Carbohydrate Polymers 101 (2014) 113–120

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Water soluble folate-chitosan nanogels crosslinked by genipin

Maite Arteche Pujana, Leyre Pérez-Álvarez*, L. Carlos Cesteros Iturbe, Issa Katime

Grupo de Nuevos Materiales y Espectroscopia Supramolecular, Departamento de Química Física, Facultad de Ciencia y Tecnología, Universidad del País Vasco (UPV/EHU), 644 Bilbao, Spain

ARTICLE INFO

Article history:
Received 18 May 2013
Received in revised form 3 September 2013
Accepted 5 September 2013
Available online 16 September 2013

Keywords:
Genipin
Chitosan
Folate
Nanogels

ABSTRACT

Folate-chitosan conjugates were prepared by a concurrent functionalization and crosslinking reaction with the natural crosslinker genipin. Genipin molecule was employed simultaneously as crosslinker agent and spacer molecule in order to allow the functionalization with folic acid for active tumor targeting. The reaction was carried out in reverse microemulsion which provided colloidal size and monodisperse particle size distribution. The water solubility of the obtained folate-genipin-chitosan nanogels was studied as function of the pH of the medium and all nanoparticles were totally dispersible at physiological pH. The enzymatic degradability of the nanogels in a lysozyme solution was evaluated at acidic and physiological pH. QELS analyses of the swelling behavior of the nanogels with the pH did not show a clear pH-sensitivity. However, the study on the loading and release capacity of 5-fluorouracil revealed an interesting pH-responsive behavior of the nanogels that makes them promising as nanodevices for targeted anticancer drug delivery.

1. Introduction

Since some specific antigens or receptors are overexpressed on the surface of cancer cells, the selectivity to tumor tissues of nanocarriers can be improved by coupling with specific targeting agents due to their preferential interaction with tumor cells. Folic acid is a low molecular weight (441 Da) vitamin whose receptors have been identified as tumor markers. Thus, folic acid has been extensively employed as a targeting moiety for various anticancer drugs through covalent conjugation with liposomes, radio imaging agents, gene transfer vectors and microgels, which have shown to be efficiently internalized within tumor cells via folate receptor-mediated endocytosis (Blanco et al., 2010; Wang & Low, 1998).

In addition, numerous reports have demonstrated that during the endocytosis process a change of pH takes place from the physiological pH of 7.4 to pH of 5.5–6.0 in endosomes and approaches pH 4.5–5.0 in lysosomes (Lee, Wang, & Low, 1996). Therefore, site-specific release within tumor cells can be achieved by mean of nanogels that show specific pH-sensitive behavior swelling in response to a lower pH. In order to this, nanogels with basic ionizable functional groups in their polymeric structure, such as chitosan, can be exploited.

Chitosan is a linear and partly acetylated (1–4)-2-amino-2-deoxy-β-D-glucan isolated from marine chitin (Muzzarelli et al., 2012). The glucosamine units are protonated at pH < 6.5 and chitosan behaves as a soluble cationic polyelectrolyte in dilute acidic solutions (Tommeraas et al., 2002). This fact is the origin of the potential pH-responsive swelling behavior of covalently crosslinked chitosan. It is well known that chitosan is a potentially useful pharmaceutical material due to its good biocompatibility, biodegradability and nontoxicity, and many favorable properties such as mucoadhesive nature, immune stimulating properties and inhibiting tumor growth (Illum, 1998; Xia, Liu, Zhang, & Chen, 2011). Additionally, chitosan can be made into a wide variety of physical forms, namely films, composites, or micro and nanogels. For all these reasons, chitosan is one of the most promising biodegradable polymers in biomedical applications (Busilacchi, Gigante, Mattioli-Belmonte, Manzotti, & Muzzarelli, 2013).

For most of the drug delivery applications chitosan should be crosslinked in order to obtain stable matrixes. Various crosslinking reagents used to crosslink covalently chitosan gels are bifunctional molecules such as PEG dicarboxylic acid, epoxy compound and glutaraldehyde (Yang & Yuan, 2001). However, since glutaraldehyde is highly cytotoxic, (Beauchamp, St Clair, Fennell, Clarke, & Morgan, 1992; Muzzarelli, 2009; Sung, Huang, Huang, & Tsai, 1999) many investigations have focused on finding out suitable crosslinking agent for chitosan use in biomedical applications (Arteche, Pérez-Álvarez, Cesteros, & Katime, 2012)

Genipin is an iridoid glucoside extracted from the fruits of Gardenia jasminoides. This compound can spontaneously react with primary amine groups to form stable crosslinks and it is about 5000–10,000 times less cytotoxic than glutaraldehyde (Sung, Huang, Huang, & Tsai, 1999).

* Corresponding author. Tel.: +34 946012711; fax: +34 946013500.
E-mail address: leyre.perez@ehu.es (L. Pérez-Álvarez).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.09.014

The aim of this work is preparing chitosan nanogels crosslinked with genipin that are functionalized with folic acid by an alternative way to the traditional direct linkage of folic acid to chitosan, where genipin acts as spacer molecule in order to improve cellular association.

2. Experimental part

2.1. Materials

Low molecular weight chitosan was purchased by Aldrich and purified as previously described (Kasaai, 2010). The average deacetylation degree of chitosan determined by ^{1}H NMR (Kasaai, Arul, Chin, & Charlet, 1999) was 79% and the viscosity average molecular weight measured by an Ubbelohde capillary viscometer (HAc 0.3 M/NaAc 0.2 M, 25 °C) (Rinaudo, Milas, & Dung, 1993) was 66,000 g/mol. Genipin, folic acid, triton X-100, 1-ethyl-3-(3-dimethylaminopropyl carbodiimide hydrochloride) (EDC) and *N*-hydroxysuccinimide (NHS) and 5-fluorouracil were provided by Sigma–Aldrich. The following solvents and solutions were used as received: Cyclohexane (for synthesis, 98%, Panreac), acetic acid (for analysis, 99.8%, Panreac), hexanol (for synthesis, 98%, Sigma–Aldrich), Folin Ciocalteu reagent solution (Sigma–Aldrich), sodium hydroxide (2 N, Panreac), hydrochloric acid (0.1 N, Panreac) and ethanol (for analysis, 96%, Panreac). Sodium carbonate (Merck), lysozyme (from hen egg white, Fluka) and potassium ferricyanide (Sigma–Aldrich) were also employed.

2.2. Folic acid modification

Folic acid was modified in order to obtain $-NH_2$ functionality. This modification was carried out with the previously synthesized monoprotected hydrophilic diamine: *N-tert*-butoxycarbonil-2,2-(ethylenedioxy)bis(ethylamine), (EDEA-BOC). Synthesis of EDEA-BOC molecule was carried out to bind the folic acid molecules to the genipin molecule monolinked to the chitosan backbone, following the procedure previously described by Sahu et al. (2010).

Modification of folic acid was conducted in three steps. Firstly, folic acid was activated using EDC and NHS in a molar ratio of 1:10:4 at pH 6.2 for 4 h. Secondly, activated folic acid was reacted with monoprotected EDEA-BOC during 24 h. Thirdly, in order to remove the protecting group from terminal amino group, the mixture was kept stirring in acidic medium (pH = 1) during 1 h. Then, the pH of the final reaction mixture was restored by adding NaOH solution. This final reaction mixture was employed as aqueous phase in the preparation of modified folic acid microemulsion (MF).

2.3. Preparation of folate-genipin-EDEA-chitosan nanogels

Folate-genipin-EDEA-chitosan nanoparticles also crosslinked with genipin were prepared by a procedure based on three microemulsions: chitosan microemulsion (MC), genipin microemulsion (MG) and modified folic acid microemulsion (MF). The microemulsions were prepared as follows: cyclohexane, *n*-hexanol and aqueous solution (chitosan, genipin or modified folic acid) were mixed in a flask in a fixed ratio of 2.75:1:1 (v/v). The aqueous solutions of MC and MG were previously prepared dissolving separately 1 g of chitosan in 100 mL of 1% v/v aqueous acetic acid solution and 1 g genipin in 540 mL respectively. Then, the microemulsion was formed by adding Triton X-100 drop by drop into the mixture while vigorous stirring until the mixture became transparent. MC and MF were mixed previously and then MG was added under magnetic stirring. Nanoparticles were separated from microemulsion by precipitating process with ethanol and the obtained nanogels were washing several times with ethanol and phosphate buffer solution (pH = 7.4). Eventually, nanogels were ultrafiltered and vacuum dried.

2.4. Characterization of nanogels

The nanogels were analyzed with ^{1}H NMR spectroscopy. NMR samples were prepared dispersing the nanogels (2% w/w) in CD_3COOD/D_2O. The spectra were recorded on a Bruker Avance 500 MHz instrument. Chemical shifts (δ) are reported relative to water ($\delta = 4.8$).

TEM observations were carried out using a Philips CM120 with Olympus SIS Morada digital camera. A drop of nanogels solution (1 mg/mL) in 1% v/v aqueous acetic acid solution was placed on a copper grid coated with carbon film and was dried by glow discharge.

Solubility of the nanogels at different pH values was measured by UV–vis spectroscopy. Nanogels were dissolved in 1% HAc solution (1 mg/mL) and the pH of the solution was adjusted adding 2 N NaOH solution. The transmittance of the solution was evaluated at 750 nm.

The quantification of bounded folic acid was also carried out by UV spectroscopy according to the previously reported Folin-Ciocalteu method (Rao, Kanjilal, & Mohan, 1978; Singleton, Orthofer, & Lamuela-Raventos, 1999). 25 mg of chitosan nanogels were dissolved in 3 mL of distilled water. Briefly 0.1 mL of nanogels solution were mixed with 7 mL of water and then 0.5 mL Folin-Ciocalteu reagent solution, (2 N). The mixture was incubated during 8 min at room temperature and then, 1.5 mL of sodium carbonate, solution (0.5 M) was added. The mixture was stored in dark at room temperature during 2 h. After this, the nanogels were separated from the mixture by centrifugation and the absorbance of supernatant solution was measured. Folic acid solutions in the specific concentration range were used to construct a calibration curve and the determined equation of the regression line was $A = 0.01293 + 0.7462\ X$ ($R^2 = 0.9951$), where A is the measured absorbance and X the concentration of folic acid (mg/mL).

X-ray diffraction patterns were obtained by a Transmision STOE Stadip X-ray diffractometer with a goniometer speed of 0.1°/90 s. The range of diffraction angle 2θ was 2.5–60°.

The hydrodynamic size of the nanogels and its dependence on the pH was examined by dynamic laser light scattering (Brookhaven BI-9000AT with an argon-ion laser operating at 514.5 nm and 522 channel digital correlator) at room temperature. The samples were prepared in Milli-Q water (1 mg/mL) under different pHs by addition of NaOH (2 N) or HCl (0.1 N) solutions. The obtained homogeneous dispersions were then diluted until the concentration was 40 mg/L.The size distribution was obtained by NNLS analysis.

The zeta potential of nanogels solutions was measured with a zeta potential analyzer (Zeta-Sizer IV, Malvern Instruments). After dispersing chitosan nanogels in acid water solution (pH = 4), the pH was adjusted by adding of 2 M NaOH solution until the final chitosan concentration was 40 mg/L and the electrophoretic mobility of each sample was measured.

2.5. In vitro release

Samples of 10 mg of nanogels were suspended in 10 mL of 5-fluorouracil solution (0.01 mg/mL) at pH = 7.4. Then, the solution was separated by centrifugation and the free 5-fluorouracil amount was estimated by the measurement of the absorbance at 264 nm. The entrapment of 5-fluorouracil into the nanogels was enhanced by the swelling of the networks when the pH was decreased until pH = 5.0. Then, the solution was removed by centrifugation and the loaded nanogels were washed with phosphate buffer at pH = 7.4

in order to eliminate the superficial drug. The free 5-fluorouracil amount delivered at pH = 5.0 and pH = 7.4 was estimated by UV–vis measurement in the corresponding supernatant solutions.

2.6. Degradation of nanogels

A known amount (10 g) of the dried nanogels were placed in amber vials containing 0.5 mg lysozyme (75,400 U/mg) and 0.5 mg NaN_3 in (10 mL) phosphate buffer solution (pH = 7.4) or acetate buffer solution (pH = 5.5), and were incubated at room temperature for 1 day. Then, the dispersion was centrifuged (9000 rpm, 3 min, 25 °C), and the supernatant was collected. The volume of the media was kept constant by adding fresh lysozyme solution after sampling. The process was repeated until the mass of the nanogels could not be visually observed. The amount of chitosan chain ends in the supernatant was determined by reducing sugar analysis following the procedure reported by Imoto (Imoto & Yagishita, 1971) after minor modifications. 0.5 g potassium ferricyanide were dissolved in 1 L of 0.5 M sodium carbonate. 4.0 mL of this color reagent solution were mixed with 3.0 mL of supernatant solution and incubated in boiling water. After cooling, the absorbance at 420 nm was measured.

3. Results and discussion

3.1. Preparation and characterization of folate-genipin-chitosan nanoparticles

The crosslinking reaction of chitosan with genipin and its functionalization with folic acid were simultaneously carried out by a multistep modification process. Thus, as described above, folic acid was modified with EDEA-BOC to obtain folate-NH_2 in order to avoid the direct linkage to the chitosan backbone. Then, folate-NH_2 was linked to genipin spacer molecule by the introduced primary amine moiety (Fig. 1). In all samples the folic acid content was fixed and genipin molar percentage in reaction mixture was varied.

The apparition of dark blue pigment (λ_{max} = 605 nm) associated with chitosan-genipin bonding was corroborated by UV–vis

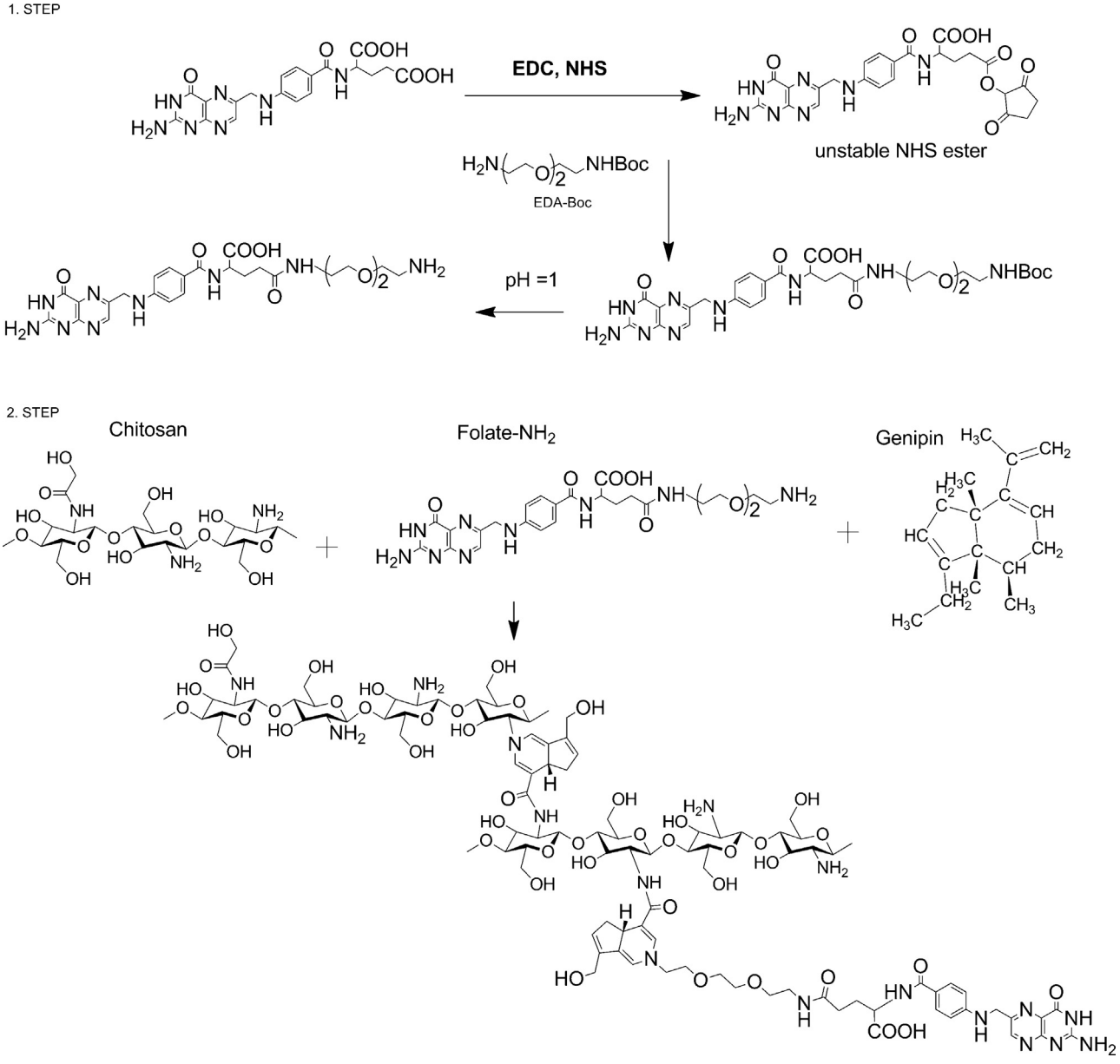


Fig. 1. Synthetic sequence of genipin-folate modification and crosslinking with genipin of chitosan.

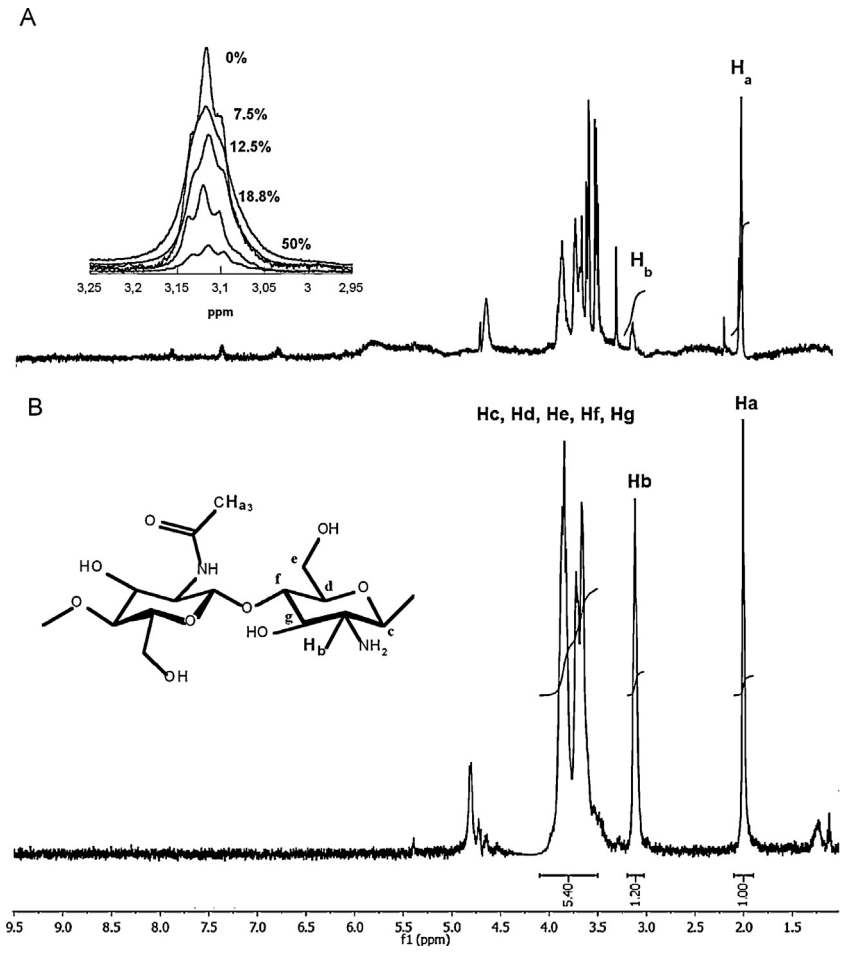


Fig. 2. ^{1}H NMR spectra of (A) folate-EDEA-genipin-chitosan, and amplified resonance signal corresponding to the H_b protons (chitosan H-2 of GlcN) of nanogels crosslinked with different genipin feeds, (B) chitosan.

spectroscopy, and the functionalization-crosslinking reaction time was fixed in 8 days.

Folin-Ciocalteu reactant was used to estimate the amount of folic acid incorporated to chitosan by a colorimetric method. A grayish color is developed when folic acid and Folin-Ciocalteu phenol reagent are mixed. This coloration presents an absorbance maximum at 760 nm, which does not overlap with the absorption band of genipin moieties. The obtained results are shown in Table 1. As expected, the efficiency of the incorporation of folic acid improved as the content of genipin in the microemulsion mixture was higher.

^{1}H NMR spectroscopy also provided the confirmation that the modification of chitosan had taken place. Fig. 2 shows ^{1}H NMR spectra of prepared nanogels in comparison with pure chitosan. Despite the low resolution of these spectra in the low frequency region, the qualitative assessment of the folic acid incorporation could be done by the appearance of resonance signal at 6.6–7.7 ppm (Fig. 2A)

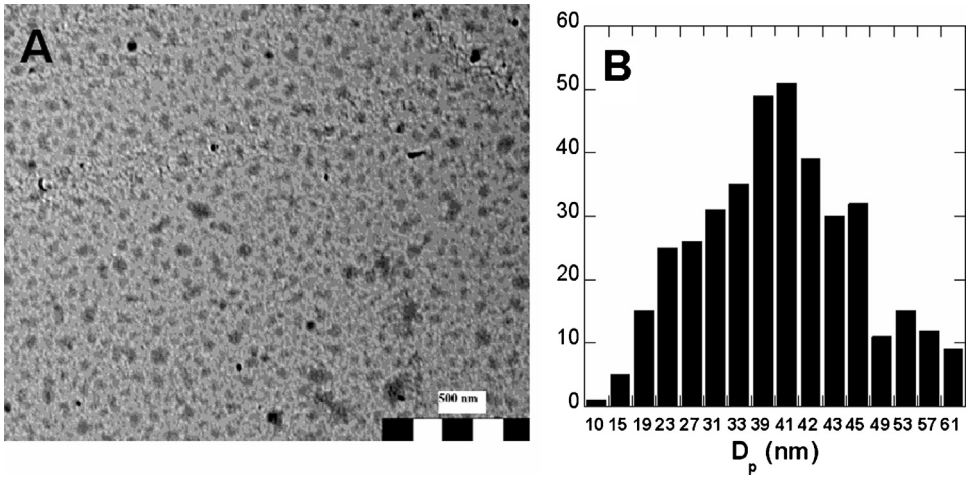


Fig. 3. (A) TEM micrograph and (B) particle size distribution of folate-EDEA-genipin-chitosan nanogels (12.5% mol genipin, 10% mol folic acid).

Table 1
Modification, crosslinking degree of folate-EDEA-genipin-chitosan nanogels and pH_{50} values.

Genipin initial feed (mol. %)	Folic acid initial feed (mol. %)[a]	Modification degree (mol. %)[b]	Crosslinking degree (mol. %)	Folic acid (mol. %)[c]	Yield of folic acid linkage (%)
7.25	10	13	5.5	2.0	30
12.50	10	23	10.0	3.0	54
18.75	10	31	14.1	2.8	51
50.00	30	65	27.0	11.0	74

[a] With respect to the total amount of amine primary in pure chitosan.
[b] Determined by ^{1}H NMR.
[c] Determined by UV–vis.

which may correspond to the aromatic protons of folic acid (Sahu, Mallick, Santra, Maiti, Ghosh, & Pramanik, 2010). In all samples a intense signal was observed at 3.2–3.5 ppm which corresponds to the resonance of the alkyl protons of the introduced EDEA.

The quantitative determination of the modification degree of chitosan was done by the decrease of the signal of H-2 proton (3.1–3.2 ppm), which is characteristic of the GlcN residues, with respect to the signal due to the three protons of *N*-acetyl glucosamine (GlcNAc) at 2.1 ppm (Kasaai, 2010). The obtained data are summarized in Table 1 and showed good correlation between genipin initial feed and final modification degree of the nanogels.

The results of ^{1}H NMR and UV–vis studies were used to estimate the crosslinking degree of the prepared nanogels (crosslinking degree = (mod. degree-folic acid)/2, Table 1).

The morphology and particle size of the prepared chitosan nanogels in the dried state was studied by TEM and are illustrated in Fig. 3. The nanogels had a similar average mean diameter of 35–45 nm. Therefore, we found that the method of combining simultaneously crosslinking with genipin and functionalization with folic acid is appropriate in preparing chitosan nanoparticles with a rather uniform size distribution. Although many authors have used genipin to crosslink macroscopically chitosan, only a few reports have focused in the nanometric scale (Arteche, Pérez-Álvarez, Cesteros, & Katime, 2013; Choubey & Bajpai, 2010; Maggi, Ciccarelli, Diociaiuti, Casciardi, & Masci, 2011).

The particle size of swollen nanogels was studied by QELS. The *z*-average of the hydrodynamic diameters of the nanogels (polidispersity index = 0.6) when the pH was varied is shown in Fig. 4. In order to reduce the presence of aggregates (Anthonsen, Varum, Hermansson, Smidsrod, & Brant, 1994), the sizes of the dispersed nanogels were measured after progressive dilution, as have been proposed by other authors to reduce the inter-particle interaction (Sorlier, Rochas, Morfin, Viton, & Domard, 2003). The expected

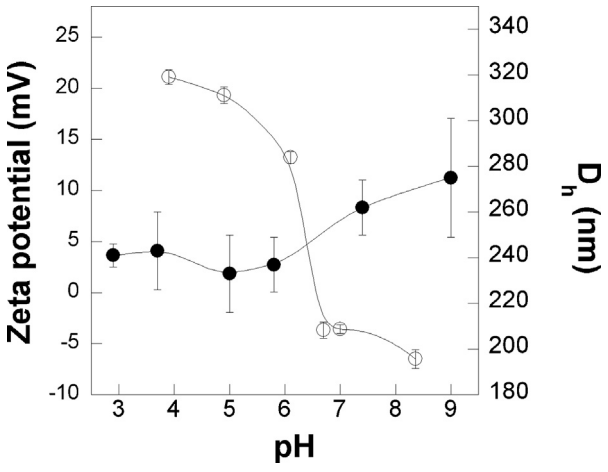


Fig. 4. (●) Average hydrodynamic diameter (D_h) (○) and zeta potential of nanogels as function of external pH (for 12.5 mol. % genipin).

pH-responsive swelling behavior of chitosan networks was not observed. At acidic pH (<5.5) the nanogels seem to swell slightly as the pH decreased. This tendency could be related to the ionization of free amine groups of chitosan backbone, which results in the swelling of the network. Certainly, zeta potential measurements confirmed that the progressive protonation of amino groups when the pH decreases takes place (Fig. 4). The value of the zeta potential increased from (−7)-(−5) to 20–22 mV when the pH was varied from 8 to 3.

On the other hand, as consequence of the rigid and compact nature of the networks due to the crosslinking with genipin, the negative effect of the elastic contribution seems to strongly influence the swelling equilibrium of the nanogels, and reduce the swelling capacity of the networks even in the ionized state.

On the contrary, when the pH was higher than 5.5, although the electrical state of the nanogels remained constant, particle size increased. It is well known that chitosan nanoparticles tend to form aggregates, so, this effect might be due to the particle aggregation resulting in a higher average hydrodynamic diameter than the real particle size of the particles (Wu, Zhou, & Wang, 1995). We also reported this swelling profile with the pH when the swelling properties of genipin-chitosan nanogels were studied (Arteche et al., 2013).

3.2. Water solubility

One of the main drawbacks of chitosan is that it is only water soluble at acidic pHs, which limits its biomedical applications. As has been extensively studied, the main explanation of chitosan insolubility is the structured network of intra/intermacromolecular hydrogen bonds that lead to the chitosan chains to be held together (Nishimura, Kohgo, Kurita, & Kuzuhara, 1991). The destruction of these intra/intermolecular hydrogen bonding interactions leads to a decrease in chitosan crystallinity, and results in an improvement of its water solubility (Vårum, Ottoy, & Smidsrød, 1994). In this way, the crystallinity and solubility of the nanogels prepared in this work could be potentially affected by the modification with folic acid and genipin.

Certainly, when the X-ray diffraction pattern of the functionalized chitosan nanogels (Fig. 5) was compared with that of the pure chitosan, it can be observed that the chemical modification resulted in the loss of crystallinity in the samples.

The water solubility was quantified by the pH_{50} parameter, which is defined as the pH when the transmittance measured at 750 nm decreased by half. The pH_{50} values measured for synthesized folate-EDEA-genipin-chitosan nanogels are shown in Fig. 6.

Taking into account the pH_{50} values previously reported for genipin-chitosan nanogels (Arteche et al., 2013), it is noted that the functionalization with folic acid improves greatly the solubility of all samples, and nanogels full water soluble were obtained at physiological pH. It should be remarked the interest of this fact due to the general insoluble character of previously reported genipin-chitosan nanogels (Arteche et al., 2013). The differences may be

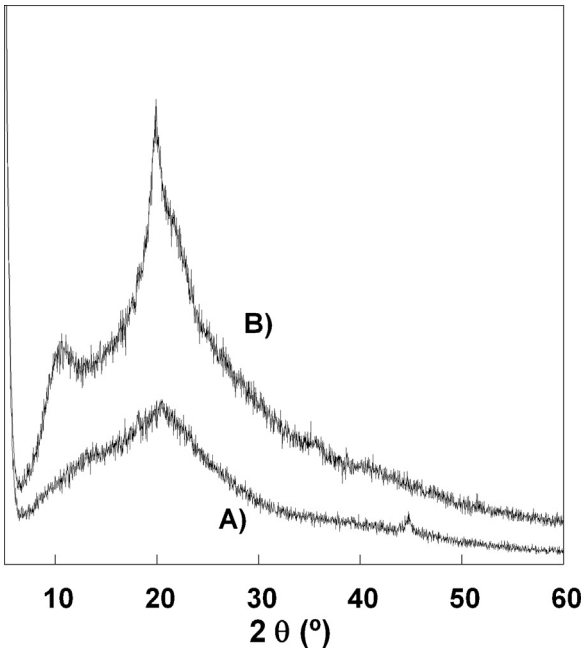
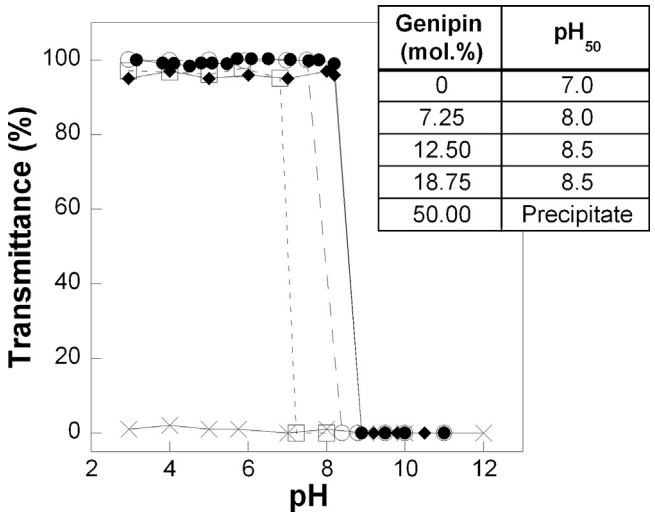


Fig. 5. X-ray diffraction pattern of (A) folate-EDEA-genipin-chitosan nanogels and (B) pure chitosan.



Genipin (mol.%)	pH_{50}
0	7.0
7.25	8.0
12.50	8.5
18.75	8.5
50.00	Precipitate

Fig. 6. Transmittance variation with external pH for (□) pure chitosan and, folate-genipin-chitosan nanogels with (○) 7.25, (•) 12.50, (♦) 18.75 and (×) 50 mol. % of genipin in feed (1 mg/50 ml in 1% HAc solution).

explained by changes in the regular intra/intermolecular hydrogen bonding organization. The random distribution of larger and hydrophobic pendant groups among amino moieties may lead to lose packing arrangement that might facilitate water molecule penetration.

Additionally, the functionalization with folic acid provides monolinked ligands and leads to more flexible and open networks than those of the unfunctionalized genipin-chitosan nanogels. All this, may provide improved water solubility properties.

3.3. *Effect of pH on biodegradation and in vitro drug release*

In view of the interest of chitosan in drug delivery research, it is essential to evaluate the degradation of chitosan and its chemically modified derivatives. Lysozyme is an enzyme which is found in various body fluids and tissues. This enzyme has β-(1–4)-glycosidic linkages of the polysaccharide backbones of chitin or chitosan as natural substrates and its degradation products are nonimmunogenic and noncarcinogenic (Rao & Sharma, 1997). For this reason, several investigations on the degradation of chitosan based macrosystems have been reported (Han, Nwe, Furuike, Tokura, & Tamura, 2012; Martins, Pereira, Leonor, Azevedo, & Reis, 2009). However literature on chitosan nanogels degradation has yet not been reported.

When chitosan are degraded by lysozyme, the cleavage of β-(1–4)-glycosidic bonds takes place, and chitosans with lower molecular weight, chitoligomers, and *N*-acetyl-D-glucosamine residues, are released into the degradation medium. In this report, the colorimetric analysis method published by Imoto and Yagishita (1971) was used to estimate the reducing sugar content in the supernatant of nanogels dispersion that underwent lysozymic degradation, as has been above described.

Fig. 7A presents the kinetic of the degradation process of the nanoparticles at physiologically healthy and endosomic pH conditions. As long as the breakage of β-(1–4)-glycosidic bonds takes place, the amount of reducing groups increases. The degradation of the nanogels was monitored until no increase in the reducing sugar content was determined and visually, the mass of nanogels

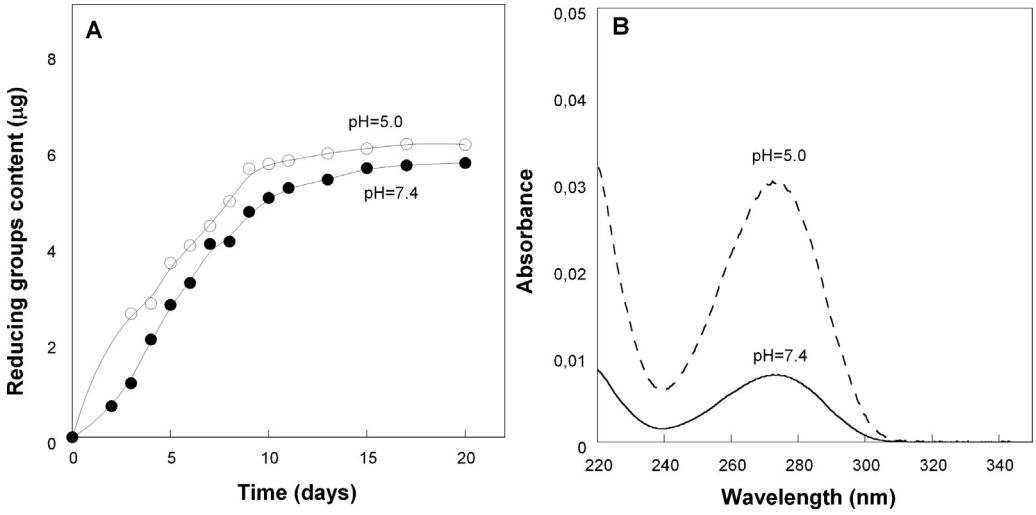


Fig. 7. (A) Biodegradation kinetic and (B) 5-fluorouracil release profile from folate-EDEA-genipin-chitosan (37.5% genipin).

disappeared. According to these experimental data the half-life of the nanogels can be estimated to be about 10 days.

It has been reported that the specificities of different lysozymes with respect to chitosan with different deacetylation degree were indistinguishable and largely independent on pH (Nordtveit, Vårum, & Smidsrød, 1996). However, as can be observed in Fig. 7A, the nanogels has a slightly faster degradation rate at pH = 5.5 than at pH = 7.4. This could be ascribed to the ionizated state of the nanogels at endosomic pH. Nanogels at acidic pHs undergoes more rapid water adsorption and their slightly higher swelling make them more accessible to lysozyme action.

Drug loading efficiency was evaluated in the ionized (pH = 5.0) and collapsed state (pH = 7.4) in order to estimate the drug loading capacity as consequence of the slight pH-responsive swelling of the nanogels. The percentage of drug loaded (total drug-free drug/total drug) at pH = 7.4 for samples of folate-EDEA-genipin-chitosan (18.8% crosslinking, 10% folate) was 1.15 mg/g. Certainly, when the pH of the incubation medium decreased until pH = 5.0, the total amount of encapsulated 5-fluorouracil increased until 1.94 mg/g, due to the swelling of the networks. This result verifies the pH-responsive swelling behavior of the nanogels that could not be evidenced by QELS. Then, nanogels suffered successive washing and centrifugation cycles in PBS buffer (pH = 7.4), so that the final drug loaded after purification was 1.01 mg/g.

Release of 5-fluorouracil from the nanogels was qualitative observed (Fig. 7B) at pH = 5.0 and pH = 7.4, indicating again a higher concentration of free drug at the endosomic pH than at healthy physiological conditions. The estimated 5-fluorouracil released amount as consequence of the change of the pH of the medium was 0.49 mg/g (25.5% respect to the final loaded 5-fluorouracil). These results endorse the potential application of the synthesized nanogels as nanocarriers for anticancer drug delivery. Despite the small and hydrophilic nature of 5-fluorouracil molecule, low percentages of released 5-fluorouracil were obtained in all cases. This could be ascribed to the closed and rigid character of the networks, as well as to the possibility of specific H-bonding formation between 5-fluorouracil and chitosan nanogels.

4. Conclusions

Linear chitosan was crosslinked with genipin and functionalized with folic acid by a multi-step modification reaction where genipin was simultaneously employed as crosslinker and spacer agent for folate conjugation. These modification reactions took place in inverse microemulsion medium that led to obtain nanometric networks with monodispersed size distribution. ^{1}H NMR and UV–vis spectroscopy verified the incorporation of genipin and folic acid into chitosan networks. The prepared folate-genipin-chitosan nanoparticles showed greatly improved water solubility at neutral and moderate basic pHs. This was due to the loss of crystallinity provided by the chemical modification of chitosan. The lysozymic degradation of the nanogels could be evaluated by a reducing sugar determination method. The release profile of 5-fluorouracil was studied. Nanogels showed high encapsulation efficiency in aqueous solution and, controlled release under different pH conditions. The data enhance the potential use of the prepared gels as biomaterial nanodevices for antitumor drug delivery.

Acknowledgements

Financial support for this work was provided by the Basque Government and the University of the Basque Country (UPV/EHU), for awarding a fellowship.

References

Anthonsen, M. W., Vårum, K. M., Hermansson, A. M., Smidsrød, O., & Brant, D. A. (1994). Aggregates in acidic solutions of chitosans detected by static laser-light scattering. *Carbohydrate Polymers*, *25*, 13–23.

Arteche, M., Pérez-Álvarez, L., Cesteros, C., & Katime, I. (2012). Water dispersible pH-responsive chitosan nanogels modified with biocompatible crosslinking-agents. *Polymer*, *53*, 3107–3116.

Arteche, M., Pérez-Álvarez, L., Cesteros, C., & Katime, I. (2013). Biodegradable chitosan nanogels crosslinked with genipin. *Carbohydrate Polymers*, *94*, 836–842.

Beauchamp, O., St Clair, M. B., Fennell, T. R., Clarke, D. O., & Morgan, K. T. (1992). A critical review of the toxicology of glutaraldehyde. *Critical Reviews in Toxicology*, *22*, 143–174.

Blanco, M., Guerrero, D., Benito, S., Teijón, M., Olmo, C., Muñíz, R., et al. (2010). Tamoxifen-loaded folate-conjugate poly[(p-nitrophenyl acrylate)-co-(N-isopropylacrylamide)] sub-microgel as antitumoral drug delivery system. *Journal of Biomedical Materials Research*, *95*, 1028–1040.

Busilacchi, A., Gigante, A., Mattioli-Belmonte, M., Manzotti, S., & Muzzarelli, R. A. A. (2013). Chitosan stabilizes platelet growth factors and modulates stem cell differentiation toward tissue regeneration. *Carbohydrate Polymers*, *98*, 665–676. http://dx.doi.org/10.1016/j.carbpol.2013.06.044

Choubey, J., & Bajpai, A. K. (2010). Investigation on magnetically controlled delivery of doxorubicin from superparamagnetic nanocarriers of gelatin crosslinked with genipin. *Journal of Materials Science Materials in Medicine*, *21*, 1573–1586.

Han, T., Nwe, N., Furuike, T., Tokura, S., & Tamura, H. (2012). Methods of N-acetylated chitosan scaffolds and its in vitro biodegradation by lysozyme. *Journal of Biomedical Science and Engineering*, *5*, 15–23.

Illum, L. (1998). Chitosan and its use as a pharmaceutical excipient. *Pharmaceutical Research*, *15*, 1326–1331.

Imoto, T., & Yagishita, K. (1971). A simple activity measurement of lysozyme. *Agricultural and Biological Chemistry*, *35*, 1154–1156.

Kasaai, M. R. (2010). Determination of the degree of N-acetylation for chitin and chitosan by various NMR spectroscopy techniques: A review. *Carbohydrate Polymers*, *79*, 801–810.

Kasaai, M. R., Arul, J., Chin, S. L., & Charlet, G. (1999). The use of intense femtosecond laser pulses for the fragmentation of chitosan. *Journal of Photochemistry Photobiology A*, *120*, 201–205.

Lee, R. J., Wang, S., & Low, P. S. (1996). Measurement of endosome pH following folate receptor-mediated endocytosis. *Biochimica et Biophysica Acta: Biomembranes*, *1312*, 237–242.

Maggi, F., Ciccarelli, S., Diociaiuti, M., Casciardi, S., & Masci, G. (2011). Chitosan nanogels by template chemical cross-linking in polyion complex micelle nanoreactors. *Biomacromolecules*, *12*, 3499–3507.

Martins, A. M., Pereira, R. C., Leonor, I. B., Azevedo, H. S., & Reis, R. L. (2009). Chitosan scaffolds incorporating lysozyme into CaP coatings produced by a biomimetic route: A novel concept for tissue engineering combining a self-regulated degradation system with in situ pore formation. *Acta Biomaterialia*, *5*, 3328–3336.

Muzzarelli, R. A. A. (2009). Genipin-chitosan hydrogels as biomedical and pharmaceutical aids. *Carbohydrate Polymers*, *77*, 1–9.

Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin. *Carbohydrate Polymers*, *87*, 995–1012.

Nishimura, S. I., Kohgo, O., Kurita, K., & Kuzuhara, H. (1991). Chemospecific manipulations of a rigid polysaccharide: Syntheses of novel chitosan derivatives with excellent solubility in common organic solvents by regioselective chemical modifications. *Macromolecules*, *24*, 4745–4748.

Nordtveit, R. J., Vårum, K. M., & Smidsrød, O. (1996). Degradation of partially N-acetylated chitosans with hen egg white and human lysozyme. *Carbohydrate Polymers*, *29*, 163–167.

Rao, S. B., & Sharma, C. P. (1997). Use of chitosan as a biomaterial: Studies on its safety and hemostatic potential. *Journal of Biomedical Materials Research*, *34*, 21–28.

Rao, G. R., Kanjilal, G., & Mohan, K. R. (1978). A direct colorimetric method for the estimation of folic acid. *The Indian Journal of Pharmacy*, 23–24.

Rinaudo, M., Milas, M., & Dung, P. L. (1993). Characterization of chitosan, influence of ionic strength and degree of acetylation on chain expansion. *International Journal of Biological Macromolecules*, *15*, 281–285.

Sahu, S. K., Mallick, S. K., Santra, S., Maiti, T. K., Ghosh, S. K., & Pramanik, P. (2010). In vitro evaluation of folic acid modified carboxymethyl chitosan nanoparticles loaded with doxorubicin for targeted delivery. *Journal of Materials Science: Materials in Medicine*, *21*, 1587–1597.

Singleton, V. L., Orthofer, R., & Lamuela-Raventos, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology*, *299*, 152–178.

Sorlier, P., Rochas, C., Morfin, I., Viton, C., & Domard, A. (2003). Light scattering studies of the solution properties of chitosans of varying degrees of acetylation. *Biomacromolecules*, *4*, 1034–1040.

Sung, H. W., Huang, R. N., Huang, L. L., & Tsai, L. L. (1999). In vitro evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation. *Journal of Biomaterials Science, Polymer Edition*, *10*, 63–78.

Tommeraas, K., Koping-Hoggard, M., Vårum, K. M., Christensen, B. E., Artursson, P., & Smidsrød, O. (2002). Preparation and characterization of chitosans with oligosaccharide branches. *Carbohydrate Research*, *337*, 2455–2462.

Vårum, K. M., Ottoy, M. H., & Smidsrød, O. (1994). Water-solubility of partially N-acetylated chitosans as a function of pH: Effect of chemical composition and depolymerization. *Carbohydrate Polymers*, *25*, 65–70.
Wang, S., & Low, P. S. (1998). Folate-mediated targeting of antineoplastic drugs, imaging agents, and nucleic acids to cancer cells. *Journal of Controlled Release*, *53*, 5339–5348.
Wu, C., Zhou, S., & Wang, W. (1995). A dynamic laser light-scattering study of chitosan in aqueous solution. *Biopolymers*, *35*, 385–392.
Xia, W., Liu, P., Zhang, J., & Chen, J. (2011). Biological activities of chitosan and chitooligosaccharides. *Food Hydrocolloids*, *25*, 170–179.
Yang, Z. K., & Yuan, Y. (2001). Studies on the synthesis and properties of hydroxal aza-crown ether-grafted chitosan. *Journal of Applied Polymer Science*, *82*, 1838–1843.