JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2013. Vol. 7(Spl. Edn.), p. 475-480

The Effects on Degradation Treatment of CMC-HLC/EDC-ADH Hydrogels using Collagenase I and Simulated Body Fluid

Xian Li[1]*, Daidi Fan[1]*, Jianjun Deng[1], Junfeng Hui[1], Xiaoxuan Ma[1], Chenhui Zhu[1] and Wenjiao Xue[2]

[1]Shaanxi Key Laboratory of Degradable Biomedical Materials, Department of Chemical Engineering, Northwest University, Taibai North Road 229, Xi'an, Shaanxi 710069, China.
[2]Shaanxi Provincial Institute of Microbiology, No. 76 Xiying Road, Xi'an 710043, China.

(Received: 03 March 2013; accepted: 14 April 2013)

A novel hydrogel for biomedical applications was successfully fabricated by human-like collagen (HLC) and carboxymethylcellulose (CMC) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and adipic acid dihydrazide (ADH) as cross-linkers. The interior morphologies of these hydrogels were also characterized before and after degradation by collagenase I and simulated body fluid (SBF). The results of in vitro degradability tests of these hydrogels that the CMC-HLC/EDC-ADH hydrogel in SBF indicated that possesses slow degradability than in collagenase I in vitro. Therefore, the results provided the possibility that CMC-HLC/EDC-ADH hydrogels are suitable for biomedical applications such as soft tissue augmentation for their good biocompatibility.

Key words: Hydrogel, Collagens I and Simulated body fluid.

The in-vitro degradation of hydrogels systems can be affected by various factors such as: chemical structure and molecular mass, formulation and morphology, and thickness of the used specimen (Tjong SC, 1998). In most situations, degradation is undesirable since the device will be intended to remain in the body for a long period of time without any change in its functional properties and without inducing any significant adverse response from the tissues (Williams DF, 1992). In other cases, however, degradation may be intentional, if either the function is transient so that the device requires elimination at an appropriate time, or if the function relies upon some degenerative process, as in enzyme solution (Miller ND, 1987; Zaikov GE, 1989). The degradation behaviour of hydrogels depends on both chemical structure and physical state, being of major importance the hydrophilicity (as in the case of starch based materials) (Bastiolli C, 1995; Soest JJG,1996). Since the aqueous environment of the body remains reasonably constant (pH=7.4 and T=37!) it should be possible to predict the susceptibility and kinetics of the in-vivo degradation process, considering hydrolysis the only mechanism present (Williams DF, 1992).

Carboxymethylcellulose (CMC), one of the major cellulose derivatives, is a water-soluble polysaccharide and has been widely applied in cosmetics due to its high water retaining ability, good biodegradability and lack of toxicity (Vasile, C, 2004; Ogushi, Y, 2007; Elkins TE, 1984). Human-like collagen (HLC), produced by high cell density cultivation of recombinant Escherichia coli (Fan DD, 2002), is a novel water soluble protein with lower immunogenicity than animal collagen and is free of pathogens; this could provide reliable compositions with predictable structure, and it is a

* To whom all correspondence should be addressed.
Tel.: +86 29 88305118; Fax: +86 29 88322585;
E-mail: li_xian1214@163.com; fandaidi@nwu.edu.cn

novel biomaterial that has successfully been used for the construction of artificial bones (Hua XF, 2005; Yang XJ, 2009) and artificial vascular scaffolds (Zhu CH, 2009). In this study, we assumed that the HLC could crosslink with CMC through amide linkages by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and adipic acid dihydrazide (ADH) as shown in the reaction scheme reported for CMC, where EDC is the carboxyl-activating agent and ADH is the cross-linker.

In order to predict the degradation behaviors of biomaterials in the human physiological environment, the in-vitro degradation tests, in simulated body fluid solutions and collagen I solution, are able to simulate the interactions between the body fluids and a biomaterial, and can allow for the understanding of its stability and degradation rate.

MATERIALS AND METHODS

Materials

The required HLC was expressed by *E. coli* with a cloned partial cDNA reversed from the mRNA coding for human collagen (Santos, 1991; Zhu, 2009) (Chinese patent number: ZL01106757.8). CMC, EDC and ADH were also supplied by Sigma Co, and all solvents and reagents were analytical grade.

Preparation of the CMC-HLC/EDC-ADH Hydrogel

The CMC-HLC/EDC-ADH hydrogel was prepared by mixing 2% (w/v) CMC with an isocratic volume of 3% HLC solution. The mixture was stirred for 20 min until complete dissolution was achieved. Then, 0.06g ADH and 0.02g EDC was added drop wise until the pH of the solution reached 7.4. The solutions (sol) were placed in room temperature for gelling.

The Porous Morphology of Hydrogels

The surface and cross-sectional morphologies of the hydrogels before and after degradation were studied by scanning electron microscopy (SEM) using a JSM-5900LV (Japan).

Bioactivity Evalution

The hydrogels samples were soaked in an m-SBF at 36.5°C up to 6 weeks for bioactivity evaluation. The m-SBF were prepared by dissolving reagent-grade chemicals of sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), potassium chloride (KCl), dipotassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), calcium chloride ($CaCl_2$), and sodium sulphate (Na_2SO_4) into $C_4H_{11}NO_3$ (Tris). 1.0 M NaOH and 1.0 M HCl was used to buffer the solution to pH 6. 0-7.4 at 36.5°C (see Table 1).

Collagenase I Degradation

Degradation of hydrogels was performed in vitro using collagenase I with an activity of 0.5mg/ml. The dried samples were weighed and sterilized by ^{60}Co irradiation and then immersed in tubes of 5mL fresh enzyme buffer. The tubes were kept static at 37.0 ± 0.5 °C in a cell culture box. After soaking for 1, 2, 3, 4, 5 or 6 weeks, the samples were withdrawn from the enzyme buffer and then rinsed with ultrapure water three times, for 30 min each time. After lyophilization, the dry weights were measured. Weight loss (W_L) was calculated according as $W_L=(W_0-W_1)/W_0\times100\%$, where W_0 and W_1 are the weights of the sample before and after soaking, respectively.

Table 1. Composition of 1.5×SBF (pH=7.4) and order of addition of the reagents to de-ionized water at 37°0

Order	Reagent	Weight (g/l)
1	NaCl	12.053
2	$NaHCO_3$	0.533
3	KCl	0.338
4	$K_2HPO_4 \cdot 3H_2O$	0.347
5	$MgCl_2 \cdot 6H_2O$	0.467
6	1M HCl	50 ml
7	$CaCl_2$	0.438
8	Na_2SO_4	0.108
9	C4H11NO3 (Tris)	9.177
10	1M NaOH	8.5 ml

RESULTS AND DISCUSSION

Structure of Hydrogels Before and after Degradation

The SEM microphotographs of the microstructure and morphology of lyophilized hydrogels before degradation are shown in Fig. 1A. The hybrid hydrogels each exhibited a different porous distribution and structure. The surface of hydrogels is full of pores and the pore size ranges

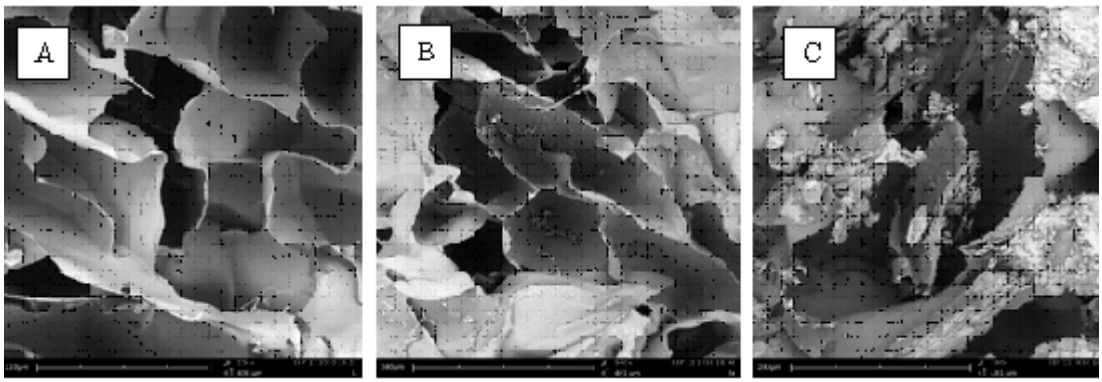

Fig. 1. SEM micrographs (×500) of CMC-HLC/EDC-ADH hydrogels at 37 °C after lyophilization (A), after degradation by collagenase I (B), after SBF degradation (C)

from several tens to several hundreds of micrometers, which could be attributed to the freeze-drying process and cross-linking degree of HLC and CMC (Fig.2). The interior morphologies of these hydrogels after enzyme degradation are shown in Fig. 1B. The surface and interior pores were most markedly disrupted by enzyme degradation, and such as many fragment of outer surface were appeared. The interior morphologies of these hydrogels after SBF degradation are shown in Fig. 1C. The surface and interior pores were most markedly changed. Moreover, the size of crystals from SBF degradation was investigated. This crystals may be come from the composition of the hydrogel, its human-like collagen will combine the Ca^{2+} and Mg^{2+} of SBF solution to chelate compound16, which forming as crystals appeared on the structure of dry hydrogels.

The Scheme of Degradation of CMC-HLC/EDC-ADH Hydrogel

In our study, the effects of cross-linking with ADH and EDC on the HLC/CMC hydrogel were evaluated. Scheme of the cross-linking processes were shown in Fig.2. The carboxylic

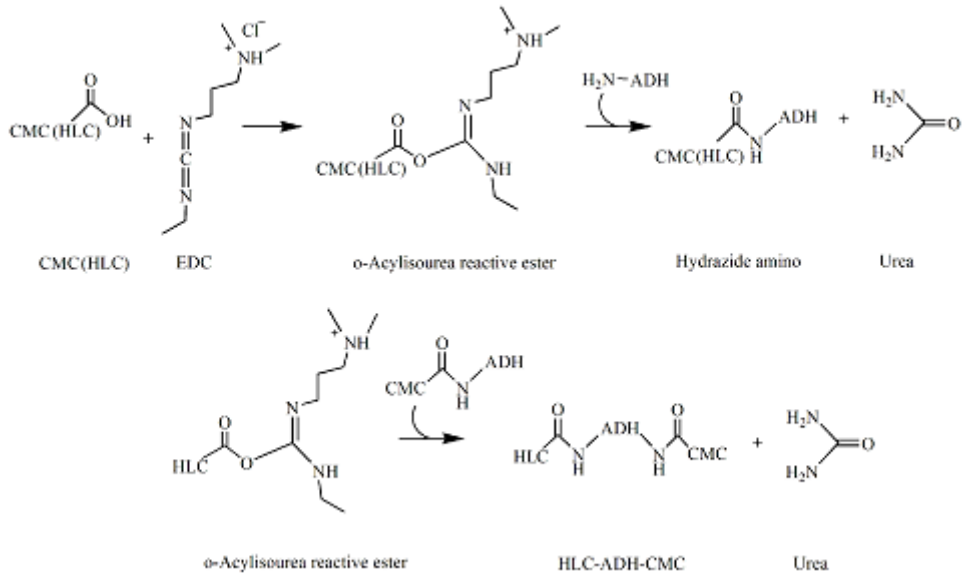

Fig. 2. Scheme of the cross-linking processes. The HLC, CMC or ADH in the figure are represented as complete molecules except for the reacted carboxylic group or amino group

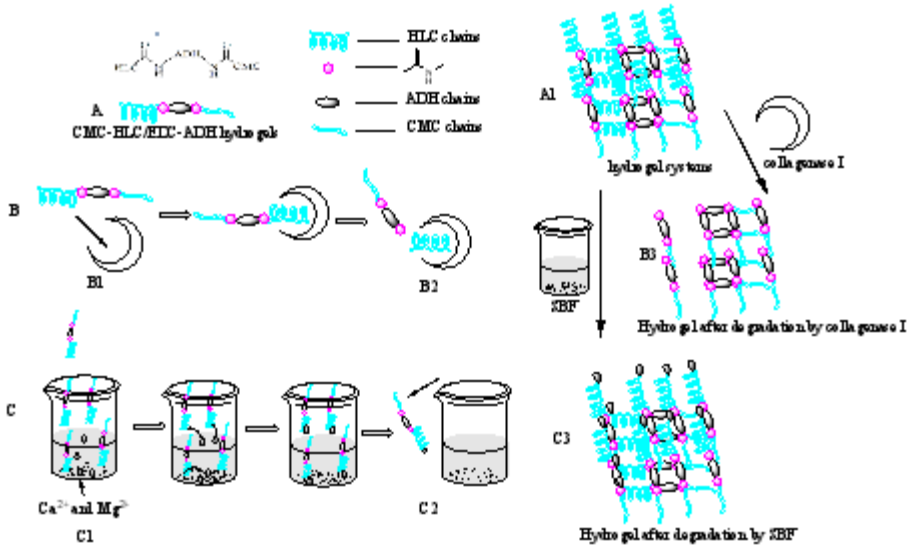

Fig. 3. Scheme of the degradation by collagenase I and SBF

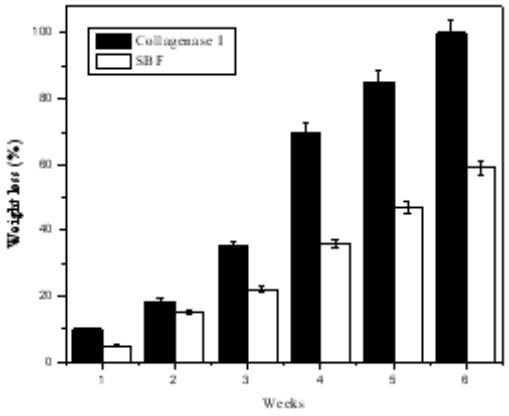

Fig. 4. The weight loss of hydrogels degradation by collagenase I and SBF

groups of CMC or HLC were activated by EDC to form the unstable intermediate O-acylisourea, which subsequently reacted with ADH to form a hydrazide amino intermediate. Furthermore, the remaining carboxylic groups of CMC and HLC activated by EDC at the same time could react with another hydrazide group of the hydrazide amino intermediate or the amino groups of HLC, leading to a highly cross-linked hydrogel of reticular structure through amide linkage between CMC and HLC. Restriction enzyme sites of Collagenase I is hydroxyproline - Leu - Gly - Pro - Ala peptides in the molecular chain of collagen(Li, et a., 2012). Therefore, the residue of hydrogels after degradation were discovered. Scheme of the degradation of CMC-HLC/EDC-ADH hydrogel by collagenase I were shown in Fig.3A. The CMC-HLC/EDC-ADH hydrogel was represent by model A, it compose by HLC chain, -CONH, -ADH chains and CMC chains. The HLC chains of hydrogels (Fig.B1) to get to the restriction enzyme sites of Collagenase I in solution, where the HLC chains were cut off by Collagenase I lead to hydrogel was disconnect to HLC chains. The residue of hydrogel (Fig.3B2) were seen in Fig.3B. while the whole hydrogels system after degradation by Collagenase I were seen in Fig. 3B3. The interior morphologies of hydrogel system have not change clearly, but remain large number of HLC chains was lost. Scheme of the degradation by SBF were shown in Fig.3C. A large number of Ca^{2+} and Mg^{2+} were exist in the SBF solution, and was easy to combine together with HLC to formation of chelate compound(Li, 2012; Yu, 2011). Therefore, the Ca^{2+} and Mg^{2+} of dissociative was connect with HLC in SBF solution were seen in Fig. 3C, the hydrogel was taken out after degradation by SBF, the hydrogel were seen in Fig. 3C. The hydrogels was not changed completely, and a lot of metalion was observer in the surface of morphologies of hydrogels (Fig. 3C).

In vitro Hydrolysis Degradation Behavior

The resistance to degradation of the hydrogels was studied in vitro by examining weight loss with time in 10% (w/v) collagenase I and SBF at 37°C. Overall, weight loss increased with time after degradation by collagenase I (Fig.4). The weight loss up to 100% after degradation by collagenase I at 6 weeks, while only half weight loss discovered in SBF degradation. This mainly due to the composition of hydrogels was degradated by physiological saline, no matter what metalion was connected to hydrogels. It also explain the CMC-HLC/EDC-ADH hydrogels is biodegradable materials.

CONCLUSION

In this study, the in-vitro degradation tests, in simulated body fluid solutions and collagen I solution were test. This hydrogel had a highly porous structure after degradation by collagenase I and the higher weight loss, while had crystal forming by SBF and the lower weight loss. Results also showed that the morphology of the injected gel resisted change for a relatively long time in vivo and degraded slowly over time.

AKNOWLEDGEMENTS

This study was financially supported by the National Natural Science Foundation of China (21276210, 21106112, 21206135, 31000019 and 21106111), China Postdoctoral Science Foundation (20110490171), China Postdoctoral Science Special Foundation (2012T50755) and Natural Science Foundation of Shaanxi, China (2011JE003). The Scientific Research Program of Shaanxi Provincial Department of Education, China (JG08181, 2010JC21, 2010JS107, 2010JS108, 2010JK876 and 2010JS109); Shaanxi Provincial Scientific Technology Research and Development Program (2007K06-03, 2010JQ2012, SJ08B03), the Specialized Research Fund for the Doctoral Program of Higher Education of China (20096101120023 and 2009610-1110014) and the NWU Graduate Innovation and Creativity Funds (YZZ12037) and Shaanxi Key Subject Program, China.

REFERENCES

1. Bastiolli C, Cerutti A, Guanella I, Romano GC, Tosin., Physical state and biodegradation behavior of starch–polycaprolactone systems. *J Environ Polym Degrad.,* 1995; **3**: 2.
2. Zhu CH, Fan DD, Duan ZG, Xue WJ, Shang LA *Journal of biomedical materials research part A.,* 2009; **89**(A): 829-840.
3. Elkins TE, Bury RJ, Ritter JL, Ling FW, Ahokas RA, Homsey C A, Malina LR., *Fertil. Steril.,* 1984; **41**: 926.
4. Fan DD, Mi Y, Song JR., High density fermentation of recombinant *E. coli* for production of human-like collagen. *Chem. Ind. Eng. (China),* 2002; **53**: 752-754.
5. Hua XF, Fan DD, Luo YE, Zhang X, Shi H., Kinetics of high cell density fed-batch culture of recombinant Escherichia coli producing human-like collagen. *Chin. J. Chem. Eng.,* 2006; **14**: 242–247.
6. Miller ND, Williams DF., On the degradation of PHB and PHB–PHV copolymers. *Biomaterials.,* 1987; **8**: 129.
7. Ogushi, Y, Sakai, S, Kawakami, K. J., Synthesis of enzymatically-gellable carboxymethyl-cellulose for biomedical applications. *Biosci. Bioeng.,* 2007; **104**: 30-33.
8. Santos PM, Winterowd JG, Allen GG, Bothwell MA, EW., Nerve growth factor increased angiogenesis without improved nerve Regeneration. *Otolaryngology Head Neck Surgery.,* 1991; **105**(1):12-25.
9. Soest JJG, Benes K, Wit D, Vliegenthart JFG., The influence of starch molecular mass on the properties of extruded thermoplastic starch. *Polymer*., 1996; **37**: 3543.
10. Tjong SC, Bei JZ., Degradation behavior of poly(caprolactone)–poly(ethylene glycol) block copolymer/low-density polyethylene blends. *Polym Eng Sci.,* 1998; **38**: 3.
11. Vasile, C, Bumbu, G. G, Dumitriu, R. P, Staikos, G. Eur., Plasma induced grafting carboxymethyl cellulose on multiwalled carbon nanotubes for the removal of UO22+ from aqueous solution. *Polym. J*., 2004; **40**: 1209-1215.
12. Williams DF., Mechanisms of biodegradation of implantable polymers. *Clin Mater*., 1992; **10**: 9.
13. Li X, Ma XX, Fan DD, Zhu CH., New suitable for tissue reconstruction injectable chitosan/ collagen-based hydrogels. *Soft matter*., 2012; **8**: 3781-3790.
14. Yang XJ, Liang CY, Cai YL, Hu K, Wei Q, Cui ZD., Recombinant human-like collagen modulated the growth of nano-hydroxyapatite

on NiTi alloy. *Mater. Sci. Eng.* C., 2009; **29**: 25-28.
15. Yu YY & Fan DD., Characterization of the Complex of Human-like Collagen with Calcium. *Biol Trace Elem Res.*, 2011; **10**: 9167-x
16. Zaikov GE. In: Jellinek HHG, editor., Degradation and stabilization of polymers, vol. 2: biodegradation of polymers: kinetics and mechanisms. *Amsterdam: Elsevier*., 1989; 469.
17. Zhu CH, Fan DD, Duan ZG, Xue WJ, Shang LA, Chen FL and Luo YE., Medium optimization based on the metabolic flux spectrum of recombinant *Escherichia coli* for high expression of human like collagen II. *J. Biomed. Mater. Res.,* 2009; **89**: 829–840.