Carbohydrate Research 345 (2010) 1774–1780

Contents lists available at ScienceDirect

Carbohydrate Research

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

Antibacterial activity and biocompatibility of a chitosan–γ-poly(glutamic acid) polyelectrolyte complex hydrogel

Ching Ting Tsao [a,†], Chih Hao Chang [b,c,†], Yu Yung Lin [a], Ming Fung Wu [d], Jaw-Lin Wang [a], Jin Lin Han [e], Kuo Huang Hsieh [a,*]

[a] Institute of Polymer Science and Engineering, College of Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei City 10617, Taiwan
[b] Institute of Biomedical Engineering, College of Engineering, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei City 10617, Taiwan
[c] Department of Orthopedics, National Taiwan University Hospital and National Taiwan University College of Medicine, No. 1, Jen-Ai Road, Taipei City 10018, Taiwan
[d] Animal Medicine Center, College of Medicine, National Taiwan University, No. 1, Jen-Ai Road, Taipei City 10018, Taiwan
[e] Department of Chemical and Materials Engineering, College of Engineering, National Ilan University, No.1, Sec. 1, Shennong Road, Ilan City, Ilan County 26047, Taiwan

ARTICLE INFO

Article history:
Received 21 April 2010
Received in revised form 31 May 2010
Accepted 6 June 2010
Available online 16 June 2010

Keywords:
Chitosan
γ-PGA
Polyelectrolyte complex (PEC)
Antibacterial ability
Biocompatibility

ABSTRACT

In this study, we prepared a polyelectrolyte complex (PEC) hydrogel comprising chitosan as the cationic polyelectrolyte and γ-poly(glutamic acid) (γ-PGA) as the anionic polyelectrolyte. Fourier transform infrared spectroscopy revealed that ionic complex interactions existed in the chitosan–γ-PGA PEC hydrogels. The compressive modulus increased upon increasing the degree of complex formation in the chitosan–γ-PGA PEC hydrogel; the water uptake decreased upon increasing the degree of complex formation. At the same degree of complex formation, the compressive modulus was larger for the chitosan-dominated PEC hydrogels; the water uptake was larger for the γ-PGA-dominated ones. Scanning electron microscopy images revealed the existence of interconnected porous structures (pore size: 30–100 μm) in all of the chitosan–γ-PGA PEC hydrogels. The chitosan–γ-PGA PEC hydrogels also exhibited antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. In addition, in vitro cell culturing of 3T3 fibroblasts revealed that all the chitosan–γ-PGA PEC hydrogels were effective in promoting cell proliferation, especially the positively charged ones (chitosan-dominated). Therefore, the chitosan–γ-PGA polyelectrolyte hydrogel appears to have potential as a new material for biomedical applications.

1. Introduction

A hydrogel is a material that exhibits the ability to swell in water and to retain a significant fraction of water within its structure.[1] Because their hydrophilic surfaces have low interfacial free energy when contacting with body fluid, giving them good biocompatibility, hydrogels have attracted much interest recently for use as drug carriers and artificial tissue scaffolds.[2] Although they possess hydrophilic polymeric backbones, hydrogels are kept from dissolution by the presence of radical, chemical, or physical crosslinks.[3] Although radical crosslinks provide a high crosslink quality, there is always the possibility of radical residues remaining in the hydrogels.[4] Chemical crosslinks involve covalent bond formation between different polymer chains; because it requires a toxic crosslinker, it is also unfavorable for biological applications.[5] The use of non-covalent interactions (physical crosslinks) removes the need for radicals or toxic chemical crosslinkers.

Several non-covalent modalities have been exploited in the design of hydrogels, including coiled–coil interactions,[6,7] hydrophobic interactions,[8] antigen–antibody interactions,[9] stereocomplex interactions,[10] and ionic interactions.[11] For ionic crosslinked hydrogels, swelling occurs as a result of ionic interactions between free ions and the charged polymer, which may feature carboxylic acid, sulfonic acid, or amino groups, thereby rendering the polymer hydrophilic and leading to its high water uptake. In this study, we exploited ionic interactions to form homogeneous polyelectrolyte complex (PEC) hydrogels from pairs of oppositely charged agents.

Chitosan, derived from chitin through alkaline deacetylation, is a polysaccharide constituted by *N*-glucosamine and *N*-acetylglucosamine units, in which the number of *N*-glucosamine units exceeds 50%.[12] Its slightly crystalline character makes chitosan insoluble when the pH is around 7 or greater. Because the free amine groups of chitosan become protonated in acidic environments, the positively charged polymer is soluble at low pH.[13,14] The net positive charge of chitosan in acidic environments allows the formation of PEC with polyanionic species.[15] In addition, some antibacterial activities have been described for chitosan and chitosan derivatives. The main factor affecting the antibacterial ability of chitosan is its molecular weight.[16,17] Chitosan oligomers are reported to be more

* Corresponding author. Tel.: +886 2 3366 3043; fax: +886 2 3366 3044.
E-mail address: khhsieh@ntu.edu.tw (K.H. Hsieh).
† These authors contributed equally to this work.

0008-6215/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2010.06.002

effective in inhibiting the growth of bacteria than polymeric chitosan. The antibacterial ability of chitosan is greatly dependent on its molecular weight, especially in the range between 5 and 10 kDa.[18,19]

γ-Poly(glutamic acid) (γ-PGA) is an unusual anionic, natural polyamide made of D- and L-glutamic acid units, connected through amide bonds between the α-amine and γ-carboxylic acid groups.[20] Because γ-PGA is water-soluble, biodegradable, and edible, it has been applied as animal feed supplement, biopolymer flocculant, humectant, or moisturizer in cosmetics,[21] and a natural bactericide or fungicide.[22] Through culturing of bacteria via fermentation, γ-PGA is already produced on an industrial scale in high yield.[23,24] The anionic nature of γ-PGA allows it to form PEC with chitosan at appropriate values of pH.

In this study, we used a simple ionic interaction to prepare homogeneous PEC hydrogels of several compositions by varying molar ratios of amine groups of chitosan to carboxylic acid groups of γ-PGA. We examined the physicochemical characteristics of these chitosan–γ-PGA PEC hydrogels using X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, and scanning electron microscopy (SEM). In terms of physical behavior, we measured the compressive modulus and the water uptake of each chitosan–γ-PGA PEC hydrogel. In terms of *in vitro* biological behavior, we investigated the antibacterial ability of chitosan–γ-PGA PEC hydrogels against *Escherichia coli* (*E. coli*, Gram-negative bacteria) and *Staphylococcus aureus* (*S. aureus*, Gram-positive bacteria). The biocompatibility of the chitosan–γ-PGA PEC hydrogels with 3T3 fibroblasts was also evaluated.

2. Experimental

2.1. Reagents

Chitosan (average molecular weights: 3×10^4 and 3×10^5 Da; degree of deacetylation: 97%) was purchased from G-HT Co. (Hsinchu, Taiwan). γ-PGA (average molecular weight: 1250 kDa) was purchased from VEDAN Co. (Taichung, Taiwan). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM F-12 medium) and 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Gibco Invitrogen (Taipei, Taiwan). Acetic acid (AcOH), sodium hydroxide (NaOH), phosphate-buffered saline (PBS), and other reagents used in the study were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA.)

Table 1
Compositions of chitosan–γ-PGA PEC hydrogels

Nomenclature	Composition		Degree of complex formation (%)	Charge of PEC hydrogel
	Weight ratio chitosan/γ-PGA	Molar ratio [$-NH_2$] of chitosan[a]/[–COOH] of γ-PGA		
Neat chitosan	4/0	100/0	0	Positive
C75P25	3.06/0.94	75/25	25	Positive
C50P50	2.09/1.91	50/50	50	Neutral
C25P75	1.07/2.93	25/75	25	Negative
Neat γ-PGA	0/4	0/100	0	Water-soluble

[a] All the chitosan–γ-PGA PEC hydrogels were fabricated with chitosan molecular weight of 3×10^5 Da, except for that a set of PEC hydrogels were fabricated with chitosan molecular weight of 3×10^4 Da for antibacterial activity test.

2.2. Preparation of PEC hydrogels

The chitosan–γ-PGA PEC hydrogels containing varied molar ratios of amine groups of chitosan to carboxylic acid groups of γ-PGA ([$-NH_2$]:[–COOH] = 75:25, 50:50, 25:75) were prepared for this study. First, chitosan powder was well dispersed in a previously prepared γ-PGA aqueous solution. The weight percentage of chitosan and γ-PGA in the prepared solution was 4, and then 1% acetic acid was added. The chitosan powder dissolved immediately due to its protonated amine groups. The homogeneous PEC hydrogel is subsequently formed through a complex formation between $-NH_2$ of chitosan and –COOH of γ-PGA. These PECs were then immersed in a 1 N NaOH aqueous solution and washed with deionized water to a pH value around 7. The neutral PEC hydrogel was further freeze-dried so a porous structure was formed; the freeze-dried PEC hydrogel was found to shrink to 75–80% of its original size. The PEC hydrogels were categorized into five groups according to the molar ratios of amine groups ($-NH_2$) of chitosan (named C) to carboxylic acid groups (–COOH) of γ-PGA (named P), and thus, the degree of complex formation was defined as complex formed in an ionic solution.

$$\text{Degree of complex formation} = [-NH_3{}^{+}{}^{-}OOC-]/([-NH_2]_C + [-COOH]_P) \times 100\% \quad (1)$$

The description of the chitosan–γ-PGA PEC hydrogels is listed in Table 1; the schematic representation of the ionic interaction formation between chitosan and γ-PGA is illustrated in Figure 1. As an example of the nomenclature used herein, C50P50 indicates that the molar ratio of amine groups ($-NH_2$) of chitosan to carboxylic acid groups (–COOH) of γ-PGA in this specimen was 50:50; the degree of complex formation = 50, which indicates that a complex formation between $-NH_2$ of chitosan and –COOH of γ-PGA was theoretically completed without free $-NH_2$ or –COOH groups in the PEC hydrogels.

The neat chitosan was prepared by the immersion–precipitation method.[25] In brief, the neat chitosan was formed with solidification by immersing the chitosan solution (4 wt % in 1% acetic acid) into 1 N NaOH. The solidified neat chitosan was then neutralized with deionized water, and freeze-dried to remove any excess water. All the specimens tested were fabricated with chitosan molecular weight of 3×10^5 Da, except that a set of specimen were fabricated with chitosan molecular weight of 3×10^4 Da for antibacterial activity test. The neat γ-PGA cannot be formed as hydrogel, as it is water soluble.

2.3. Characterization of PEC hydrogels

2.3.1. FTIR Spectroscopy

FTIR spectroscopy (Fourier transform infrared spectroscopy, Perkin–Elmer Spectrum RX1 System) was used to examine the signal variations of the amine and carboxylic acid groups of the

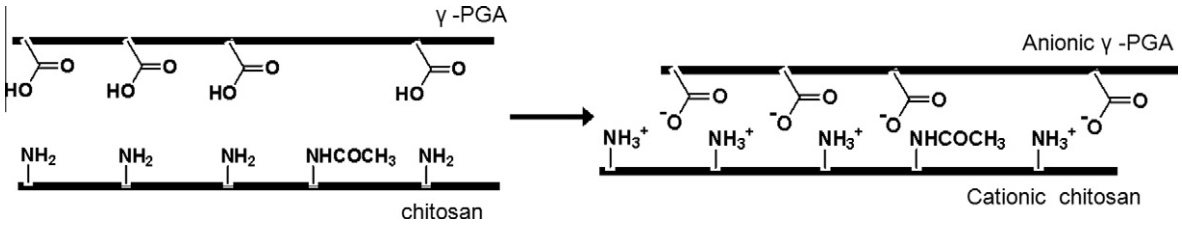

Figure 1. Schematic representation of the ionic interaction formation between chitosan and γ-PGA.