Food Chemistry 110 (2008) 923–926

Contents lists available at ScienceDirect

Food Chemistry

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

Influence of hyaluronidase addition on the production of hyaluronic acid by batch culture of *Streptococcus zooepidemicus*

Long Liu [a,b], Guocheng Du [a,b,*], Jian Chen [b,c,*], Maio Wang [d], Jun Sun [e]

[a] School of Biotechnology, Jiangnan University, Wuxi 214122, China
[b] Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China
[c] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[d] School of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[e] Institute of Information Technology, Jiangnan University, Wuxi 214122, China

ARTICLE INFO

Article history:
Received 22 November 2007
Received in revised form 18 February 2008
Accepted 27 February 2008

Keywords:
Streptococcus zooepidemicus
Hyaluronic acid
Batch culture
Hyaluronidase

ABSTRACT

Enhancement of hyaluronic acid (HA) production by *Streptococcus zooepidemicus* through increasing oxygen transfer rate via degradation of HA by hyaluronidase was investigated. Dissolved oxygen (DO) level became a limiting factor for HA production during 8–16 h, and thus hyaluronidase (0.05, 0.10, 0.15, 0.20, 0.25 g/l) was added at 8 h to degrade HA. Oxygen transfer rate coefficient and DO level during 8–16 h increased with increased hyaluronidase concentration. Compared to 5.0 ± 0.1 g/l of the control without hyaluronidase addition, HA production was increased from 5.0 ± 0.1 g/l to 6.0 ± 0.1 g/l when hyaluronidase concentration was 0.15 g/l. Further increase of hyaluronidase concentration had no effect on HA production. The molecular weight of HA decreased with the increased hyaluronidase concentration and decreased to 21 kDa when hyaluronidase concentration was 0.25 g/l from 1300 kDa of the control. The prepared low molecular weight HA (LMW-HA) could function as potential anti-angiogenic substances, antiviral and anti-tumor agents to possibly be used as functional food ingredients.

1. Introduction

Hyaluronic acid (HA) is a linear glycosaminoglycan composed of 2000–25,000 disaccharides of glucuronic acid and *N*-acetylglucosamine joined alternatively by β-(1 → 3) and β-(1 → 4) glycosidic bonds. With unique biocompatibility, hydrophilicity and viscoelasticity, HA has been widely applied in wound healing (Tian & Zhang, 2005), osteoarthritis treatment (Peyron, 1993), drug delivery (Esposito, Menegatti, & Cortesi, 2005), cosmetics and healthcare fields (Alkayali, 2006).

Conventionally HA was extracted from animal tissues like rooster combs but nowadays HA produced by microbial fermentation leads to lower production cost and more efficient purification. HA is synthesized as an extracellular capsule by pathogenic group A and C *streptococci*. HA fermentation belongs to high-viscosity aerobic fermentation where HA production is limited by low oxygen transfer rate due to the high broth viscosity (Fong Chong, Blank, Mclaughlin, & Nielsen, 2005). Much work has been done in order to optimize HA fermentation. Maxblend fermentor was designed and applied in HA production (Hasegawa, Nagatsuru, Shibutani, Yamamoto, & Hasebe, 1999). The energy and redox status of *S. zooepidemicus* was improved through the overexpression of NADH oxidase to enhance HA production (Fong Chong & Nielsen, 2003). Finally, a novel cell growth-based approach was developed to improve HA production in an industrial scale (Kim, Park, & Kim, 2006).

Since high broth viscosity and low oxygen transfer rate limit HA production (Fong Chong et al., 2005), it would be expected that the decrease of broth viscosity and the increase of oxygen transfer rate could enhance HA production. In fact, broth viscosity could be decreased by degrading high molecular weight HA (HMW-HA) into low molecular weight HA (LMW-HA). HA can be degraded by hyaluronidase (Mahoney, Aplin, Calabro, Hascall, & Day, 2001), oxidants (Šoltés et al., 2007), ultrasonication (Miyazaki, Yomota, & Okada, 2001), and UV and γ-irradiation (Reháková, Bakoš, Soldán, & Vizárová, 1994). The low molecular weight HA could a ct as potential anti-angiogenic substances and antiviral agents. HA degradation by hyaluronidase is attracting an increasing interest due to its high efficiency and mildness of reaction conditions.

This study aimed to enhance HA production by *S. zooepidemicus* through increasing oxygen transfer rate via degradation of HA by hyaluronidase. Firstly, the addition time of hyaluronidase was determined based on the kinetics of HA batch fermentation. Afterwards the effects of hyaluronidase addition on broth rheology and

* Corresponding authors. Address: School of Biotechnology, Jiangnan University, Wuxi 214122, China. Tel./fax: +86 510 85918309 (G. Du); Tel.: +86 510 85913661; fax: +86 510 85910799 (J. Chen).
E-mail addresses: gcdu@jiangnan.edu.cn (G. Du), jchen@jiangnan.edu.cn (J. Chen).

0308-8146/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2008.02.082

oxygen transfer rate were studied. Finally, the effects of HA degradation by hyaluronidase on HA fermentation were discussed.

2. Materials and methods

2.1. Microorganism and medium

Streptococcus zooepidemicus WSH-24 was used in this study. Fresh slants were cultured at 37 °C for 12 h and were used for inoculation.

Seed culture medium consisted of (g/l): sucrose 20, yeast extract 20, $MgSO_4 \cdot 7H_2O$ 2.0, $MnSO_4 \cdot 4H_2O$ 0.1, KH_2PO_4 2.0, $CaCO_3$ 20 and 1 ml trace elements solution ($CaCl_2$ 2.0 g/l, $ZnCl_2$ 0.046 g/l, $CuSO_4 \cdot 5H_2O$ 0.019 g/l).

The medium of batch fermentation contained (g/l): yeast extract 25, sucrose 70, K_2SO_4 1.3, $MgSO_4 \cdot 7H_2O$ 2.0, $Na_2HPO_4 \cdot 12H_2O$ 6.2, $FeSO_4 \cdot 7H_2O$ 0.005, and 2.5 ml of trace element solution (pH 7.2). The culture medium was sterilized at 121 °C for 15 min. Sucrose and $MgSO_4 \cdot 7H_2O$ solutions were prepared together and autoclaved separately with other culture medium to avoid the reaction of sucrose with amino acids.

2.2. Batch fermentation in 7-l fermentor

One loop of cells from a fresh slant was transferred to 50 ml of seed culture medium and cultured on a rotary shaker at 200 rpm and 37 °C for 12 h. The seed culture was inoculated into a 7-l fermentor (Model KL-7l, K3 T Ko Bio Tech, Korea) containing 4.0 l fermentation medium. Agitation was provided by three six-bladed disk turbines (diameter ratio of turbine and fermentor was 1:2.5). The pH was automatically controlled at 7.0 by adding NaOH solution (5 mol/l) and temperature was maintained at 37 °C. Aeration rate and agitation speed was 0.5 vvm and 200 rpm, respectively.

2.3. Analytical methods

Five millilitres of culture broth were mixed with two folds of ethanol, followed by centrifugation at 560*g* for 10 min. Supernatant was collected for further analysis of lactic acid, acetic acid and residual sucrose. Lactic acid and acetic acid concentrations were determined by an Agilent 1100 high-pressure liquid chromatograph (Agilent Technologies Co. Ltd., Palo Alto, CA, USA) equipped with an Agilent G1313A injector and an Agilent G1314A detector at 215 nm. The supernatant was filtered with 0.2 μm pore-size filter prior to injection into a C_{18} column (4.6 × 200 mm, Thermo Electron Corporation, Waltham, MA, USA). 0.5 mol/l KH_2PO_4 (pH 2.5) was used as mobile phase at a flow rate of 1 ml/min. The temperature of the column was maintained at 30 °C.

Sucrose concentration was determined using the resorcinol method (Ning, 1998). Cell concentration was measured from optical density (OD) of culture broth at 660 nm with a spectrophotometer (722s spectrophotometer, Shanghai Xiaoguang Co. Ltd., Shanghai, China). The correlation of OD with dry cell weight (DCW, in g/l) was DCW = 75.44 × OD + 1.85.

HA molecular weight and polydispersion index were determined by high performance gel filtration chromatography (HPGFC) with a multi-angle laser light scattering detector (MALLS). Solution was filtered with 0.2 μm pore-size filter prior to injection into a chromatograph column of Ultrahydrogel™ (300 mm × 7.8 mm i.d., Waters Corporation, Milford, MA, USA). For mobile phase, 0.1 mol/l $NaNO_3$ at a flow rate of 0.9 ml/min was used. The temperature of the column was maintained at 45 °C. The standard samples for the standard curve of molecular weight are as the following: DextranT-2000 (molecular weight: 2000 kDa), DextranT-580 (molecular weight: 580 kDa), DextranT-190 (molecular weight: 188 kDa), DextranT-70 (molecular weight: 70 kDa), DextranT-10 (molecular weight: 10 kDa), and DextranT-3 (molecular weight: 2.5 kDa).

The precipitate from above centrifugation was washed twice using distilled water, and then re-dissolved in water for analysis of HA. HA concentration was measured by the carbazole method based on uronic acid determination (Bitter & Muir, 1962). Five millilitre of borate–sulphuric acid solution were introduced into a test tube which was then cooled to 4 °C. One millilitre of HA sample was mixed thoroughly with cooled borate–sulphuric acid solution and then the mixed solution was heated for 10 min in boiling-water bath. Finally, 0.2 ml of carbazole solution was added to the tube and was heated for 15 min in a boiling-water bath. The concentration of glucuronic acid was measured by the optical density of the solution at 530 nm, at which there exists a fittest linear relationship between the concentration of glucuronic acid (the solution takes on pink due to the action of glucuronic acid with carbazole) and optical density. Oxygen mass transfer coefficient K_La was measured according to the dynamic method (Chisti & Jauregui-Haza, 2002).

2.4. Statistical analysis

Under the same condition, at least three experiments were carried out and the data were expressed as means ± standard deviation (SD).

3. Results and discussion

3.1. The kinetic analysis of HA batch fermentation

Batch fermentation of HA by *S. zooepidemicus* was carried out and the results are shown in Figs. 1 and 2. The main fermentation products of *S. zooepidemicus* were HA and lactic acid. Fig. 1 shows that HA and lactic acid concentration reached a maximum value of 5.0 ± 0.1 g/l and 53 ± 0.1 g/l at 16 h, respectively. Fig. 2 shows that, apparent viscosity of culture broth reached the highest value of 372 ± 5.0 mPaS at 16 h. Dissolved oxygen (DO) level decreased to zero at 8 h due to the increased broth viscosity and then maintained at 0–1% of air saturation during 8–16 h. DO level became a limiting factor for HA fermentation during 8–16 h and it was expected that the alleviation of DO limitation during this phase would be beneficial for the improvement of HA production.

Hyaluronidase (E.C.3.2.1.36, 999 units/mg solids) was added into culture broth (hyaluronidase concentration in culture broth: 0.05, 0.10, 0.15, 0.20, 0.25 g/l) at 8 h to degrade HA and further to increase oxygen transfer rate and DO level. Fig. 3 shows that broth viscosity decreased and DO level increased significantly with the increase of hyaluronidase concentration during 8–16 h. The average DO level during 8–16 h increased to about 1.5%, 3%, 5%,

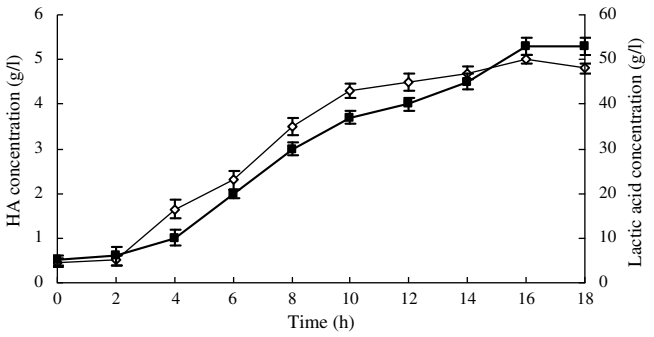

Fig. 1. Time courses of HA concentration and lactic acid concentration during HA fermentation. ◇: HA; ■: lactic acid.

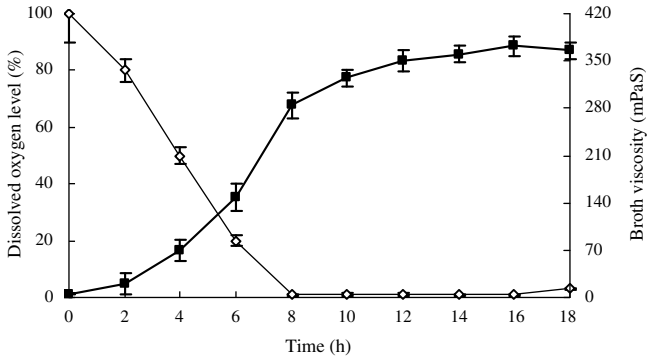

Fig. 2. Time courses of dissolved oxygen level and broth viscosity during HA fermentation. ◇: Dissolved oxygen; ■: broth viscosity.

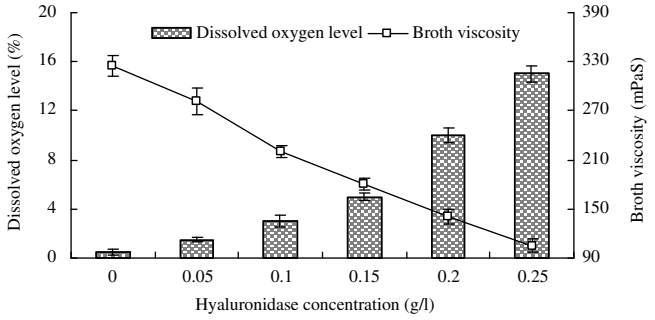

Fig. 3. The effects of hyaluronidase concentration on the dissolved oxygen level and broth viscosity during 8–16 h.

10%, and 15% when the hyaluronidase concentration was 0.05, 0.10, 0.15, 0.20 and 0.25 g/l, respectively. The increase of DO level may result from two aspects: the decrease of cell activity or the increase of oxygen mass transfer. Compared with the control (without hyaluronidase addition), biomass concentration in batch fermentation with hyaluronidase addition increased (Table 1), indicating that the increased DO level stimulated cell growth. Thus the increased DO level resulted from the increased oxygen transfer rate. Therefore, the effects of hyaluronidase addition on broth rheology and oxygen transfer rate were studied further.

3.2. The effects of hyaluronidase addition on broth rheology and oxygen transfer rate

Two representative parameters of Non-Newtonian broth rheology are consistency index K and flow behavior index n. The increase of K and the decrease of n indicate the increased broth viscosity and the strengthened Non-Newtonian characteristics. In fermentation without hyaluronidase addition, K increased and n decreased during HA fermentation (Fig. 4). Index K decreased and

Table 1
The effects of hyaluronidase addition on HA fermentation

Hyaluronidase concentration (g/l)	0	0.05	0.10	0.15	0.20	0.25
Biomass concentration (g/l)	13.6	14.2	15.3	16.7	18.9	19.0
Residual sucrose concentration (g/l)	8.6	7.3	6.2	5.3	4.7	3.1
HA concentration (g/l)	5.0	5.3	5.6	6.0	6.0	6.0
Lactic acid concentration (g/l)	53	48	43	36	30	25
Acetic acid concentration (g/l)	5.2	6.3	7.6	8.9	10.2	12.3
Molecular weight (kDa)	1300	500	200	45	32	21
Polydispersion index	1.8	1.7	1.5	1.4	1.3	1.2

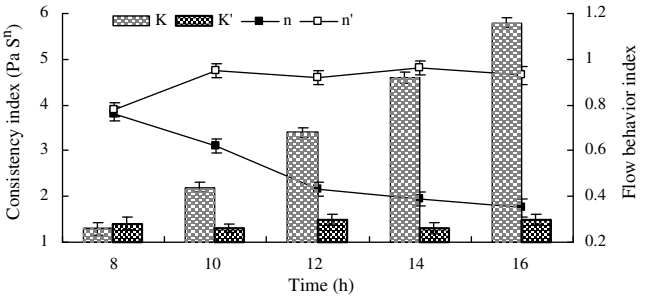

Fig. 4. The effects of hyaluronidase addition on the consistency index K and flow behavior index n of culture broth (K: Consistency index of the control; n: flow behavior index of the control; K': consistency index of the fermentation with hyaluronidase concentration of 0.25 g/l; n': flow behavior index of the fermentation with hyaluronidase concentration of 0.25 g/l).

n increased with the increase of hyaluronidase concentration, indicating the decreased broth viscosity and Non-Newtonian characteristics. Particularly, the increase of flow behavior index to about one indicated that the broth transformed into a kind of Newtonian fluid from a typical Non-Newtonian fluid when hyaluronidase concentration was 0.25 g/l (Fig. 4). Fig. 5 shows that oxygen transfer rate coefficient K_La increased significantly with the increase of hyaluronidase concentration and increased to $320 \pm 12\ h^{-1}$ with hyaluronidase concentration of 0.25 g/l from $10 \pm 1.0\ h^{-1}$ of the control. The expected increase of oxygen transfer rate coefficient K_La by HA degradation was achieved and then the effects of the increased K_La on HA fermentation were studied further.

3.3. The effects of hyaluronidase addition on HA fermentation

Table 1 shows that biomass concentration increased and residual sucrose concentration decreased with the increase of hyaluronidase concentration, indicating increased oxygen transfer rate and DO level stimulated the cell growth and the metabolic ability. The decrease of lactic acid concentration and the increase of acetic acid concentration were observed with the increased hyaluronidase concentration. The increased oxygen transfer rate inhibited the synthesis of lactic acid. The molecular weight of HA and polydispersion index (PD) of molecular weight decreased with the increase of hyaluronidase concentration. When the hyaluronidase concentration was 0.25 g/l, molecular weight decreased to 21 kDa from 1300 kDa of the control. As expected, the alleviated DO limitation resulted in the increase of HA production and HA concentration increased with the increase of hyaluronidase concentration ranging from 0 to 0.15 g/l. HA production was increased to

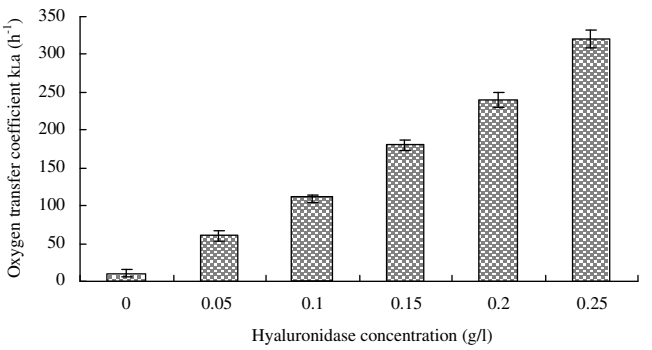

Fig. 5. The effect of hyaluronidase addition on oxygen transfer coefficient K_La during 8–16 h.

6.0 ± 0.1 g/l with the hyaluronidase concentration of 0.15 g/l from 5.0 ± 0.1 g/l of the control. However, HA production was not affected by hyaluronidase concentration from 0.15 to 0.25 g/l, indicating that there maybe existed a critical DO level for HA production, and then the effects of DO level on HA production were studied by DO-controlled fermentation. DO level was controlled automatically at a certain value (0%, 1%, 3%, 5%, 10%, and 15%) by varying agitation speed. HA production increased with increased DO level when DO level was lower than 5% of air saturation, and HA production was almost not affected by DO level when DO level was higher than 5% of air saturation. It was indicated that there existed a critical DO level of 5% air saturation for HA synthesis, in accordance with what reported by Huang, Chen, and Chen (2006). As mentioned above, the average DO level during 8–16 h was 5% when hyaluronidase concentration was 0.15 g/l, thus the further enhancement of HA production with the increase of hyaluronidase concentration was not observed. It was reported that HA synthesis could protect cells from the damage of oxygen-derived free radicals (Cleary & Larkin, 1979). In other words, an appropriate DO level could stimulate the synthesis of HA and when DO concentration was higher than critical level, 5% of air saturation, the function of stimulation was not so significant.

The prepared LMW-HA also could be used as potential anti-angiogenic substances and antiviral agents. The addition of hyaluronidase during HA fermentation not only increases the production of LMW-HA, but improves the efficiency of the purification process due to the low broth viscosity.

4. Conclusions

In the present study, HA production was enhanced by increasing oxygen transfer rate through the degradation of HA by hyaluronidase. The average DO level during 8–16 h increased to about 1.5%, 3%, 5%, 10%, and 15% when hyaluronidase concentration was 0.05, 0.10, 0.15, 0.20 and 0.25 g/l, respectively. HA production was increased to 6.0 ± 0.1 g/l with the hyaluronidase concentration of 0.15 g/l from 5.0 ± 0.1 g/l of the control. HA production was not affected by hyaluronidase concentration above 0.15 g/l. The molecular weight of HA and polydispersion index decreased with increased hyaluronidase concentration. The molecular weight of HA decreased to 45, 32, 21 kDa when hyaluronidase concentration was 0.15, 0.20, 0.25 g/l, respectively.

Acknowledgements

This project was financially supported by Program for Changjiang Scholars and Innovative Research Team in University (No. IRT0532), the National Science Fund for Distinguished Young Scholars of China (No. 20625619), and 973 Project (2007CB714306).

References

Alkayali, A. (2006). Preparation of low molecular weight hyaluronic acid as a food supplement. US patent: 20060183709.
Bitter, H., & Muir, H. M. (1962). A modified uronic acid carbazole reaction. *Analytical Biochemistry, 4*, 330–334.
Chisti, Y., & Jauregui-Haza, U. J. (2002). Oxygen transfer and mixing in mechanically agitated airlift bioreactors. *Biochemical Engineering Journal, 10*, 143–153.
Cleary, P. P., & Larkin, A. (1979). Hyaluronic acid capsule: Strategy for oxygen resistance in group A *Streptococci*. *Journal of Bacteriology, 140*, 1090–1097.
Esposito, E., Menegatti, E., & Cortesi, R. (2005). Hyaluronan-based microspheres as tools for drug delivery: A comparative study. *International Journal of Pharmaceutics, 288*, 35–49.
Fong Chong, B., Blank, L. M., Mclaughlin, R., & Nielsen, L. K. (2005). Microbial hyaluronic acid production. *Applied Microbiology and Biotechnology, 66*, 341–351.
Fong Chong, B., & Nielsen, L. K. (2003). Amplifying the cellular reduction potential of *Streptococcus zooepidemicus*. *Journal of Biotechnology, 100*, 33–41.
Hasegawa, S., Nagatsuru, M., Shibutani, M., Yamamoto, S., & Hasebe, S. (1999). Productivity of concentrated hyaluronic acid using maxblend fermentor. *Journal of Bioscience and Bioengineering, 88*, 68–71.
Huang, W. C., Chen, S. J., & Chen, T. L. (2006). The role of dissolved oxygen and function of agitation in hyaluronic acid fermentation. *Biochemical Engineering Journal, 32*, 239–243.
Kim, S. J., Park, S. Y., & Kim, C. W. (2006). A novel approach to the production of hyaluronic acid by *Streptococcus zooepidemicus*. *Journal of Microbiology and Biotechnology, 16*, 1849–1855.
Mahoney, D. J., Aplin, R. T., Calabro, A., Hascall, V. C., & Day, A. J. (2001). Novel methods for the preparation and characterization of Hyaluronic oligosaccharides of defined length. *Glycobiology, 11*, 1025–1033.
Miyazaki, T., Yomota, C., & Okada, S. (2001). Ultrasonic depolymerization of hyaluronic acid. *Polymer Degradation and Stability, 74*, 77–85.
Ning, Z. X. (1998). *Analysis of food composition*. Beijing: China Light Industry Press.
Peyron, J. G. (1993). A new approach to the treatment of osteoarthritis: Viscosupplementation. *Osteoarthritis and Cartilage, 1*, 85–87.
Reháková, Milena., Bakoš, Dušan., Soldán, Maroš., & Vizárová, Katarína. (1994). Depolymerization reactions of hyaluronic acid in solution. *International Journal of Biological Macromolecules, 16*, 121–124.
Šoltés, L., Valachová, K., Stankovská, M., Mendichi, R., Kogan, G., Arnhold, J., et al. (2007). Solution properties of high-molar-mass hyaluronans: The biopolymer degradation by ascorbate. *Carbohydrate Research, 342*, 1071–1077.
Tian, W. M., & Zhang, C. L. (2005). Hyaluronic acid hydrogel as Nogo-66 receptor antibody delivery system for the repairing of injured rat brain: In vitro. *Journal of Controlled Release, 102*, 13–25.