J Mater Sci: Mater Med (2008) 19:1127–1132
DOI 10.1007/s10856-007-3224-1

Fabrication of a PLGA-collagen peripheral nerve scaffold and investigation of its sustained release property in vitro

Bin Liu · Shao-Xi Cai · Kai-Wang Ma · Zhi-Ling Xu · Xiao-Zhen Dai · Li Yang · Cai Lin · Xiao-Bing Fu · KL. Paul Sung · Xiao-Kun Li

Received: 20 September 2006 / Accepted: 18 June 2007 / Published online: 15 August 2007

Abstract This study deals with the fabrication of a peripheral nerve scaffold prepared with poly (lactic acid -*co*- glycolic acid) [PLGA] and acellularized pigskin collagen micro particles and the investigation of its sustained release property in vitro. We took bovine serum albumin [BSA] as model drug to investigate the sustained-release property of the scaffold in vitro. The results showed the scaffold could release BSA steadily with a rate of 6.6 ng/d (r = 0.994) or so. In a 1-month test period, the accumulative release ratio of BSA from the scaffold was up to 43%, and the shape of the scaffold was still originally well kept. In addition, the scaffold outcome non-immunogenicity, good cell adhesion and biodegradability. The results indicated a scaffold constructed by this technique would be a potential implanting support with prolonged sustained release function, such as for the use of nerve scaffold.

B. Liu · S.-X. Cai · K.-W. Ma · Z.-L. Xu · X.-Z. Dai · L. Yang
College of Bioengineering, Chongqing University, Chongqing 400044, P.R. China

B. Liu
College of Life Science, Southwest University, Chongqing 400716, P.R. China

B. Liu · X.-K. Li (✉)
Engineering Research Center of Bioreactor, Ministry of Education, JiLin Agricultural University, Changchun 130118, P.R. China
e-mail: lixiaokun2@tom.com

Z.-L. Xu · L. Yang · KL. P. Sung
China–USA International Collaborative Lab, Chongqing University and UCSD, Chongqing 400044, P.R. China

C. Lin
Department of Burn, The First Affiliated Hospital of Wenzhou Medical College, Wenzhou 325000, P.R. China

X.-B. Fu
Wound Healing and Cell Biology Laboratory, Institute of Basic Medical Science, PLA General Hospital, Postgraduate Medical College, Beijing 100853, P.R. China

KL. P. Sung
Department of Orthopaedics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0412, USA

KL. P. Sung
Department of Bioengineering, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0412, USA

X.-K. Li
School of Pharmacy, Wenzhou Medical College, Wenzhou 325027, P.R. China

Introduction

The human being peripheral nerve has a strong regeneration potentiality. The disrupted peripheral nerve could successfully regenerate [1–4] if a proper environment were provided. At present, to recover the disrupted nerve is clinically carried out by autograft, but there still exist certain limits and disadvantages: the donator source limitation for autograft, and the accompanying risks in harvesting autograft [1–4]. To overcome those disadvantages, artificial nerve scaffold has been developed and showed great potentiality [3, 5–10]. Moreover, studies confirm the addition of nerve growth factors [NGFs] into artificial nerve scaffold can promote its regeneration and repair [11–15], however, due to the very short half-life time of NGFs in vivo [16], thus sustained release of NGFs is required for them to function better. PLGA, a polymer with excellent

biocompatibility and biodegradability as well as proper mechanical property, has been extensively used in surgery and tissue engineering, and as sustained release supporter [3, 17–21]. Meanwhile, collagen has been used in peripheral nerve repair for it was considered to promote cell proliferation and tissue healing [5, 6, 16, 22–25].

Bovine serum albumin [BSA] and NGFs are of protein category, and BSA has usually been taken as a model drug to investigate the sustained release property of protein from matrix [26–28]. NGFs, due to its very short half-life time, may not be an appropriate model drug for testing the sustained release property of supporter. In addition, study [29] found increase of ionic strength could enhance the thermo-stability of BSA in PBS solution. Based on the findings, in this study, we took PLGA and cell-free pigskin collagen micro particle as dual-components to construct a peripheral nerve scaffold. To pre-investigate its' property of sustained release in vitro, BSA was chosen as model drug to carry the experiment out.

Materials and methods

Preparation of cell-free pigskin collagen

The preparation method was similar to that reported in the literature [30] with modification. Briefly:

First, fresh pigskin purchased from market was cleaned in warm water (<40 °C) and then immersed in 1.0 M NaOH at 37 °C for 4–6 h to degrease, repeating the process for 3 times.

Second, the treated pigskin was immersed in 1.0 M $NaHCO_3$ for 8–10 h at 37 °C to further degrease.

Third, the treated pigskin was flushed in water for 2 days.

Fourth, the treated pigskin was immersed in the water solution (0.5% trypsin [purchased from Sigma] + 0.02 % EDTA + 0.5 million unit penicilin + 0.5 million unit phytomycin) with slightly shaking at 37 °C for 24 h.

Fifth, the treated pigskin was immersed in the buffer (10 mM PH = 8.0 Tris–HCl [purchased from Sigma] + 1-million unit antibiotics) with slightly shaking at 37 °C for 24 h.

Sixth, the treated pigskin was immersed in detergent (5.0 mM Tris–HCl pH 8.0 + 2% Triton ×–100 [purchased from Sigma] + 0.5 million unit penicilin + 0.5 million unit phytomycin) with slightly shaking at 37 °C for 3 days.

Seventh, flushing the treated pigskin in water for 3 days thus made the cell-free pigskin ECM.

The ECM was underwent H-E Staining and SEM (AMRAY 1000B, USA) to observe its structure. Then the ECM was homogenized and lyophilized (Flexi-Dry μP, FTS SYSTEM, USA), following degreased in $CHCl_3$ at room temperature for 6 h, finally the ECM was shattered and sieved through 400-mesh grit, thus cell-free pigskin collagen micro particles was prepared. The microparticles underwent ethanol sterilization before use.

Fabrication of drug-preloaded collagen support

BSA was pre-diluted to make stock solution (1.0 mg/10 mL), and then pipetted the stock solution into cell-free pigskin collagen micro particle suspension, finally mixed for uniform dispersion and then following lyophillization. Thus made drug-preloaded collagen support.

Fabrication of the scaffold

First, quantitive PLGA was dissolved into di-chloromethane (DCM); Second, addition of quantitive drug-preloaded cell-free pigskin collagen micro particle into the PLGA-DCM solution, and then following uniform dispersion of it in the solution; Third, Using a small cylinder stick as tooting, then dipping the tooting into the solution; Finally, taking the tooting out to remove solvent by air-dry.

Repeating the "dipping-drying" process for 12–15 times thus made the scaffold (See Fig. 1), and then the scaffold was vacuated at reduced pressure to remove residual solvent (DCM).

Preparation of standard curve

It was carried out according to the literature [31]. Briefly, the 96-well plates were coated with anti-BSA IgG (monoclonal, purchased from Sigma) diluted with 0.05 mol/L bicarbonate buffer, incubated at 4 °C for overnight and washed with PBS-Tween for three times. Gradient concentrations of standard BSA (>99%, purchased from Sigma) were prepared and loaded into wells respectively, while the blanks with PBS-Tween. After

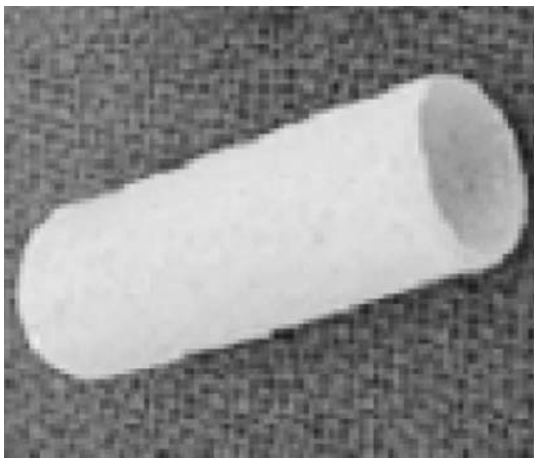

Fig. 1 The scaffold Length × Thickness × Diameter ≈ 20 mm(L) × 0.2 mm(T) × 4 mm(D)

incubation at 37 °C for 1 h, the plates were washed with PBS for three times. Then standard HRP-labeled anti-BSA IgG (monoclonal, purchased from Sigma) were added respectively and incubated at 37 °C for 1 h, while the blanks with PBS-Tween only. After washing with PBS for three times, the substrate added and incubated at room temperature away from light for 40 min, 2 mol/L H_2SO_4 was then added to terminate the reaction and the absorbance at 490 nm was recorded. The standard curve was plotted according to the absorbance and the content of samples evaluated.

Investigation on the sustained-release property of the scaffold in vitro

Weighing the scaffold (0.1 mg precisely), measuring its thickness, then immersing it into a culture flask with 10 mL PBS buffer solution containing 0.9% NaCl, finally the flask was placed upon a constant temperature swing-bed with 40 rmp at 37 °C to carry the experiment out. First, sampling 1 mL solution out of the flask and supplying 1 mL the buffer solution into the flask in the first 12 h with a 2-h interval respectively, then sampling with a 12-h interval in the following 2 days respectively, finally sampling once a day in the remaining days. The experimental period was 1 month. 1 mL solution was sampled out and 1 mL buffer solution was supplied in for each sampling. When the experiment ended, the scaffold was taken out to observe its appearance and weigh its dry weight. The absorption value was assayed as described in the section of "*Preparation of standard curve*", and at each sampling point, the record was repeatedly done for three times to get the mean value.

Cell adhesion test

Co-culturing the scaffold with SC cells (from mus' sciatic nerve) for 15 days and then observing its characteristics for SC cell adhesion to evaluate its biocompatibility.

Allergy sensitization test [32]

First, preparing the scaffold free from BSA in accordance with the process as was described in the section of "*Fabrication of the scaffold*"; second, 2 g of this scaffold was added into 30 mL saline, then following homogenation and centrifugalization, thus supernate was obtained as test sample.

Six Japanese albino rabbits were taken as test animal (female and male in half, 2 kg ± 200 g). Each rabbit was intraperitoneally injected once a day with 0.5 mL of the supernate in the first 6 days with 1-day interval. On the 14th day and the 21th day after the first injection, three of the rabbits were intravenously injected with 2 mL of the supernate from their ears, then observing those rabbits' symptoms such as scratching nose, sneezing, piloerecting, retching or coughing over three times, convulsing, reddening, gatism (urinary incontinence), shock and death.

Results

Microstructure of cell-free pigskin ECM

The well-treated cell-free pigskin ECM was stained by H-E as shown in Fig. 2. From the H-E staining result, it can be confirmed there were no cells left in the matrix.

Sustained-release performance in vitro

The total original mass of the scaffold was 48.4 mg (containing 1.2 μg BSA) with a mean thickness of 0.22 mm, while with a end residual mass of 39.8 mg after the experiment ended, thus the mass loss of the scaffold was about 18%(=(48.4–39.8)/48.4 × 100%). On the 30th day, the scaffold still kept its original shape.

Recording the A of each sample (done three parallel records and calculated their mean value), and then looking up the BSA content of each sample from the standard curve as made in the section of "*Preparation of standard curve*", finally converting the BSA content of each sample into the accumulative releasing ratio thus made a curve of accumulative releasing ratio of BSA with time as shown in Fig. 3. The formula of accumulative releasing ratio was:

$$r_i = \frac{V\sum_{i=1}^{n} C_{i-1} + C_i V_0}{m_0} \times 100\%$$

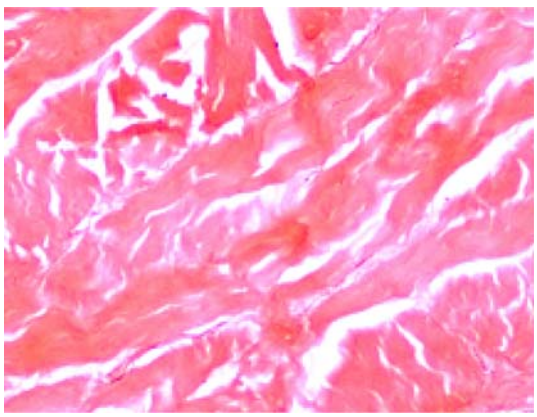

Fig. 2 Microstructure of completely acellularized pigskin ECM, stained by H-E. No cells left in the ECM

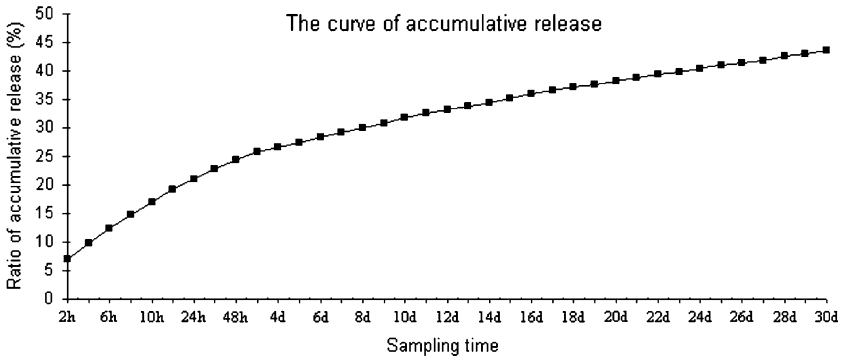

Fig. 3 Accumulative releasing ratio of BSA with time (30 days; $\bar{x}$, three parallel records for each sample)

where m_0 is the initial total *m*ass of BSA in the scaffold; n is the total sampling *n*umber of times;

i is the *i*ndex of sampling point; V_0 is the initial total Volume of degradation solution;

V is the Volume of each sampling; C_0 is the initiate Concentration of BSA in degradation solution;

C_i is the Concentration of BSA in degradation solution at each sampling point;

r_i is the accumulative ratio of BSA released from the scaffold into the degradation solution at each sampling point.

In this study, $C_0 = 0$ ng/mL, $V = 1$ mL, $V_0 = 10$ mL, $n = 37$, $m_0 = 1\ 200$ ng.

From Fig. 3 it could be seen there exists a burst release phase in the first 2 h and the burst ratio of BSA took up to 7%, then the release rate slow down. Among 2–48 h, the release rate was about 21.7 ng per sampling point ($r = 0.996$), then the release rate kept going down to a steady rate about 6.6 ng/d ($r = 0.994$). In the 30-day experimental period, the accumulative release ratio of BSA from the scaffold was up to 43%.

SC cell adhesion test

Co-culturing the scaffold with SC cell suspension for 15 days then underwent SEM scanning. The SEM outcome was shown in Fig. 4. It could be observed SC cells can adhere to the matrix and extend, thus indicates the matrix is biocompatible.

Allergy sensitization test

The rabbits were raised in cages, when shouting at the rabbits to frighten them on the 14th day and the 21th day respectively after the first injection, no symptoms as described in the section "*Allergy sensitization test*" appeared. According to the Chinese Government Standard [32], the scaffold was considered not to cause allergy sensitization.

Discussions

The human peripheral nerve has a strong regeneration potentiality [1–4], which is the clinical prerequisite for the repair of peripheral nerve lesions. Due to the limit [1] of autograft, the development of artificial nerve scaffold is clinically in demand. In previous studies, the materials for fabricating those artificial scaffolds were focused on artificial polymers with excellent biocompatibility and biodegradability or native giant molecule materials [5–8, 16, 17, 22–28]. Moreover, studies indicated the addition of NGFs into and/or the seeding of Schwann cell upon scaffolds could promote the nerve regeneration [11–13, 33–36]. However, the successful nerve regeneration was influenced by many factors, e.g. the scaffold provides a proper route and environment for the regeneration of neuraxon, while drug or nerve growth factor [11–14] can also promote its regeneration. Therefore, active compound nerve scaffold would function better than those of inactive and/or simplex ones.

In this study, we used PLGA and cell-free pigskin collagen microparticle, which have been considered excellent biocompatibility and biodegradability, to fabricate a artificial nerve scaffold in which PLGA provides mechanical strength and sustained release performance while collagen plays a role on adjusting the mechanical strength and degradation property as well as preloading hydro-soluble drug. From the sustained release curve in vitro (See Fig. 3), there exists a burst phase for the scaffold, which was a common phenomena upon encapsulating hydro-soluble drug for the purpose [37], the reason may be: BSA physically binding to the surface of the scaffold or/and the micro-pores, which could be easily flushed away in water solution, thus resulted in the burst phase at early stage. By improving the fabricating technology, the burst level could be lowered down. Subsequently, the releasing rate slowed down to a steady one, this may result from the permeation/diffusion process and/or the degradation of the scaffold. In the 1-month testing period in vitro, the ratio of

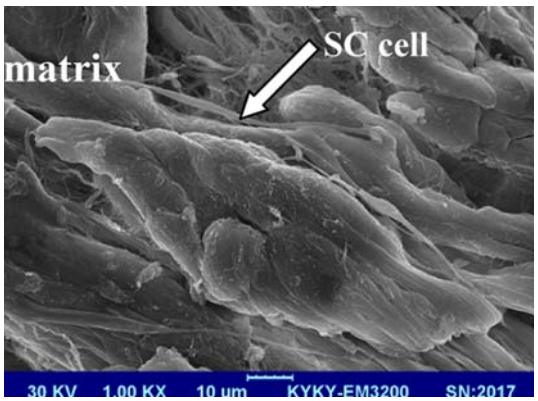

Fig. 4 SEM results of the scaffold seeded with SC cells for 15 days. SC cells adhering to the matrix and extending

accumulative release was up to 43% and the mass loss of scaffold was up to 18%. According to the hypothesis that PLGA was uniformly hydrolyzed and degradated in vivo and in vitro [37], it could be predicted that the scaffold maybe completely degradated in 3–5 months, which matched the period well with one reported by Tasuo Nakamura [5] that the optimal period for disrupted nerve repair was in the 1st–3rd month, and the repair should be completed in 6 months.

In our fabricating process, the water-soluble protein was dissolved in water at room temperature or lower, and then allowed the cell-free pigskin collagen microparticles to be uniformly dispersed in the solution, following by vacuum freeze-drying, thus could ensure the homogeneity of water-soluble protein dispersing in the matrix. Because those removed by vacuum freeze-drying are only moisture and/or evanescent solvents, the process could also achieve the quantitation of protein in the matrix, as well as avoid the denaturalization and deactivation of thermal-sensitive drug molecules, which is difficult to achieve by other technologies such as emulsification method. Meanwhile, Drying at reduced pressure could produce micropores in the scaffold, which were considered as an essential property for nerve scaffold [4, 38].

From Fig. 2, it can be ensured that no cells were left in the treated matrix. Klaus Kallenbach [30] et al has studied the property of acellularized porcine matrix. They used H-E staining and SEM to prove a completely acellularized matrix, however, they found even if the matrix was completely cell free there is still cell debris left such as protein, DNA and porcine endogenous retrovirus, and they thought no technique can achieve the goal of no cell debris left in acellularized matrix. Fortunately, from their study they found the acellularized matrix does not cause significant immunogenic response and does not transmit porcine endogenous retrovirus to the host though there was still cell debris left.

The immunogenicity evaluation for tissue-engineering scaffold could be carried out by allergization test [39]. According to the result of allergy sensitization test and the Chinese Government Standard [32], the scaffold could be considered to be non-immunogenic or clinically acceptable.

Cytotoxic test has been generally accepted for evaluating the biocompatibility of biomaterials [40]. From Fig. 4, it was shown SC cell could adhere to the matrix and extend, therefore, the scaffold could be considered biocompatible.

The scaffold constructed in this study could release the BSA steadily, if BSA were to be replaced with other water-soluble NGFs or the scaffold were to be seeded with Schwann cells [11–14, 33–36], the scaffold might become an active compound one.

Conclusion

The active compound nerve scaffold constructed by this technique possesses proper mechanical property, sustained release performance, degradation behavior, non-immunogenicity and biocompatibility, which may make it to be a potential implanting support with prolonged sustained release effect.

Acknowledgement This work got the support of the National Basic Research Program of China (973 Grant No. 2005CB522603) and "111-Project" (College of bioengineering, Chongqing University, P.R. China)

References

1. R. V. WEBER and S. E. MACKINNON, *Clin. Plast. Surg.* **32** (2005) 605
2. J. S. TARAS, V. NANAVATI and P. STEELMAN, *J. Hand Ther.* **18** (2005) 191
3. R. Y. KANNAN, H. J. SALACINSKI, P. E. M. BUTLER and A. M. SEIFALIAN, *Biotechnol. Appl. Biochem.* **41** (2005) 193
4. C. E. SCHMIDT and J. B. LEACH, *Annu. Rev. Biomed. Eng.* **5** (2003) 293
5. T. NAKAMURA, Y. INADA, S. FUKUDA, M. YOSHITANI, A. NAKADA, S. ITOI, S. KANEMARU, K. ENDO and Y. SHIMIZU, *Brain Res.* **1027** (2004) 18
6. K. MATSUMOTO, K. OHNISHI, T. KIYOTANI, T. SEKINE, H. UEDA, T. NAKAMURA, K. ENDO and Y. SHIMIZU, *Brain Res.* **868** (2000) 315
7. T. TOBA, T. NAKAMURA, Y. SHIMIZU, K. MATSUMOTO, K. OHNISHI, S. FUKUDA, M. YOSHITANI, H. UEDA, Y. HORI and K. ENDO, *J. Biomed. Mater. Res.* **58** (2000) 622
8. A. HAGIWARA, S. NAKASHIMA, T. ITOH, C. SAKAKURA, E. OTSUJI, H. YAMAGISHI, S. OKAJIMA, K. KUSUZAKI, H. HASE, S. KUBO, J. SOH, T. MIKI, T. TOBA, T. NAKAMURA and Y. SHIMIZU, *Gan To Kagaku Ryoho*. **29** (2002) 2202
9. X. WANG, W. HU, Y. CAO, J. YAO, J. WU and X. GU, *Brain* **128** (2005) 1897

10. Y. INADA, S. MORIMOTO, Y. TAKAKURA and T. NAKAMURA, *Neurosurgery* **55** (2005) 640
11. L. M. LEE, M. C. HUANG, T. Y. CHUANG, L. S. LEE, H. CHENG and I. H. LEE, *Life Sciences* **74** (2004) 1937
12. K. IWAYA, K. MIZOI, A. TESSLER and Y. ITOH, *Neurosurgery* **44** (1999) 590
13. M. S. RAMER, J. V. PRIESTLEY and S. B. MCMAHON, *Nature* **403** (2000) 312
14. Z. SAHENK, H. N. NAGARAJA, B. S. MCCRACKEN, W. M. KING, M. L. FREIMER, J. M. CEDARBAUM and J. R. MENDELL, *Neurology* **65** (2005) 681
15. C. DEISTER and C. E. SCHMIDT, *J. Neural Eng.* **3** (2006) 172
16. A. K. KITAHARA, Y. SUZUKI, P. QI, Y. NISHIMURA, K. SUZUKI, T. KIYOTANI, Y. TAKIMOTO, T. NAKAMURA, Y. SHIMIZU and K. ENDO, *J. Plast. Reconstr. Surg. Hand. Surg.* **33** (1999) 187
17. S. SAHOO, H. OUYANG, J. C. GOH, T. E. TAY, and S. L. TOH, Transactions -7th World Biomaterials Congress, (2004) p. 8
18. R. YANG, T. CHEN, H. CHEN, and W. WANG, in Proceedings of SPIE-The International Society for Optical Engineering, v 5345, Microfluidics, BioMEMS, and Medical Microsystems II, (2004) p. 130
19. J. PARK, M. MELHEM, MURAD, and D. PANKAJ, in "Advances in Bioengineering—Vol. 55", (American Society of Mechanical Engineers, Bioengineering Division (Publication) BED, 2003) p. 11
20. Y. DONG and S.-S. FENG, *Biomaterials* **26**, (2005) 6068
21. S. DUVVURI, K. G. JANORIA and A. K. MITRA, *J. Control. Release* **108** (2005) 282
22. G. KEILHOFF, F. STANG, G. WOLF and H. FANSA, *Biomaterials* **24** (2003) 2779
23. S. YOSHII and M. OKA, *Brain Res.* **888** (2001) 158
24. M.R. AHMED, S. VAIRAMUTHU, M. SHAFIUZAMA, S. H. BASHA and R. JAYAKUMAR, *Brain Res.* **1046** (2005) 55
25. F. STANG, H. FANSA, G. WOLF, M. REPPIN and G. KEILHOFF, *Biomaterials* **26** (2005) 3083
26. F. KANG and J. SINGH, *Int. J. Pharmaceut.* **260** (2003) 149
27. M. VAN DE WEERT andW. E. HENNINK, W. JISKOOT, Pharm. Res. 17, (2000) 1159
28. G. JIANG, B. H. WOO, F. KANG, J. SINGH and P. P. DELUCA, *J. Control. Release* **79** (2002) 137
29. M. YAMASAKI, H. YANO and K. AOKI, *Int. J. Biol. Macromol.* **12** (1990) 263
30. K. KALLENBACH, R. G. LEYH, E. LEFIK, T. WALLES, M. WILHELMI, S. CEBOTARI, A. SCHMIEDL, A. HAVERICH and H. MERTSCHING, Biomaterials **25**, (2004) 3613
31. M. A.AKASHI, The Research Foundation for Microbial Disease of OSAKA University, (1999) p. 10
32. GB/T 16886.10, Standard Press of China, (2005)
33. S.-H. HSU, C.-J. CHANG, C.-M. TANG and F.-T. LIN, *J. Biomat. Appl.* **19** (2004) 163
34. S. RAIMONDO, S. NICOLINO, P. TOS, B. BATTISTON, M. G. GIACOBINI-ROBECCHI, I. PERROTEAU and S. GEUNA, *J. Comp. Neurol.* **489** (2005) 249
35. K. YU and J. D. KOCSIS, *J. Neurophysiol.* **94** (2005) 1519
36. A. I. GRAVVANIS, A. LAVDAS, A. E. PAPALOIS, I. FRANCESCHINI, D. A. TSOUTSOS, M. DUBOIS-DALCQ, R. MATSAS and J. D. IOANNOVICH, *Microsurgery* **25** (2005) 423
37. A. KAMIJO, S. KAMEI, A. SAIKAWA, Y. IGARI and Y. OGAWA, *J. Control. Rel.* **40** (1996) 269
38. C. -J. CHANGA andS.-H. HSU, Biomaterials **27** (2006) 1035
39. F. DELUSTRO, R. A. CONDELL, M. A. NGUYEN and J. M. MCPHERSON, *J. Biomed. Mater. Res.* **20** (1986) 109
40. S. STIVASTAVA and D. G. STEPHEN, *Biomaterials* **11** (1990) 133