eudragit® E PO microgels showed a cell growth inhibition close to 90%.

SK-MEL-28 cell line was used to mimic the basal skin carcinoma that can be treated with topically administered 5-FU formulations (Ceilley & Del Rosso, 2006). Furthermore, 5-FU is used in solution for the treatment of actinic keratosis or precursor lesions related to melanoma in order to avoid their progression (Paolino et al., 2008). In the cytocompatibility assay carried out with SK-MEL-28 cells, only 5000 cells were seeded per well and 5-FU concentration of 10 μM was used as IC_{50}, following previously reported protocols (Paolino et al., 2008). Cell growth inhibition of the anticancer solution in EMEN culture medium with a concentration of 10 μM was 54.2% after 72 h, which is in agreement with previous reports (Paolino et al., 2008).

Placebo microgels exerted low cytotoxic effect, with cell growth inhibition at 72 h of 24.5% and 29.9% for 5 uncoated and triply coated microgels, respectively (Fig. 8). According to Lim and coworkers, a polymer can be considered cytocompatible if the cell growth inhibition is below 30% after 72 h, compared to the control (Lim, Yaacob, & Halim, 2010). Taking this in mind, placebo uncoated and triply coated chitosan microgels may be considered cytocompatible. 5-FU-loaded chitosan microgels caused greater cell growth inhibition of SK-MEL-28 cells than the free drug at all time points (82.9% and 91.1% for 3 and 5 microspheres versus 73.7% and 78.4% for 5-FU solutions). Triply coated microgels showed the same effect; the cytotoxic effect of the antitumoral drug being promoted by the polysaccharide. Chitosan and Eudragit® E PO semi-IPN microgels

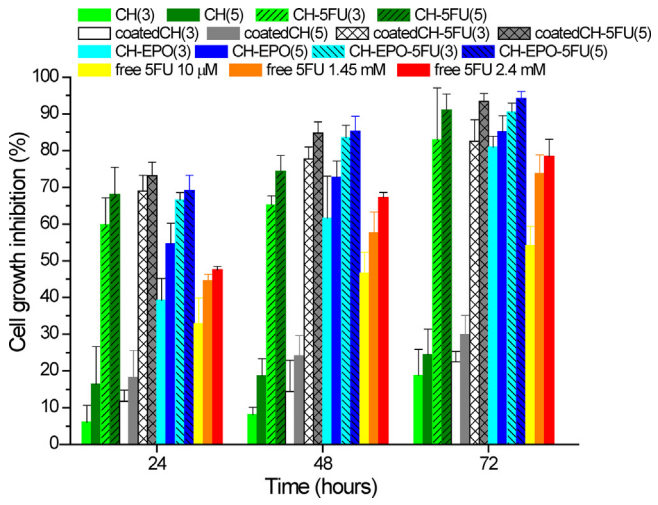

Fig. 8. Growth inhibition of SK-MEL-28 cells caused by chitosan 1.5% (CH), triply-coated chitosan 1.5% (CH coated) and chitosan 1.5%-Eudragit® E PO 2.5% (CH-E PO) microgels (3 or 5) with and without 5-FU 0.5%, and of free drug solutions.

resulted cytotoxic for SK-MEL-28 cells, although to a lesser extent than in the case of HeLa cell line.

4. Conclusions

The bioinspired methodology based on the use of superhydrophobic surfaces is suitable for environmental-friendly preparation of microgels with a variety of polysaccharides, such as chitosan, sodium hyaluronate or pectin, or acrylic copolymers (for instance different Eudragit® varieties) that can be used in the drug delivery field. Microgels containing the antitumoral drug 5-FU with an encapsulation efficiency of 100% had diameters in the 200–600 μm range, which is the lowest size obtained until now using the methodology of the superhydrophobic surfaces. The microgels showed pH-dependent release rate, being slower at acid pH than at pH 7.4. Coating with pectin of microgels on the superhydrophobic surfaces prevented premature leakage of the drug during the process as it occurs in the solid/air interface, and compared to classical coating methods (e.g. spraying) requires less consumption of energy and materials. Pectin coating improved the controlled release of 5-FU in acid medium. Cytotoxicity studies performed with epithelial adenocarcinoma of cervix and malignant melanoma cells revealed that the microgels promoted the cytotoxic effect of 5-FU. In sum, microgels obtained could be useful for mucosal and topical treatments and also, in case of pectin-coated microgels, for the site specific delivery of antitumor agents to colon cancer cells.

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