Chinese Journal of Polymer Science Vol. 34, No. 3, (2016), 280–287

Chinese Journal of Polymer Science

Design and Preparation of pH-responsive Curdlan Hydrogels as a Novel Protein Delivery Vector*

Ze-chuan Yang[a, b], Yan-chun Li[c], Fan Li[b], Qing-rong Huang[d], Guo Zhang[a**] and Tong-fei Shi[b**]

[a] *Institute of Materials Science and Engineering, Jilin University, Changchun 130012, China*
[b] *State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China*
[c] *Department of Pediatric Respiratory Medicine, First Hospital of Jilin University, Changchun 130021, China*
[d] *Food Science Department, Cook College Rutgers University, New Brunswick, NJ 08901-8520, United States*

Abstract New pH-responsive saccharide hydrogels were designed and prepared using curdlan derivatives (curdlan-Boc-histidine, CUR-HIS). The CUR-HIS hydrogels possessed highly porous structures. The swelling ratios of CUR-HIS hydrogels increased with the degree of substitution of Boc-histidine groups. And the addition of 0.5 mol/L NaCl provoked a sharp reduction of swelling ratio of CUR-HIS hydrogels. Bovine serum albumin (BSA) can be efficiently encapsulated into CUR-HIS hydrogels. Moreover, the release profiles of BSA at different pH values from CUR-HIS hydrogels were significantly different. These hydrogels showed good biocompatibility in the cytotoxicity assays. The CUR-HIS hydrogels are of great potential in biomedical applications such as protein delivery systems.

Keywords: pH-responsive; Hydrogel; Protein; Delivery.

Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s10118-016-1757-9.

INTRODUCTION

Interest in proteins and peptides has recently been growing because of their powerful and selective therapeutic activities. However, physical and chemical instability, large size and enzymatic degradation represent unique issues that affect the pharmacokinetics and the pharmacodynamics of protein drugs[1]. Stimuli-reactive hydrogels, also referred to as 'smart-hydrogels'[2], have been investigated as a formulation strategy to increase solubility, bioavailability, and stability of protein drugs. But the majority of stimuli-responsive hydrogels were created using conventional methods of synthesis[3–5] of a relatively small number of both natural and synthetic polymers[6, 7].

Curdlan is a natural linear beta-1,3-glucan produced by microorganism such as *Alcaligenes faecalis*[8]. Curdlan is soluble in diluted base solutions (0.25 mol/L NaOH), dimethylsulfoxide (DMSO), and formic acid, but it is not soluble in alcohols or in water due to the existence of extensive intra/intermolecular hydrogen bonds[9]. Curdlan can self-assemble into the triple helix formation and form elastic hydrogels upon heating in aqueous suspension[10]. Upon different preparation protocols, curdlan exhibits three crystal structures (forms I, II,

* This work was financially supported by the National Natural Science Foundation of China (Nos. 51028301 and 21174146) and the Special Funds for National Basic Research Program of China (No. 2009CB930100).
** Corresponding authors: Guo Zhang (张国), E-mail: guozhang@jlu.edu.cn
Tong-fei Shi (石彤非), E-mail: tfshi@ciac.ac.cn
Received August 13, 2015; Revised October 14, 2015; Accepted November 15, 2015
doi: 10.1007/s10118-016-1757-9

and III)[11]. Curdlan form I consists of a right-handed 6/1 single helix[12] with large amount of water. Curdlan forms II and III are the triple helical structures composed of right-handed 6/1 helices. These two forms with triple helices are different in the degree of hydration and fiber repeats. Curdlan and its derivatives are proven to have an important biological activity such as the anti-tumor, anti-HIV, and immunomodulatory effects[13–15]. Some papers reported the synthesis of the support matrices for the enzyme immobilization or of the gels/microparticles as carriers for protein/vaccine/enzyme/drug[16–18]. However, the original curdlan has a poor solubility in water and its gelation condition is so harsh that may lead to the premature degradation of its protein cargos. This phenomenon could be prevented by increasing the water-solubility of the curdlan and introducing ionic groups. Histidine is a biocompatible and eco-friendly amino acid. Its imidazole side group possesses pH-responsive properties. Histidine based pH-responsive materials have been extensively investigated[19–21], but its application in saccharide hydrogels was little.

In this paper, new pH-responsive hydrogels based on curdlan derivatives were obtained by conjugating curdlan with Boc-histidine. The morphologies of the hydrogels were analyzed by scanning electron microscopy and the chemical composition was studied by FTIR spectroscopy. The main characteristics of these new hydrogels such as the swelling degree in various pH solutions and the exchange capacity were also discussed. These new pH-responsive hydrogels could be used as potential carriers for protein drug delivery. In order to verify this supposition, the hydrogels were loaded with BSA, which was taken as a model protein drug. The release profiles were studied in various pH media to simulate the physiological body fluids (pH = 7.4 for cytoplasmic fluid and pH = 4 for lysosomal fluid). The biocompatibility was also investigated.

EXPERIMENTAL

Materials

1,1-Carbonyldiimidazole (CDI, 99%), Boc-histidine, dimethyl sulfoxide (DMSO, anhydrous 99.9%), 4-Pyrrolidinopyridine (PYP), cell counting kit-8 (CCK-8) were obtained from Sigma-Aldrich Chemical Co. β-(1→3)-Glucan (curdlan) was obtained from Sigma Aldrich and dried under vacuum at 40 °C overnight prior to use. Diethyl ether and methanol were purchased from Sinopharm Co. HepG2 cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Synthesis of Boc-histidine Conjugated Curdlan

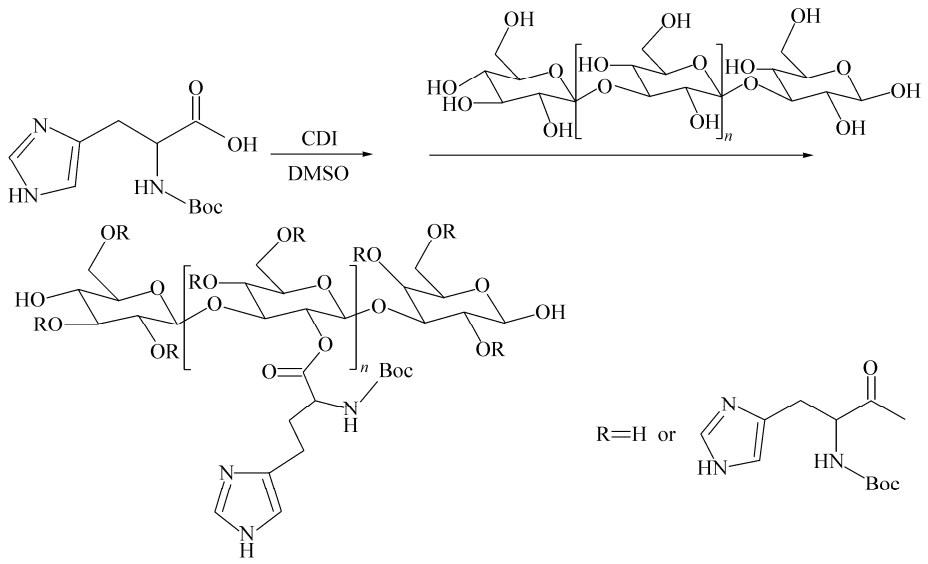
The synthetic strategy is shown in Scheme 1. In a general procedure, dried curdlan (0.6 g, 3.6 mmol) was weighed into a 50 mL round-bottom flask. 10 mL anhydrous DMSO was added to the flask, and the contents were stirred at room temperature for 1 h before use. In a separate flask, Boc-histidine (0.95 g) was weighed into a 50 mL schlenck flask with 10 mL anhydrous DMSO. After Boc-histidine was dissolved completely, 0.54 g CDI was added to the flask and then the reaction system was degassed with three freeze-evacuate-thaw cycles. The flask was immersed into an oil bath at 50 °C for 3 h to produce the acylimidazole. The flask was flushed with dry argon in the whole procedure. Next, the activated Boc-histidine solution was injected into the curdlan solution. 0.049 g PYP was added to the mixed solution as catalyst. The reaction was performed at 50 °C for 24 h after purging with nitrogen. The final reaction mixture was precipitated with methanol. Finally, the CUR-HIS was dried in vacuum overnight.

NMR and FTIR Spectroscopy

The ^{1}H-NMR spectra of curdlan and CUR-HIS was obtained using a Bruker AV400 instrument. Samples were dissolved in DMSO-d_6 and analyzed. The FTIR spectra obtained were recorded and compared using a Nicolet Nexus 470 spectrometer at frequencies ranging from 400 cm^{-1} to 4000 cm^{-1}. Samples were thoroughly mixed with dried KBr powder and pressed into pellet form.

Determination of Degree of Substitution (DS)

The degree of substitution (DS), defined as the number of Boc-histidine contents per hundred anhydroglucose units of curdlan, was determined by elemental analyses (EA). The nitrogen contents of the polymer were performed by a Perkin Elmer 2400 II analyzer.



Scheme 1 Synthetic routes of CUR-HIS

X-ray Diffraction Studies

The raw materials were subjected to X-ray diffraction (D8 ADVANCE, Bruker, Germany) using Cu Kα radiation generated at 40 kV and 40 mA, the range of diffraction angle was 5°–40° (2θ).

Scanning Electron Microscopy (SEM)

The morphology of the CUR-HIS was observed under a scanning electron microscope (Hitachi, Model: S-4800). The hydrogels were allowed to reach equilibrium swelling and cut into small pieces to expose the inner surface. Then, the hydrogels were lyophilized. Before imaging, the freeze-dried hydrogels were taped on SEM stub and sputter coated with a thin layer of gold under vacuum.

Swelling Property of the Hydrogel

The swelling characteristics of the prepared hydrogels were determined by immersing the xerogels in PBS buffer solutions of pH 4 and 7.4 at 20 °C. Then, the hydrogels were weighted at specific time intervals. The swelling of samples were calculated from the following formula:

$$\text{Swelling} = \frac{W_\tau - W_0}{W_0}$$

where 'W_τ' is the weight of the swollen hydrogels and 'W_0' is the weight of the xerogels.

In vitro BSA Encapsulation and Release Studies at Different pH

The model protein BSA was loaded into the hydrogels using the swelling-diffusion method: BSA was dissolved in PBS solution (10 mg/mL, pH 4). Then, the xerogels were placed into 5 mL of BSA solution and allowed to reach equilibrium swelling for 48 h at 20 °C. Then, after a quick washing with fresh PBS solution, the BSA-loaded hydrogels were lyophilized to dry powder for subsequent use.

To evaluate the BSA release profile, CUR-HIS xerogels were immersed in PBS solution at different pH (*i.e.*, pH 4 and 7.4). At specific time intervals, an aliquot of the sample (200 μL) was taken and the corresponding concentration of the released BSA was determined by UV-Vis spectroscopy following standard Lowry's protein assay at 595 nm[22].

In vitro Cytotoxicity Test

Cytotoxicity of the CUR-HIS hydrogels was evaluated using the CCK-8 assay in HepG2 cell line. The method was reported previously by our group with minor modification[23]. The cells were cultured in Dulbecco's

modified eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), under 5% CO_2 and 95% relative humidity atmosphere. The HepG2 cells were seeded in a 96-well plate at a density of about 18000 cells/well in 100 μL of growth medium and incubated for 24 h, after which time the growth medium was replaced with DMEM medium containing different concentrations of the CUR-HIS hydrogels. The samples were sterilized by filtering with 0.22 μm syringe filters, and different volumes of prepared solution were added into the DMEM medium. The cells were further incubated for 24 h under the same conditions, after which 10 μL of CCK-8 solution was added to each well, and the plate was incubated for 2 h. Absorbance was measured at 450 nm (PERLONG DNM-9602 plate reader). The cell viability (%) relative to control cells cultured in media without CUR-HIS hydrogels was calculated from:

$$\text{Cell viability (\%)} = \frac{A_{\text{test}}}{A_{\text{control}}} \times 100\%$$

where A_{test} and A_{control} are the absorbance values of the wells (with the CUR-HIS hydrogels) and control wells (without the CUR-HIS hydrogels), respectively. For each sample, the final absorbance was the average of those measured from four wells in parallel.

RESULTS AND DISCUSSION

Synthesis of CUR-HIS

The CUR-HIS was obtained by partial esterification of the hydroxyl groups of the polysaccharide with Boc-histidine. *N*,*N*-Carbonyldiimidazole (CDI) was chosen as esterification reagent in our present work. Because CDI and its by-products are non-toxic, the imidazole and CO_2 formed during the reaction can be easily removed from the polymer, and side reaction (*e.g.* moffatt reaction) can be avoided during the esterification[24]. The conversion is followed as a two-stage process. CDI is first reacted with the Boc-histidine to yield the acylimidazole within 6 h at room temperature. Thereby, the tendency of cross-linking initiated by unreacted CDI is avoided. Sequencely, the curdlan with a catalyst amount of 4-pyrrolidinopyridine (PYP) was added to the acylimidazole to form the required ester. The degree of substitution (DS) of CUR-HIS is shown in Table 1.

Table 1. Characterization of CUR-HIS by elemental analyses

Sample name	N (%)	Composition mole ratio (glucose units:Boc-his)	Conv. (%)	Per 100 glucose units
CUR-HIS1	1.160	1:0.1	47	4.7
CUR-HIS2	2.280	1:0.3	33.7	10.1
CUR-HIS3	5.780	1:1	33.1	33.1

The data were the average of two measurements.

Figure 1 shows the FTIR spectra for the underivatized curdlan and CUR-HIS3. In the FTIR spectrum of CUR-HIS3, the stretching vibration of carbonyl in Boc-histidine appeared at 1740 cm^{-1}, but not in that of its precursor, which indicates that the Boc-histidine was successfully grafted onto curdlan chains by esterification.

The CUR-HIS was further confirmed by ^{1}H-NMR spectrum (Fig. 2). In case of DMSO-d_6, the DMSO-d_6 multiplet at $\delta = 2.5$ was selected as the reference standard. The chemical shift in the range of $\delta = 1.3$, $\delta = 4.7$ and $\delta = 7.6$ could be attributed to the methyl protons of Boc-histidine groups, the anomeric protons of the glucose units and the protons of double bond in imidazole groups respectively.

X-ray Diffraction Analysis

X-ray diffraction diagrams of curdlan and its derivative are shown in Fig. 3. Native curdlan showed diffraction peaks at 2θ = 12° and 20° which were assigned to the crystal form. The diffraction peaks of CUR-HIS3 hydrogels were similar to the peaks of native curdlan. These results indicated the crystalline structure of curdlan was not disrupted after Boc-histidine modification.

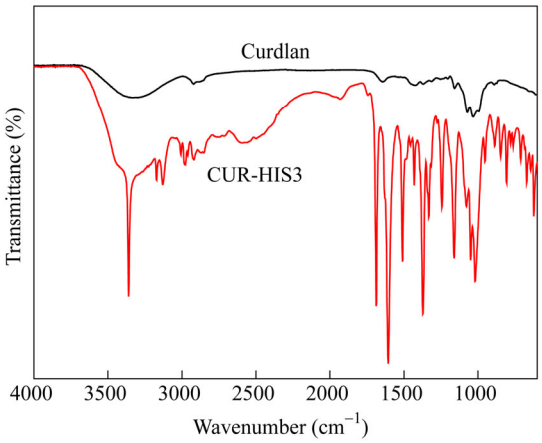


Fig. 1 FTIR spectra of curdlan and CUR-HIS3

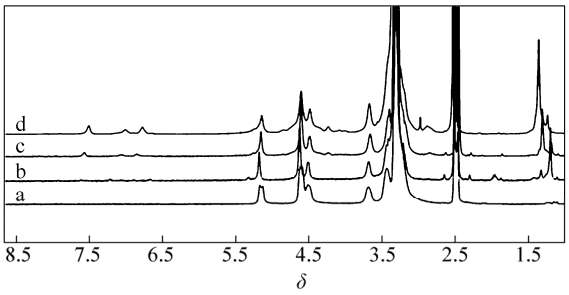


Fig. 2 The ^{1}H-NMR spectra of (a) curdlan, (b) CUR-HIS1, (c) CUR-HIS2 and (d) CUR-HIS3 (in DMSO-d_6)

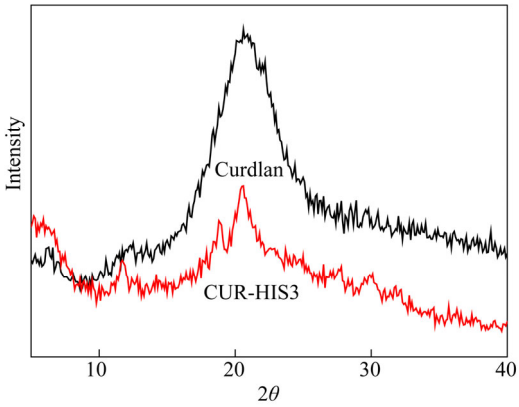


Fig. 3 X-ray diffraction patterns for curdlan and CUR-HIS3

Scanning Electron Microscopy (SEM) of Hydrogels

The SEM images of curdlan xerogel and CUR-HIS3 xerogel are shown in Fig. 4. These xerogels possessed highly porous structures. These porous architectures should be beneficial for encapsulation of drugs and protect the encapsulated drugs from the protease degradation[25]. Moreover, porous structure enables their fast swelling, thereby allowing the hydrogels to quickly entrap drugs.

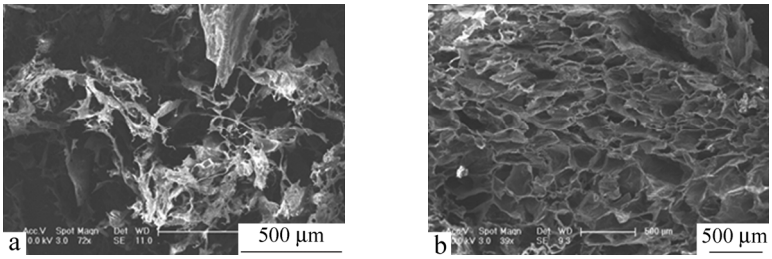


Fig. 4 SEM micrographs of (a) curdlan and (b) CUR-HIS3

Swelling Behavior of Hydrogels

The swelling behavior of the hydrogels has been studied in different conditions of pH values and salt concentrations. At pH = 4, CUR-HIS hydrogels swelled to a large extent by keeping a transparent and soft aspect (Fig. 5). And the swelling ratios of CUR-HIS hydrogels increased with the DS of Boc-histidine groups. This phenomenon was probably derived from the ionization of imidazole groups at low pH. When imidazole groups of the hydrogels get ionized, Boc-histidine groups in the hydrogel network are positively charged. These positive charges cause electrostatic repulsion between polymer chains which results in the expansion of the gel network. Also, these positive charges induce the increased concentration of counter ions inside the network, this leads to a net osmotic pressure difference between the inner and outer environments of the hydrogel network[26]. However, at pH = 7.4 (Fig. S1), the swelling degree of CUR-HIS hydrogels decreased, even nonswelling. Because the pKa of the imidazole groups was about 6.0, above this pH, in fact, the Boc-histidine was prevailing in the neutral form, this led to a strong reduction of the net charges of the CUR-HIS polymer chains.

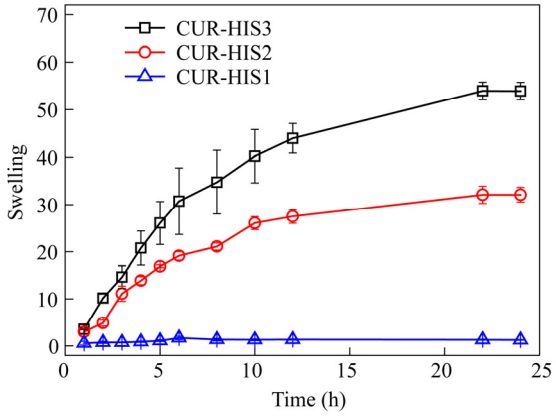


Fig. 5 Swelling kinetics of the hydrogels at 20 °C (pH = 4)

Figure 6 shows the degree of swelling of CUR-HIS hydrogels in relation to the concentration of NaCl. The addition of 0.5 mol/L NaCl provoked a sharp reduction of swelling ratio of CUR-HIS hydrogels. This is attributed to the polyelectrolyte nature of the polymer network. The salt shielded the positive charges of the imidazole groups. As a consequence, the electrostatic repulsion between the chains reduced, which resulted in the collapsing of the gel network.

Drug Release

BSA was used as a water-soluble model protein to evaluate the properties of the hydrogels as protein delivery vectors. The BSA release profiles of BSA@CUR-HIS hydrogels are shown in Fig. 7. The release rate of BSA from hydrogels significantly increased at higher pH (pH 7.4). It implies that the BSA release profiles are dependent on the pH environment, which is both in relation to the degree of swelling of the hydrogels and in relation to the positive charged status of Boc-histidine groups.

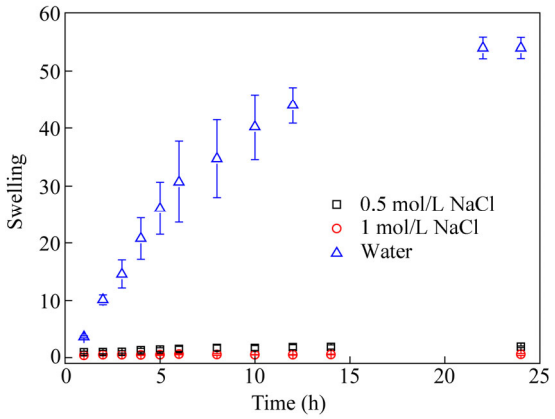


Fig. 6 Swelling kinetics of gel CUR-HIS3 in relation to the concentration of salt at 20 °C (pH = 4)

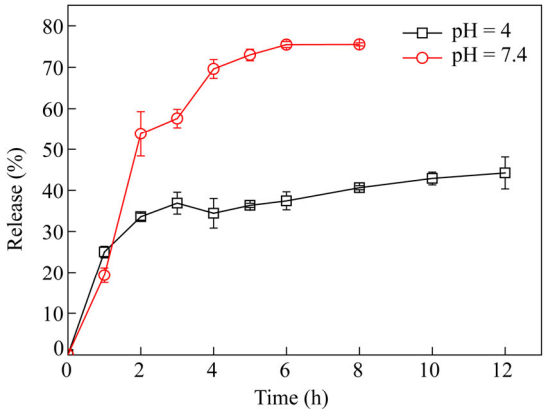


Fig. 7 The *in vitro* release profiles for BSA from CUR-HIS3 hydrogels at different pH (T = 20 °C)

In vitro Cytotoxicity

The biological and biomedical applications of CUR-HIS hydrogels are highly dependent on their biocompatibility. We investigated the toxicity of CUR-HIS hydrogels at different concentrations. In this case, the *in vitro* cytotoxicity of the CUR-HIS hydrogels was obtained using the CCK-8 assay on the HepG2 cell line. Here, we use CCK-8 for the determination of cytotoxicity rather than MTT, due to its higher sensitivity for detection[27]. The wells that contained only the media were used as the negative control, and the wells containing untreated cells were used as the positive control. Each experiment was performed in quadruplicate and cell viability was obtained by CUR-HIS hydrogels treated cells/absorbance of positive control. As shown in Fig. 8, their cytotoxicity values were compared to those of the untreated cells. At very low concentration (50 μg/mL), the cell viability of CUR-HIS2 and was found to be about 106%. Increasing the concentration of CUR-HIS hydrogels up to 800 μg/mL did not cause a significant increase in the toxicity of CUR-HIS2, and the cell viability was about 98%. Similarly, at various concentrations (from 50 μg/mL to 800 μg/mL), the cell viability of CUR-HIS3 was found to be above 90%. It is apparent from the graph that the cytotoxicity is very low for CUR-HIS hydrogels. The CUR-HIS hydrogels may be suitable for use in some potential biomedical applications.

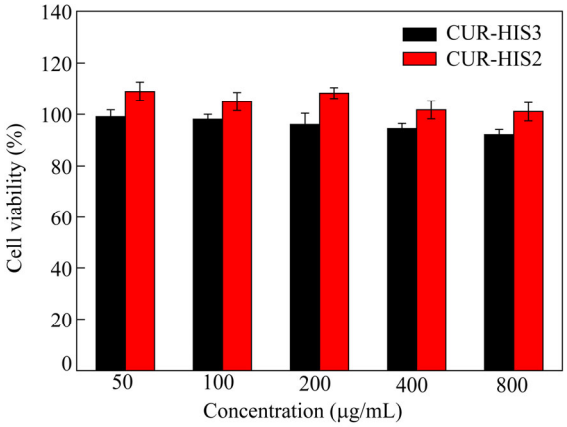


Fig. 8 Cytotoxicity of CUR-HIS hydrogels in HepG2 cells, mean ± standard derivation (n = 4)

CONCLUSIONS

In summary, the new curdlan hydrogels were synthesized by esterificating curdlan with Boc-histidine. A better water absorbency was obtained for CUR-HIS hydrogels, and the swelling ratios of CUR-HIS hydrogels increased with the DS of Boc-histidine groups. And the addition of NaCl provoked a sharp reduction of swelling ratio of CUR-HIS hydrogels. This is attributed to the salt shielding the positive charges of the imidazole groups, which results in the collapsing of the gel network. The drug release experiments performed at different pH values indicated that the drug release rate increased with the pH value. The CUR-HIS hydrogels revealed low cytotoxicity against the HepG2 cell line. Above all, these results allow us to propose these materials as useful candidates for biomedical applications such as protein delivery systems.

REFERENCES

1 Mizuno, N., Niwa, T., Yotsumoto, Y. and Sugiyama, Y., Pharm. Rev., 2003, 55: 425
2 Putnam, A.J. and Mooney, D.J., Nat. Med., 1996, 2: 824
3 Zeng, Y.F., Tseng, S.J., Kempson, I.M., Peng, S.F., Wu, W.T. and Liu, J.R., Biomaterials, 2012, 33(36): 9239
4 Casolaro, M., Cini, R., Bello, B.D., Ferrali, M. and Maellaro, E., Biomacromolecules, 2009, 10: 944
5 Casolaro, M., Bottari, S. and Ito, Y., Biomacromolecules, 2006, 7: 1439
6 Hu, Y.F., Liu, Y.F., Qi, X., Liu, P., Fan, Z.Y. and Li, S.M., Polym. Int., 2012, 61(1): 74
7 Cai, M.T., Zhang, J.X., Chen, Y.W., Cao, J. and Leng, M.T., Chinese J. Polym. Sci., 2014, 32(2): 236
8 Harada, T., Misaki, A. and Saito, H., Arch. Biochem. Biophys., 1968, 124: 292
9 Mclntosh, M., Stone, B.A. and Stanisich, V.A., Appl. Microbiol. Biotechnol., 2005, 68: 163
10 Funami, T., Funami, M., Yada, H. and Nakao, Y., Food Hydrocolloid., 2000, 14(5): 509
11 Kasai, N. and Harada, T., ACS Symp. Ser., 1980, 141: 363
12 Okuyama, K., Otsubo, A., Fukuzawa, Y., Ozawa, M., Harada, T. and Kasai, N., Carbohydr. Chem., 1991, 10: 645
13 Bohn, J.A. and BeMiller, J.N., Carbohydr. Polym., 1995, 28: 3
14 Bacic, A., Fincher, G.B. and Stone, B.A., "Chemistry, biochemistry, and biology of 1-3 beta glucans and related polysaccharides", Academic Press, New York, 2009, p.563
15 Gordon, M., Deeks, S., De, M.C., Goodgame, J., Guralnik, M., Lang, W., Mimura, T., Pearce, D. and Kaneko, Y., J. Med. Chem., 1997, 28: 108
16 Kim, B., Jung, I., Kim, J., Lee, J.H., Lee, I. and Lee, K., Biotechnol. Lett., 2000, 22 (14): 1127
17 Na, K., Park, K.H., Kim, S.W. and Bae, Y.H., J. Controlled Release, 2000, 69: 225
18 Li, P.L., Tan, H.N., Xu, D.Q., Yin, F.X., Cheng, Y.N., Zhang, X.K., Liu, Y.H. and Wang, F.S., Carbohydr. Polym., 2014, 110: 446
19 Thomas, J.J., Rekha, M.R. and Sharma, C.P., Mol. Pharm., 2012, 9(1): 121
20 Gaspar, V.M., Marques, J.G., Sousa, F., Louro, R.O., Queiroz, J.A. and Correia, I.J., Nanotechnology, 2013, 24(27): 275101
21 Fullenkamp, D.E., He, L.H., Barrett, D.G., Burghardt, W.R. and Messersmith, P.B., Macromolecules, 2013, 46(3): 1167
22 Bradford, M.M., Anal. Biochem., 1976, 72: 248
23 Li, F., Pei, D.F., Huang, Q.R., Shi, T.F. and Zhang, G., Carbohydr. Polym., 2014, 99: 728
24 Heinze, T., Liebert, T., Heublein, B. and Hornig, S., Polysaccharides II, 2006, 205: 199
25 Yin, L.C., Ding, J.Y. and Fei, L.K., Int. J. Pharm., 2008, 350: 220
26 Kim, S.J., Lee, K.J., Kim, I.Y. and Kim, S.I., J. Macromol. Sci. A, 2003, 40(5): 501
27 Wu, S.N., Xing, D., Gao, X.J. and Chen, W.R., J. Cell. Physiol., 2009, 218(3): 603