БИООРГАНИЧЕСКАЯ ХИМИЯ, 2015, том 41, № 5, с. 627–632

SYNTHESIS AND CHARACTERIZATION OF POLYSIALIC ACID/CARBOXYMETHYL CHITOSAN HYDROGEL WITH POTENTIAL FOR DRUG DELIVERY

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Received September 10, 2014; in final form March 3, 2015

A novel hydrogel was prepared from polysialic acid (PSA) and carboxymethyl chitosan (CMCS) using glutaraldehyde as the cross-linking agent. The resulting PSA–CMCS hydrogel exhibited pH sensitivity, in which the swelling ratio under acidic conditions was higher than those under neutral or alkaline conditions. The swelling ratio of PSA–CMCS hydrogel at equilibrium depended on the medium pH, the cross-linking agent concentration, and the ratio of PSA to CMCS (w/w). Bovine serum albumin (BSA) and 5-fluorouracil (5-FU) were used as model drugs to prepare hydrogel delivery systems. The loading efficiencies of the hydrogel for BSA and 5-FU were 26.25 and 36.74%, respectively. Release behaviors of BSA and 5-FU were influenced by the pH. MTT assays confirmed that PSA–CMCS hydrogel has no cytotoxicity toward the NIH-3T3 cell line; in fact, the 100% aqueous extract of the PSA–CMCS hydrogel enhanced cell growth. These results suggest that PSA–CMCS hydrogel may be a promising pH-sensitive delivery system, especially for hydrophobic chemicals.

Keywords: *polysialic acid, carboxymethyl chitosan, hydrogel, drug delivery.*

DOI: 10.7868/S0132342315040132

INTRODUCTION

Polysialic acid (PSA) is a negatively charged homopolymer of α-2,8-linked sialic acids (*N*-acetylneuraminic acid) found at the *N*-glycan terminal of neural cell adhesion molecule (NCAM) [1]. PSA can be easily produced in large-scale setups by fermentation with *Escherichia coli* [2] and presents great potential in biomedical applications. PSA exhibits prospective use as a biomaterial because of its biodegradability, biocompatibility, and non-immunogenicity [3]. Certain pathogenic bacteria, such as *Neisseria meningitis*, *E. coli* K1, *Haemophilus ducreyi*, and *Pasteurella heamolytica*, can synthesize PSA, and the polysaccharide can help these pathogens escape from mammalian immunological recognition and killing through mimicking the host's cellular oligosaccharides [4]. Based on this observation, Gregoriadis et al. [5] developed a polysialylation method for modifying therapeutic proteins to improve protein pharmacokinetics and stability. Several groups have employed this technology to modify catalase [6], asparaginase [7, 8], insulin [9], CuZn-superoxide dismutase [10], and butyrylcholinesterase [11]. Some of these proteins have been used in clinical studies, and their functions were observed to be similar to those of PEGylation. PSA may also be employed in nerve-cell tissue engineering because of the similarity of its structure to NCAM [12–14].

Functional groups, such as amino, hydroxyl, and carboxyl groups, can react with other macromolecules to produce nanoparticles, hydrogels, and microcapsules for drug delivery. In 2009, Bezuglov et al. [15] mixed PSA with insulin, interferon, and rhG-CSF to produce nanoparticles and found that PSA–insulin nanoparticles could prolong hypoglycemic ability. In 2011, Bader et al. [16] used PSA and decylamine to produce micelles that could encapsulate hydrophobic cyclosporin by self-assembly. Similarly, a PSA–epirubicin conjugate prolonged the half-life of epirubicin in vivo with low cytotoxicity [17]. In 2012, Zhang et al. [18] prepared PSA-*N*-trimethyl chitosan nanoparticles that exhibited excellent slow-release characteristics for methotrexate. Wilson et al. [19] employed PSA and polycaprolactone to prepare a self-assembled micelle for encapsulation of cyclosporin.

PSA, as a carrier in drug delivery, may improve pharmacokinetics and stability as well as anti-immunogenicity for therapeutic drugs [20]. Hydrogels are an important formulation in the development of new drug delivery technologies. In this work, PSA and car-

Abbreviations: BSA, bovine serum albumin; CMCS, carboxymethyl chitosan; GA, glutaraldehyde; 5-FU, 5-fluorouracil; PSA, polysialic acid; RGR, relative growth rate.

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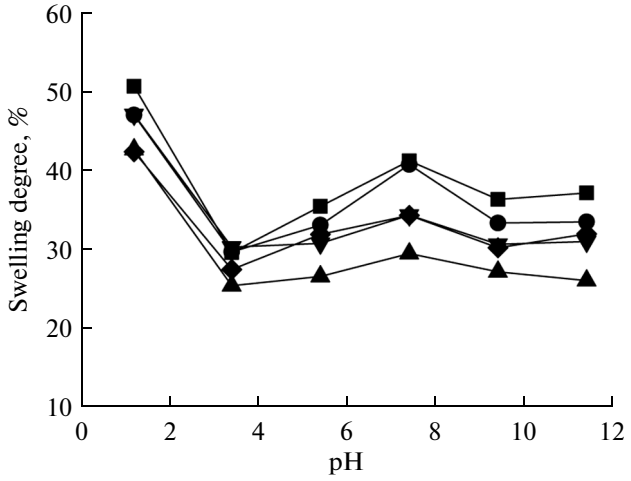

Fig. 1. Influence of weight ratio of PSA to CMCS on the pH-sensitivity of hydrogel: $m_{PSA} : m_{CMCS}$ = 1 : 10 (■), 1 : 4 (●), 1 : 2 (▲), 1 : 1(▼), 5 : 2 (◆).

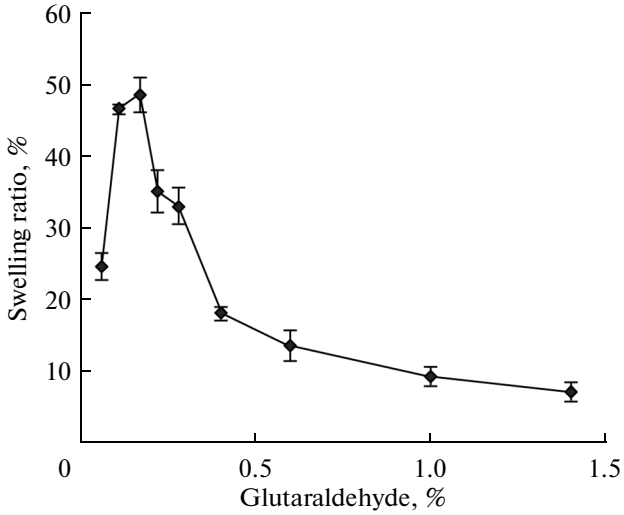

Fig. 2. Influence of glutaraldehyde on the swelling ratio of PSA–CMCS hydrogel (the $m_{PSA} : m_{CMCS}$ was set as 1 : 1, pH 7.4, 40 mM PBS).

boxymethyl chitosan (CMCS) are used to synthesize a novel functional hydrogel that may potentially be used as a drug carrier.

RESULTS AND DICUSSION

Chitosan and its derivatives are sensitive to pH because of their characteristic chemical structure. As shown in Fig. 1, PSA–CMCS hydrogel with various weight ratios showed different swelling ratios in media of different pH. The swelling ratio of the hydrogel was the highest at pH 1.2, and no linear dependence in the concentrations of PSA and CMCS were observed. This finding may be explained by protonation of the amino group ($-NH_2$) in CMCS, which increases the electrostatic repulsion and hydrophilicity of the hydrogel. At pH 3.4, CMCS was close to its isoelectric point, and the swelling ratio reached minimal levels. In this situation, the charges of $-COO^-$ and $-NH_3^+$ were equivalent, and electrostatic interactions reduced the free ions. Ionic bonding between the amino and carboxyl groups could also lead to the compact structure of the PSA–CMCS hydrogel.

When the pH was increased, the swelling ratio was improved. The swelling ratio of the hydrogel under pH 7.4 reached about 40% for the case of $m_{PSA} : m_{CMCS}$ = = 1 : 10 and $m_{PSA} : m_{CMCS}$ = 1 : 4, which indicates that relatively high amounts of CMCS render the hydrogel more liable to pH shift. Less protonated $-NH_2$ and more hydrophobic –COOH led to the loose structure of the hydrogel. At pH beyond 8.0, the swelling ratio of the PSA–CMCS hydrogel slightly decreased. In all cases, however, the hydrogel with more CMCS achieved slightly higher swelling ratios.

Glutaraldehyde (GA) was used as the cross-linking agent, and its concentration influenced the hydrogel property [21]. As shown in Fig. 2, the swelling ratio increased and then decreased as the amount of cross-linking agent increased. When the GA dose was 0.05% (w/w), the hydrogel did not form readily and the gelling time was long. When the GA dose was increased to 0.1%, the polymers formed a three-dimensional (3D) network and absorbed water, leading to a higher swelling ratio. At 0.15% GA dose, the highest swelling ratio was obtained. When the cross-linking agent was increased further, the connection points in the network could be improved, and the distances between these connections could decrease, leading to smaller pores in the hydrogel network. The resulting swelling ratio decreased with less water adsorption. The optimal dose of GA was thus determined as 0.15%.

A colorless and transparent hydrogel was prepared using PSA and CMCS. In the scanning electron microscopy (SEM) images shown in Fig. 3, the PSA–CMCS hydrogel was interconnected and showed a 3D porous structure. The distribution of pores with various sizes was heterogeneous. As a potential biomaterial, the interconnections of the pore structure could favor water and nutrient transportation. Adsorption of water into the pores would also endow the hydrogel with a high swelling rate.

When analyzed by IR spectroscopy, the spectra of chitosan (CS) and CMCS showed no significant difference (Fig. 4). In CMCS, an absorbance peak at 1608 cm^{-1}, which indicates carboxyl groups, was observed. The absorbance of –NH in CMCS at 1427 and 1334 cm^{-1} showed a slight shift compared with those of chitosan. Peaks at 1334–1600 cm^{-1} indicate the characteristic absorbance of $-NH_2$ group of CMCS. With regard to PSA, peaks at 1684 and 1402 cm^{-1} reflect the absorbance of stretching vibrations of –COOH. For the PSA–CMCS hydrogel, peaks of $-NH_2$ vibrations

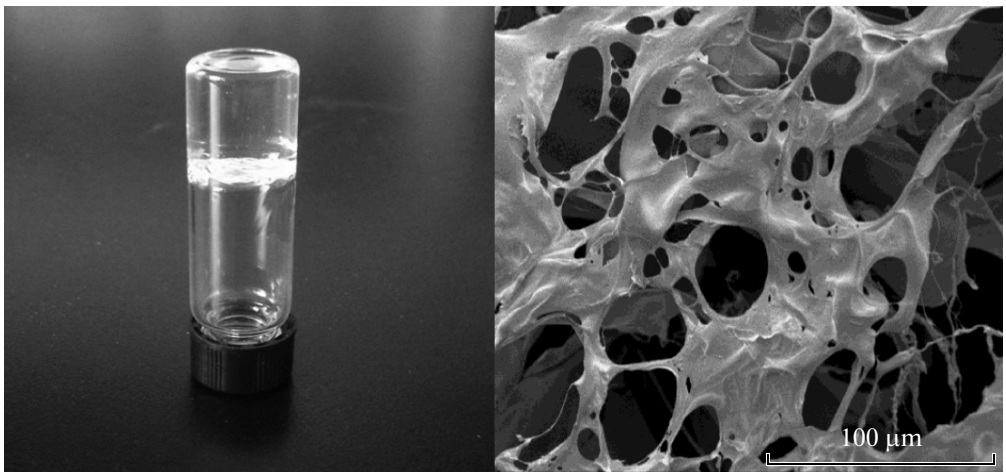

Fig. 3. Morphology and scanning emission microscopy of PSA–CMCS hydrogel.

at 1334–1600 cm^{-1} weakened. Peaks at 1684 and 1402 cm^{-1}, which indicate the –COOH of PSA, also weakened. The absorbance peak of $-NH_3^+ \cdot COO^-$ appeared at around 1620 cm^{-1}. At 1420 cm^{-1}, the peak indicating $-COO^-$ symmetrical stretching vibrations was enhanced, which illustrates the interaction of PSA and CMCS in the gelling process.

In a typical digestion process, drugs stay in the stomach for about 1 h and then move to the intestinal tract, where they stay for some time. In this work, we attempted to simulate the gastric juice (HCl at pH 1.2) and body fluid (PBS at pH 7.4) as simulative intestinal juices for in vitro testing of the loading and release behaviors of bovine serum albumin (BSA) in the hydrogel. Result showed that the drug-loading rate is 26.25 ± 0.82% (n = = 3). In terms of drug-release rate, the release rate of BSA from the hydrogel under acidic conditions occurred faster than that under neutral conditions (Fig. 5). At pH 1.2, the release rate of BSA occurred rapidly during initial stages and reached 75.78% after 8 h of incubation, which is double that at pH 7.4. At later stages, the BSA release rate stabilized to 85.86% at 24 h, likely because $-NH_2$ in the hydrogel may have become protonated and the repulsive effect of $-NH_3^+$ improved the pore size of the hydrogel, leading to the release of BSA. Under neutral conditions, only a small number of amino groups are protonated, and hydrogen bonding between amino and carboxyl groups may be en-

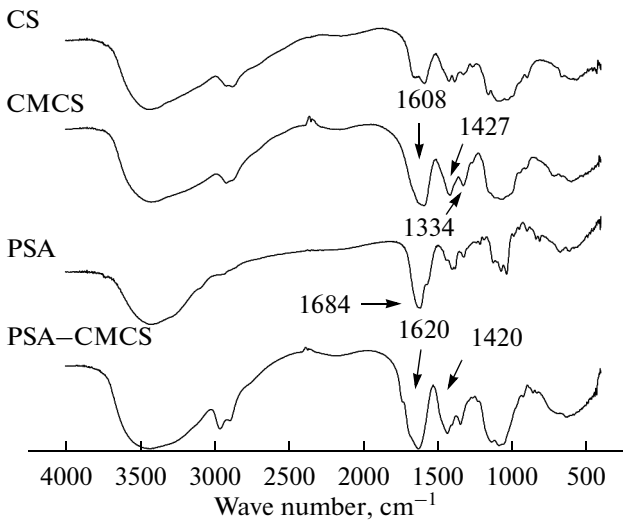

Fig. 4. Infrared spectroscopy of PSA–CMCS hydrogel.

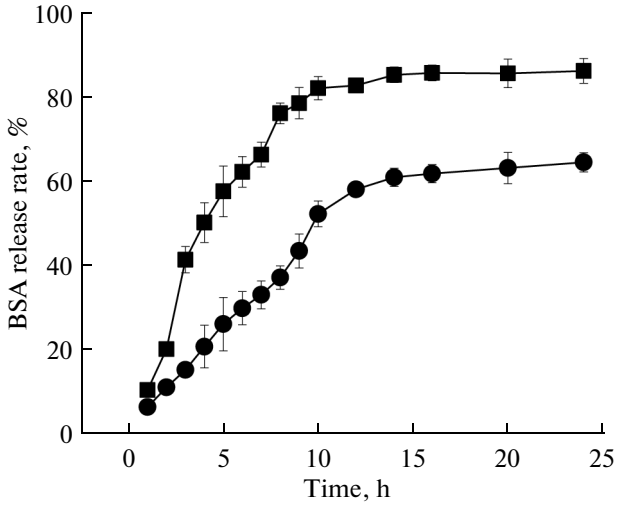

Fig. 5. Fractional release of BSA from the PSA–CMCS hydrogel as a function of time: pH 1.2 (■),7.4 (●); $m_{PSA} : m_{CMCS}$ = 1 : 1; cross-linking agent 2.5%, 0.17% GA in hydrogel.

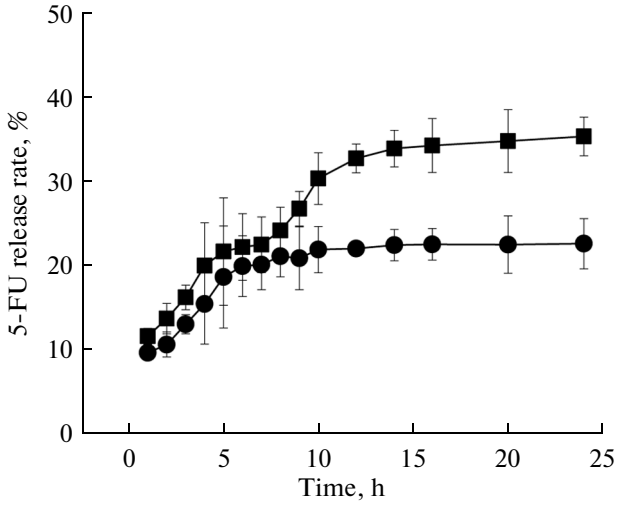

Fig. 6. Fractional release of 5-FU from the PSA–CMCS hydrogel as a function of time: pH 1.2 (■), 7.4 (●); $m_{PSA} : m_{CMCS} = 1 : 1$; cross-linking agent 2.5%, 0.17% GA in hydrogel.

hanced, leading to a compact hydrogel structure and slow BSA release rate. Consequently, the BSA release rate reached 36.74% at 8 h and 64.12% at 24 h in vitro.

The lyophilized PSA–CMCS hydrogel was soaked with 5-fluorouracil (5-FU) solution to test its loading and release rate in vitro. As shown in Fig. 6, the PSA–CMCS hydrogel showed excellent slow-release effects for low-MW chemicals; in particular, the 5-FU release rate was less than 40% after 24 h of incubation. The 5-FU release rate from the hydrogel was slightly higher under acidic conditions than that under neutral conditions. After 10 h of incubation, this difference was more prominent. 5-FU is slightly soluble in water and can be dissolved in acidic and alkaline solutions. Thus, under neutral conditions, the 5-FU release rate was fairly low.

MTT assays were performed using the NIH-3T3 cell line to determine the effect of the PSA–CMCS hydrogel on cytotoxicity and cell growth. As shown in Table, the relative growth rate (RGR) in medium with 100% aqueous extracts of hydrogel was higher than 100% (the cell growth of NIH-3T3 in the control medium was set as 100%). In medium with 50% aqueous extracts, the RGR was higher than 75%. In medium with 25% aqueous extracts, the RGR was higher than 50%. In addition, the cross-linking agent concentration used and the final GA titer, which ranged from 0.0 to 1.4%, did not show any toxicity toward cell growth. These results indicate that high titers of hydrogel aqueous extracts enhance cell growth. Thus, the MTT assays verified that the PSA–CMCS hydrogel has good biocompatibility.

This work is the first to prepare and characterize PSA-containing hydrogels for drug delivery. When PSA was combined with CMCS, the resulting hydrogel showed apparent pH sensitivity, similar to the property of chitosan and its derivatives. In contrast to other hydrogel systems [22–24], the PSA–CMCS hydrogel achieved a large swelling ratio under acidic conditions but not under neutral conditions. In addition, the physiochemical properties of the drugs to be loaded showed significant influences on the drug-loading and release rates of the hydrogel. Hydrophobicity favored the drug-loading rate and decreased the drug release rate in PSA–CMCS hydrogel. We further found that the pH of the drug release buffer affects the in vitro drug-release rate. Compared with neutral conditions at pH 7.4, acidic conditions (pH 1.2) led to quicker drug release. Taken together, the results indicate that the PSA–CMCS hydrogel may potentially be used as a gastric retention drug release system for hydrophobic drugs.

RGR and cell toxicity grades of the PSA–CMCS hydrogel

GA titer, %	100% aqueous extract		50% aqueous extract		20% aqueous extract	
	RGR	toxicity grade*	RGR	toxicity grade	RGR	toxicity grade
0.05	134.4 ± 9.1	0	87.8 ± 9.4	1	87.8 ± 9.4	1
0.1	140.1 ± 0.9	0	88.8 ± 8.7	1	88.8 ± 8.7	1
0.15	146.8 ± 17.5	0	93.6 ± 8.4	0	93.6 ± 8.4	1
0.2	165.7 ± 31.9	0	90.6 ± 8.9	1	90.6 ± 8.9	1
0.3	220.3 ± 21.8	0	142.9 ± 15.5	0	142.9 ± 15.5	0
0.4	129.7 ± 8.4	0	89.5 ± 9.2	1	73.9 ± 6.3	2
0.6	135.8 ± 21.5	0	85.0 ± 4.9	1	75.5 ± 5.0	1
1.0	147.6 ± 3.9	0	88.2 ± 2.4	1	79.5 ± 8.1	1
1.4	178.7 ± 18.0	0	89.1 ± 10.0	1	57.9 ± 8.5	2

Gelling conditions: $m_{PSA} : m_{CMCS} = 1 : 1$; cross-linking agent 2.5%, 0.17% GA in hydrogel.

* RGR and toxicity grade: grade 0–2 (no cytotoxicity), grade 3–5 (cytotoxic). The higher grade means higher toxicity for cells.

EXPERIMENTAL

Activation of polysialic acid. Exactly 100 mg of PSA (100 kDa, produced in our laboratory using *E. coli* K235) was dissolved in 10 mL of sodium periodate (0.1 M), and the resultant mixture was incubated for 15 min in the dark at room temperature. Thereafter, 20 mL of ethylene glycol was added to terminate the reaction, and the solution was shaken for 30 min. After dialyzing for 24 h in 0.02% ammonium carbonate (4°C) through a dialysis bag with a molecular weight cut-off of 120 kDa, the dialysate was lyophilized to obtain the activated PSA [10].

Preparation of PSA–CMCS hydrogel. Activated PSA solution (10 mg/mL) and CMCS solution (20 mg/mL; carboxymethyl degree >80%; viscosity 10–80 cps; Dalian Melonepharma) were mixed according to mass ratios of 1 : 10, 1 : 4, 1 : 2, 1 : 1, and 5 : 2. After incubation at 4°C for 20 min, a small amount of GA was added to obtain the PSA–CMCS hydrogel. The hydrogel was then immersed in PBS (pH 7.4, 40 mM) and deionized water sequentially for 24 h (the solution was renewed every 12 h). The hydrogel obtained was lyophilized for characterization.

Determination of the swelling ratio. The lyophilized PSA–CMCS hydrogel was weighed accurately and immersed into buffers of various pH (1.2, 3.4, 5.4, 7.4, 9.4, and 11.4). The samples were weighed at intervals until the weight of the sample reached equilibrium. Based on a preliminary experiment, the sample immersed into the buffers reached equilibrium after 24 h. Finally, the swelling ratio was calculated using the following equation:

$$\text{Swelling ratio } (\%) = 100\% \times (w_{24} - w_0)/w_0,$$

where w_{24} is the weight of the samples after immersion for 24 h and w_0 is the initial weight of the samples.

Morphology of the PSA–CMCS hydrogel. The lyophilized PSA–CMCS hydrogel was surface-treated with spray-gold and observed by SEM (S-4000, Hitachi, Tokyo, Japan).

IR spectroscopy. The PSA–CMCS hydrogel was extruded into slices with potassium bromide and then scanned by an IR-440 (Shimadzu, Japan) spectrometer in the range of 4000–400 cm^{-1}. The chitosan with degree of deacetylation of 85% (200–800 cps, Sigma, St.igma, 200n of 8) was used as control.

Characterization of adsorption and release ability in the PSA–CMCS hydrogel. PSA–CMCS hydrogel was weighed, immersed into a certain amount of 5 mg/mL BSA or 5-FU, and then left to stabilize for 24 h at 4°C. After determining the volume and BSA (or 5-FU) concentration in the residual solution, the drug-loading rate in the PSA–CMCS hydrogel was calculated.

To determine the drug release behavior of the hydrogel, the water on the surface of the balanced hydrogel was removed. The hydrogels were then immersed in 50 mL of HCl solution (pH 1.2) or PBS (pH 7.4, 50 mM). At specific intervals, 5 mL of sample was obtained to determine the BSA (or 5-FU) concentration. The drug release rate of the PSA–CMCS hydrogel was calculated using the initial and final concentrations of BSA (or 5-FU).

Analytical methods. BSA was determined by bicinchoninic acid assays [25] and 5-FU was determined according to the method described by Nair et al. [26]. The glutaraldehyde was assayed by a simple method described by Frigerio et al. [27].

Cytotoxicity assays. The PSA–CMCS hydrogel was dialyzed for 48 h and lyophilized to obtain sponge-like products. The hydrogel was then sterilized by γ-radiation for 20 s in a low-energy electron accelerator (AB5.0, Wuxi El Pont, China). Subsequently, the hydrogel was immersed into DMEM medium with 10% fetal bovine serum for 48 h to obtain an aqueous extract. DMEM medium with 10% fetal bovine serum was used as a control.

Exponential-phase NIH-3T3 mouse embryonic fibroblast cells (China Academy of Science) were diluted to produce an 8×10^4 CFU cell suspension. The cell suspension was then inoculated in 96-well plates at 100 μL aliquots for each well. After cultivation for 24 h at 37°C, the supernatant was discarded. Thereafter, 100 μL of the control solution, the negative control solution of samples with various titers, the positive control solution of sample, and solutions with 100, 50, and 20% aqueous extracts were added to the wells. The plate was cultivated for another 48 h. The supernatant was again discarded and 100 μL of DMSO was added to the wells. After mixing for 10 min, the absorbance at OD_{570} was recorded to calculate the RGR.

ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of Jiangsu Province No. BK2011158, the National Natural Science Foundation of China No. 31201384, and the Program of Introducing Talents of Discipline to Universities (111-2-06).

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Сдано в набор @@.@@.2015 г. Подписано к печати @@.@@.2015 г. Дата выхода в свет @@@@ Формат $60 \times 88^1/_8$
Цифровая печать Усл. печ. л. 15.0 Усл. кр.-отт. 2.9 тыс. Уч.-изд. л. 15.0 Бум. л. 7.5
Тираж 190 экз. Зак. 1117 Цена свободная

Учредитель: Российская академия наук,
Институт биоорганической химии им. академиков М.М. Шемякина и Ю.А. Овчинникова РАН

Издатель: Российская академия наук. Издательство “Наука”, 117997, Москва, Профсоюзная ул., 90
Оригинал-макет подготовлен МАИК “Наука/Интерпериодика”
Отпечатано в ППП “Типография “Наука”, 121099, Москва, Шубинский пер., 6