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RESEARCH PAPER

The Implantable and Biodegradable PHBHHx 3D Scaffolds Loaded with Protein-Phospholipid Complex for Sustained Delivery of Proteins

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ABSTRACT

Purpose PHBHHx (poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)) is an excellent biomaterial for tissue repair. Here, we aim to develop a PHBHHx-based three-dimensional (3D) scaffold system for sustained delivery of proteins (insulin serves as a model protein).

Methods The insulin-phospholipid complex (INS-PLC) was prepared to enhance the insulin lipophilicity. INS-PLC loaded PHBHHx 3D scaffolds (INS-PLC-SCAs) containing PEG-2000 were fabricated by lyophilization. *In vitro* release was performed in the medium with or without lipase. The bioactivity of INS-PLC-SCAs was measured in diabetic rats.

Results *In vitro* release shows that the release rate of INS-PLC-SCAs was very slow (~6% of total insulin was released within 120 days), and PEG-2000 or lipase had no effect on its release pattern. The bioactivity test shows that the hypoglycaemic effect of insulin was maintained after formulated into scaffolds. After subcutaneous (s.c.) implantation, its therapeutic effect lasted for over 130 h, and its bioavailability was enhanced by 4-fold.

Conclusions PHBHHx based 3D scaffold has a great potential for sustained delivery of proteins, especially growth factors. When growth factors are incorporated, it can serve as a bifunctional system that provides a porous skeleton for cells attachment and proliferation, as well as a matrix for long term release of the loaded growth factors.

KEY WORDS drug delivery · insulin · PHBHHx scaffold · phospholipid complex · tissue engineering

ABBREVIATIONS

3D	three-dimensional
AAC	area above the curve
B-SCAs	blank PHBHHx 3D scaffolds
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
HPLC	high performance liquid chromatography
INS-PLC	insulin-phospholipid complex
INS-PLC-SCAs	INS-PLC loaded PHBHHx 3D scaffolds
PA	pharmacological availability
PEG	polyethylene glycol
PHBHHx	poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
s.c.	subcutaneous
SEM	scanning electronic microscopy
STZ	streptozotocin

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INTRODUCTION

In the past few decades, increasing attentions have been paid to developing novel biodegradable materials based systems for protein delivery or tissue engineering use (1–3). Growth factors, a kind of bioactive protein, have been considered as critical for tissue repair because they play important roles in stem cells growth and differentiation (4–7). Growth factors are like some other proteins, such as insulin, whose concentration is needed to maintain within a certain range for a long period. Unfortunately, the sustained delivery of growth factors seems difficult since these factors delivered with a bolus injection will be cleared rapidly from the injection site (8,9). Therefore, in order to make tissue repair highly efficient, it is necessary to develop a bifunctional system that can not only serve as a skeleton for stem cells attachment and growth, but also as a matrix for sustained delivery of growth factors. Such a bifunctional system should be based on an appropriate biomaterial that can match the above requirements.

Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), a member of microbial polyhydroxyalkanoates (PHA) biopolyester family, has been widely studied on its biological properties and applications (10,11). It also has been extensively used as either films or scaffolds for proliferation and differentiation of various stem cells in the field of tissue engineering due largely to its adjustable mechanical properties, nice biocompatibility and biodegradability, as well as non-cytotoxicity (12–18). In addition, our previous work has demonstrated its ability to control insulin release in a sustained manner (19). Hence, PHBHHx based drug delivery system is potential to prolong the release profile and improve the low bioavailability of proteins caused by instability and rapid removal from the local site (20–23), and growth factors loaded PHBHHx three-dimension (3D) scaffold can serve as an above mentioned bifunctional system.

As for this bifunctional system, the homogenous protein dispersion throughout the scaffold is an essential requirement (24,25), as the uniformity of protein dispersion influences its release pattern. It can be predicted that the non-uniform dispersion of protein in the biodegradable scaffold may probably lead to an unpredictable pulse-like release which has risks of inducing side effects. Nevertheless, the remarkable difference in chemical properties between proteins (hydrophilic for majority of proteins) and lipophilic PHBHHx makes it difficult to fabricate PHBHHx scaffolds with homogenous protein dispersion. One approach to solve this problem is to incorporate drug loaded nano-/microparticles into the scaffolds (26,27), but this method will also lead to an increase in fabrication difficulty and a decrease in drug loading capacity due to the introduction of particles. Another possible approach is to enhance the lipophilicity of the loaded protein. Our previous study has demonstrated that the lipophilicity of insulin was significantly enhanced by formation of insulin phospholipid complex (19). The increased lipophilicity of protein can enhance its affinity to PHBHHx and thus be beneficial to its uniform distribution throughout the PHBHHx scaffolds.

To our best knowledge, the PHBHHx 3D scaffolds loaded with protein phospholipid complex has never been reported. Therefore, the aim of this present study is to construct a novel platform for sustained delivery of proteins by incorporating the protein phospholipid complex (insulin is served as the model protein) into the PHBHHx 3D scaffolds, and thus to provide an efficient strategy for sustained delivery of growth factors.

MATERIALS AND METHODS

Materials

Phospholipids (soybean lecithin, with phosphatidylcholine content of 70–97%) were purchased from Shanghai Tai-wei Pharmaceutical Co. Ltd. (Shanghai, China). Pure crystalline porcine insulin was purchased from Xuzhou Wanbang Bio-Chemical Co. Ltd. (Jiangsu, China). PHBHHx (M_W=380,000) containing 11 mol% of R-3-hydroxyhexanoate (HHx) was kindly provided by Prof. Guo-Qiang Chen (Department of Biological Science and Biotechnology, Tsinghua University, Beijing, China). Streptozotocin (STZ), fluorescein isothiocyanate (FITC), and Coumarin 6 were purchased from Sigma-Aldrich (St. Louis, USA). All other chemical reagents were of analytical grade or better.

Preparation of Insulin Phospholipid Complex (INS-PLC) Solution

INS-PLC was prepared according to our previous method (19) with a bit of modification. Briefly, at a molar ratio of 1:120 insulin and phospholipid were dissolved in DMSO (dimethylsulfoxide) containing 5% (v/v) of acetic acid. The mixture was magnetically stirred at 30°C for 24 h. The resultant INS-PLC solution was subsequently used to fabricate 3D scaffolds without any other treatment.

Preparation of INS-PLC Loaded PHBHHx 3D Scaffolds (INS-PLC-SCAs)

INS-PLC-SCAs were fabricated by lyophilization method (28,29). Initially, 20% (w/v) PHBHHx solution was prepared by dissolving PHBHHx in DMSO with magnetic stirring at 60°C for 30 min. After equilibrating the PHBHHx solution and INS-PLC solution prepared above to 37°C, the two solutions were mixed together with equal

volume under continuous stirring at 37°C. Ten minutes later, the resultant mixture was added into a 96-well plate with 250 μl per well and lyophilized. The obtained INS-PLC-SCAs were sealed hermetically and stored at room temperature. In order to investigate the effect of a hydrophilic substance contained in the scaffolds on the release pattern, PHBHHx scaffolds containing different concentration of PEG-2000 (0%, 1.5%, 3%, w/v, presented as INS-PLC-SCAs-0%, INS-PLC-SCAs-1.5% and INS-PLC-SCAs-3%, respectively.) were prepared. In addition, the blank scaffolds (B-SCAs) without INS-PLC were also prepared using the same procedure described above.

Content Determination of Insulin Loaded in INS-PLC-SCAs

The three types of INS-PLC-SCAs were dissolved in 2 ml of chloroform, respectively. After removing the organic solvent by evaporation at 40°C under vacuum, 1.5 ml of extracting solution containing acetonitrile, water and trifluoroacetic acid with a volume ratio of 32:68:0.1 was added in, followed by sonication for 3 min to extract insulin. The resulting suspension was centrifuged at 8,000 rpm for 5 min and the supernatant was collected. The extraction process was performed three times, and the supernatant was combined and diluted to 5 ml followed by filtration through a membrane (0.45 μm). The filtrate was injected into a HPLC system to measure the insulin content. Three scaffolds for each type were used for test.

Drug Dispersion Throughout PHBHHx 3D Scaffolds

In order to observe the drug dispersion throughout PHBHHx scaffolds, fluorescent dyes FITC and Coumarin 6 were used to represent hydrophilic and lipophilic molecules, respectively. The FITC loaded scaffolds (FITC-SCAs) and Coumarin loaded scaffolds (Coumarin-SCAs) were prepared as follows: FITC (300 μg/ml) and Coumarin (100 μg/ml) were dissolved in DMSO, respectively. PHBHHx solution in DMSO (10%, w/v) was prepared under stirring at 60°C and then cooled down to 37°C. Subsequently, FITC/PHBHHx mixture and Coumarin/PHBHHx mixture were prepared by adding 4 drops of FITC solution and 2 drops of Coumarin solution to 1 ml of PHBHHx solution under stirring, respectively. The mixture was transferred to a 96-well cell culture plate with 250 μl per well followed by lyophilization for 24 h. The obtained scaffolds were transversely cut into small pieces and the drug dispersion was observed using a fluorescence imaging system (LT-99D2 Illumatool Dual Light System, Lightools Research, Encinitas, CA) with a built-in CCD camera.

In Vitro Release of INS-PLC-SCAs

The INS-PLC-SCAs with different concentration of PEG-2000 were placed in centrifuge tubes and 3 ml of release medium (PBS with pH of 7.4) was added in. The tubes were shaken in a horizontal shaker under controlled conditions (70 rpm, 37°C). At fixed time intervals, the release medium was collected and replaced with 3 ml of fresh medium. After appropriate dilution the collected medium was filtered through a membrane (0.45 um) and stored at −80°C till further analysis. The insulin content was measured using the ELISA Kit according to the manufacturer's instruction. Additionally, the release of INS-PLC-SCAs in the presence of 0.2 mg/ml of lipase (type II from porcine pancreas, Sigma) was performed to investigate the effect of lipase on PHBHHx degradation and release pattern *in vitro*. All the studies were performed in triplicate.

In Vitro Degradation of INS-PLC-SCAs in the Release Medium

Following the *in vitro* release studies the INS-PLC-SCAs were collected and lyophilized. The size of these INS-PLC-SCAs was measured using a vernier calliper, followed by calculating the volume loss ratio. The morphology was observed by scanning electronic microscopy (SEM), and compared with the INS-PLC-SCAs before the *in vitro* release studies.

Scanning Electronic Microscopy (SEM)

The morphology of the INS-PLC-SCAs and B-SCAs was observed using SEM (JSM-5900LV, JEOL Ltd. Japan). The samples were coated with gold using an ion sputter and observed at an accelerating voltage of 20 kV.

In Vivo Studies in Rats

Healthy male Sprague–Dawley (SD) rats (200–250 g) were purchased from Laboratory Animal Centre of Sichuan University (Chengdu, China). All the animal experiments including the use of arterial clamp on rats were approved by Institutional Animal Care and Use Committee of Sichuan University. Rats were housed in cages (5 rats per cage) under controlled conditions (25°C, 55% air humidity) with free access to water and standard rat chow. The rats were acclimatized for at least 7 days before use.

In Vivo Degradation of Blank PHBHHx Scaffolds (B-SCAs) in Normal Rats

The *in vivo* degradation studies were performed by subcutaneously inserting B-SCAs into the normal rats according to the instruction of ISO 10993: International Standard

Biological evaluation of medical devices, Part 6: Tests for local effects after implantation 2007.

The rats were divided into three groups (3 rats in each group) and inserted with B-SCAs-0%, B-SCAs-1.5% and B-SCAs-3%, respectively. The implantation was operated as follows: the rats were anesthetized with ether vapour. A small incision (~7 mm) was made along the dorsal line of each rat and a subcutaneous pocket was created by blunt dissection of the connective tissue. The B-SCAs were inserted into these created pockets (one whole scaffold for each rat) and the wound was closed using artery clamps which were removed 1 week later. Ninety days later, the rats were sacrificed and the B-SCAs were collected carefully followed by lyophilization. The size of the lyophilized B-SCAs was measured with the vernier calliper.

Bioactivity of Insulin

The bioactivity of insulin loaded in INS-PLC-SCAs was tested in diabetic rats. The STZ (streptozotocin) induced diabetic rats were divided into 4 groups with 5 rats in each group. Group 1, serving as a control group, was inserted with B-SCAs-3% to investigate the effect of scaffold matrix PHBHHx and PEG-2000 on blood glucose control. Group 2, 3, 4 were inserted with INS-PLC-SCAs-0%, INS-PLC-SCAs-1.5% and INS-PLC-SCAs-3% at an insulin dose of 4 IU/kg, respectively. At fixed time intervals, the blood glucose level was measured using a Blood Glucose Monitoring System (ACCU-CHEK® Integra blood glucose meter, Roche Diagnostics GmbH) according to the manufacturer's instruction. The glucose level change was presented as a blood glucose level-time curve. The area above the curve (AAC) was used to evaluate the pharmacological availability (PA) according to the following equation.

$$PA = (AAC_a/Does_a)/(AAC_b/Does_b) \times 100\%$$

where the subscript "a" and "b" represented different groups.

Statistical Analysis

The data were presented as mean ± s.d. (standard deviation). The one-way analysis of variance (ANOVA) was used for comparison between any two groups. The difference between two groups with the p value less than 0.05 was considered to be statistically significant.

RESULTS

Preparation and Characterization of PHBHHx 3D Scaffolds

All the INS-PLC-SCAs are white in colour and cylindrical in shape, with height, diameter and volume of ~7 mm, ~6 mm and ~200 mm^3, respectively (Supplementary Material Figure S1a and Figure S2). The surface and cross section morphology of INS-PLC-SCAs are shown in Figs. 1a and 2a, respectively. It is clear that the original INS-PLC-SCAs possess a porous structure with mean pore size of ~100 μm. These pores may provide pathways for the flow and diffusion of liquid medium and play an important role in the release and transport of loaded insulin.

The insulin content determined by HPLC is 9.43±0.13, 9.61±0.38 and 9.67±0.57 IU for INS-PLC-SCAs-0%, INS-PLC-SCAs-1.5% and INS-PLC-SCAs-3%, respectively.

Drug Dispersion Throughout the PHBHHx Scaffolds

As shown in Fig. 3, fluorescence distribution is not uniform in FITC-SCA (Fig. 3b), but much more uniform in Coumarin-SCA (Fig. 3c), suggesting that enhancing the lipophilicity of the loaded molecule is beneficial to its homogeneous dispersion throughout the PHBHHx scaffolds. This result also demonstrates the necessity of formation of INS-PLC.

In Vitro Release of INS-PLC-SCAs

The *in vitro* release profiles of INS-PLC-SCAs are shown in Fig. 4. The insulin release in the absence of lipase was very slow, with cumulative release amount within 120 days of only 6.14%, 5.92% and 5.87% for INS-PLC-SCAs-0%, 1.5% and 3%, respectively (Fig. 4a). It is interesting to find out that the similar results were obtained when the *in vitro* release studies were performed in the medium containing lipase (Fig. 4b). The insulin release rate was also quite slow with cumulative release amount within 120 days of only 6.18%, 5.66% and 5.59% for INS-PLC-SCAs-0%, 1.5% and 3%, respectively. Furthermore, the statistical analysis shows that there is no significant difference between any two release profiles, indicating that the *in vitro* release pattern of insulin from INS-PLC-SCAs was neither influenced by PEG-2000 loaded in scaffolds when PEG content was not above 3%, nor by the lipase contained in the release medium.

In Vitro Degradation of INS-PLC-SCAs in the Release Medium

INS-PLC-SCAs showed little change in shape or appearance after the *in vitro* release studies in the absence (Supplementary Material Figure S1b) or presence of lipase (Supplementary Material Figure S1c). However, an obvious change in surface morphology was observed (Fig. 1), which was probably due to a small part of matrix exfoliation from the scaffolds surface. In contrast, little change was found in the inner structure of INS-PLC-SCAs (Fig. 2), indicating that the degradation of PHBHHx matrix was quite low *in vitro*. The low degradation of INS-PLC-SCAs could be further demonstrated by their size change. Only 9%~10% of volume loss in all types of INS-

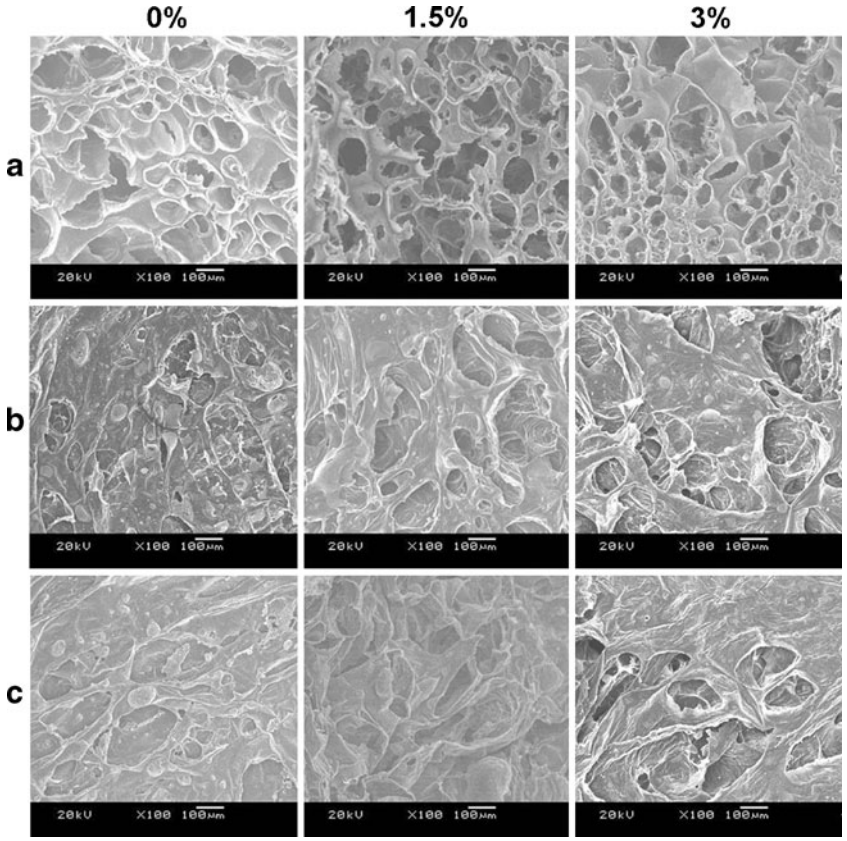

Fig. 1 SEM images of INS-PLC-SCAs (surface). (**a**) Original INS-PLC-SCAs; (**b**) 120 days after the *in vitro* release in the absence of lipase; (**c**) 120 days after the *in vitro* release in the presence of lipase. Scale bar: 100 μm.

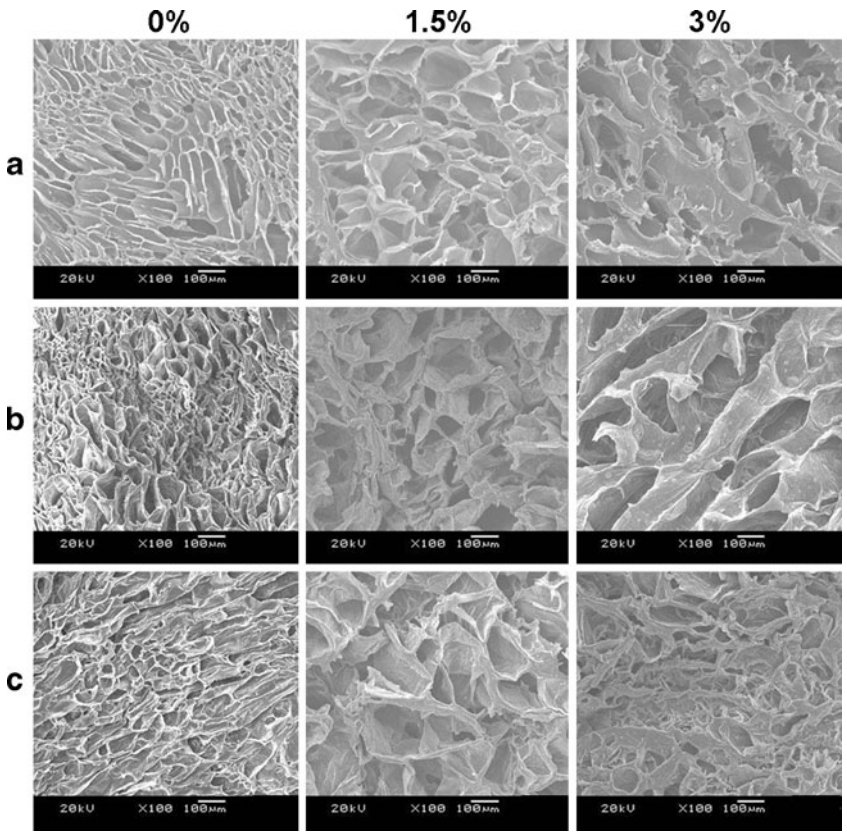

Fig. 2 SEM images of INS-PLC-SCAs (cross section). (**a**) Original INS-PLC-SCAs; (**b**) 120 days after the *in vitro* release in the absence of lipase; (**c**) 120 days after the *in vitro* release in the presence of lipase. Scale bar: 100 μm.

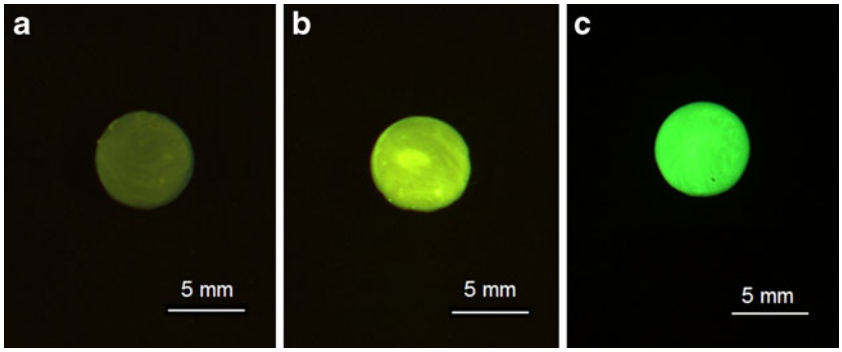

Fig. 3 Drug distribution throughout the PHBHHx 3D scaffolds observed by fluorescent imaging system. (**a**) Blank scaffold (B-SCA); (**b**) FITC loaded scaffold (FITC-SCA); (**c**) coumarin loaded scaffold (Coumarin-SCA).

PLC-SCAs was observed despite of the presence of lipase (Fig. 5), and the volume loss was probably caused by the matrix exfoliation from the scaffolds surface. In addition, the statistical analysis shows that there is no significant difference in volume loss ratio *in vitro* for all involved INS-PLC-SCAs, which is consistent with the *in vitro* release results.

In Vivo Degradation of B-SCAs in Normal Rats

The *in vivo* degradation was studied to preliminarily observe the degradation speed of PHBHHx scaffolds in physiological environment and compare with the *in vitro* degradation. The operation wound on rat skin was healed completely after removal of artery clamps at 1 week post-implantation. Three months later, the implanted B-SCAs were connected with s.c. tissue and maintained their skeleton (Supplementary Material Figure S3), but showed significant changes in appearance and size (Supplementary Material Figure S4). As shown in Fig. 5, the volume loss ratio of B-SCAs *in vivo* after implantation for 3 months is 20–35% without significant difference among them, but significantly higher than that *in vitro* ($p<0.05$), indicating that the degradation of PHBHHx scaffolds was faster *in vivo* probably due to the presence of various degradation enzymes in physiological environment.

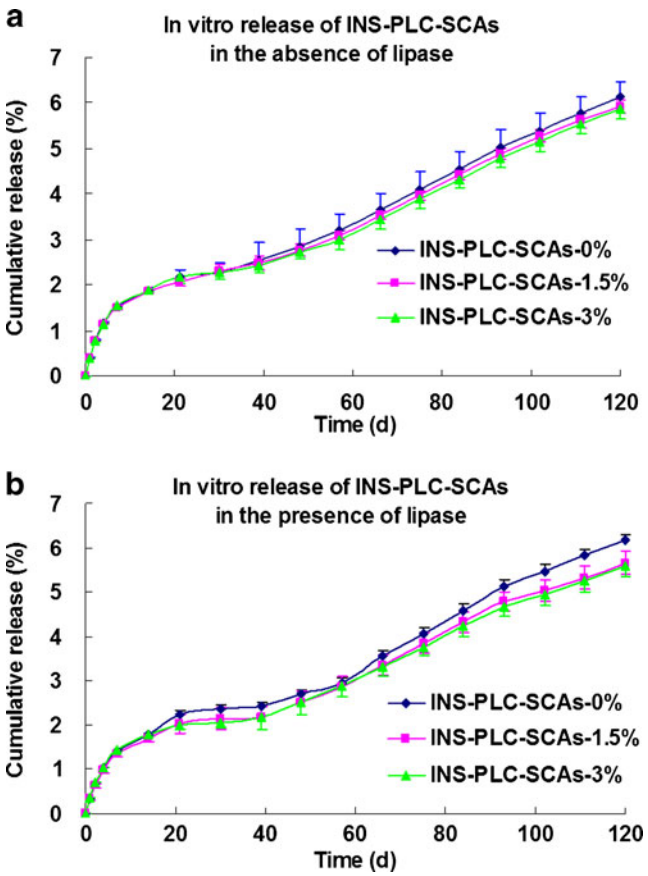

Fig. 4 *In vitro* release profiles of INS-PLC-SCAs in PBS (pH 7.4) at 37°C, (**a**) in the absence of lipase; (**b**) in the presence of lipase (0.2 mg/ml). Data presented as mean ± s.d. ($n=3$).

Bioactivity of Insulin in Diabetic Rats

Maintaining the bioactivity of the loaded drug is essential in developing a drug delivery system. As shown in Fig. 6, B-SCAs showed no hypoglycemic effect throughout the study, indicating that the scaffold matrix had no bioactivity in blood glucose control. In contrast, the diabetic rats showed similar responses after s.c. implantation of 3 types of INS-PLC-SCAs (4 IU/kg), i.e. the blood glucose level decreased rapidly and significantly. The relative blood glucose level (RBGL, percentage of the blood glucose level at any time

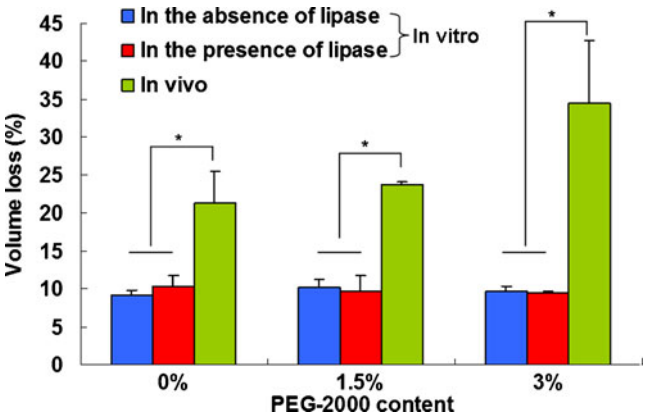

Fig. 5 Volume loss of PHBHHx scaffolds *in vitro* and *in vivo*. Data presented as mean ± s.d. ($n=3$). Statistically significant difference between *in vitro* and *in vivo* groups: $*p<0.05$.

relative to the initial level) reached 70% (this value is considered as the threshold of therapeutic effect (19)) within 2 h post-implantation. Furthermore, the therapeutic effect of all INS-PLC-SCAs lasted for more than 130 h (Table I), indicating that INS-PLC-SCAs also showed a sustained release property *in vivo*. Statistical analysis shows that there is no significant difference in blood glucose level among the three types of INS-PLC-SCAs at any time point, suggesting that PEG-2000 had no effect on insulin release *in vivo* when its content in scaffolds was not above 3% (w/v). Additionally, the AAC (the area above the curve) of all INS-PLC-SCAs was higher than 70 (Table I), while the AAC of insulin solution after s.c. injection at a dose of 1 IU/kg was only 3.86 (See our previous work (19)). As such, the pharmacological availability (PA) of INS-PLC-SCAs relative to insulin solution was higher than 400% (Table I). These results suggest that INS-PLC-SCAs can not only maintain the bioactivity of insulin, but can also significantly enhance the bioavailability of insulin. Therefore, PHBHHx 3D scaffold may be a promising system for sustained delivery of proteins.

Table I Pharmacodynamic Parameters after S.C. Implantation of B-SCAs and INS-PLC-SCAs into Diabetic Rats ($n=5$)

Parameters[a]	B-SCAs	INS-PLC-SCAs-0%	INS-PLC-SCAs-1.5%	INS-PLC-SCAs-3%
Dose (IU/kg)	–	4	4	4
$RBGL_{min}$ (%)	71.65	12.69	16.18	16.72
T_{min} (h)	12	24	24	6
PT-70% (h)	0	1.5–158.5	1.5–136.5	1–138.5
$AAC_{0-168\ h}$ (h)	–	93.92	73.08	81.51
PA (%)	–	608.29	473.32	527.91

[a] $RBGL_{min}$ represents the minimum relative blood glucose level. T_{min} represents the time to reach $RBGL_{min}$. PT-70% represents the period of time, during which the RBGL is lower than 70%. $AAC_{0-168\ h}$ represents the area above the curve during 0–168 h. PA (%) represents the pharmacological availability relative to insulin solution (AAC of insulin solution (1 IU/kg) is 3.86 (19))

DISCUSSION

In the present study, the shape of the PHBHHx 3D scaffolds is cylindrical (Supplementary Material Figure S1), as the 96-well cell culture plates were used as the moulds. In another word, according to the requirements the protein contained 3D scaffolds with various shapes can be fabricated using the moulds with corresponding shape, which makes the application of this system more extensive.

In the study of drug dispersion throughout the PHBHHx scaffold, labelling insulin and INS-PLC with fluorescent molecules would have been the most direct method. Labelling insulin is relatively easy, while labelling INS-PLC seems somewhat difficult. It has been demonstrated that the drug-phospholipid complex is formed by weak physical interactions, like hydrogen bonds and/or Van der Waals forces (30,31), which would probably be broken during labelling reactions. On the other hand, if the already labelled insulin is used for PLC formation, it is not sure that phospholipid interacts only with insulin moiety since the fluorescent molecule also posses active reaction groups, like –OH and C=O. Therefore, in order to avoid the undesired effects caused by labelling, an indirect method (i.e. fluorescent dyes were used as surrogate molecules) was used to study the drug distribution throughout the scaffold. It is shown in Fig. 3 that enhancing the lipophilicity of the loaded molecule is helpful for its homogeneous dispersion throughout the PHBHHx scaffolds. This is the reason for formation of INS-PLC which can significantly increase the lipophilicity of insulin (19). The homogeneous dispersion of bioactive agents is essential for the scaffolds based delivery system (24,25). One can predict that in the case of non-homogeneous dispersion of bioactive agent throughout the scaffolds, an unpredictable pulse-like release pattern would occur following continuous scaffolds degradation. The burst release of active agent from time to time has a high risk of causing severe side effects. Our present work demonstrates an easy way, i.e. formation of phospholipid complex, to achieve homogeneous dispersion of hydrophilic proteins throughout the hydrophobic scaffolds.

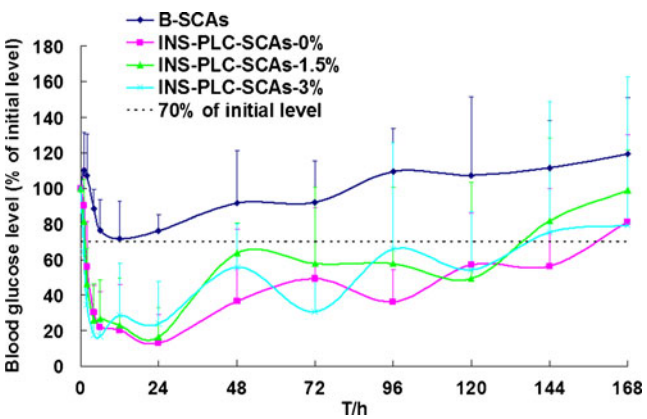

Fig. 6 Blood glucose level-time curve after s.c. implantation of B-SCAs and INS-PLC-SCAs (4 IU/kg) to male diabetic rats. Each data presented as mean ± s.d. ($n=5$).

It is shown in Fig. 4 that the cumulative release amount of insulin from the INS-PLC-SCAs was only ~6% within 120 days despite of the PEG content in the scaffolds or the lipase in the release medium. Such release rate of insulin from PHBHHx scaffolds was much slower than that from other systems including nano/micro-particles and hydrogels (32–34). At the beginning stage of release, the insulin located on the external surface of INS-PLC-SCAs was released at a relatively fast speed due to the direct touch of INS-PLC-SCAs to the release medium. This is also the main reason for the

initial relatively faster *in vitro* release (Fig. 4) as well as the rapid and significant glucose level decrease after implantation (Fig. 6). During the sustained release stage, insulin was released *via* two possible pathways. In the first route, the release medium diffused into the internal space of INS-PLC-SCAs through the pores and solubilized the insulin located on the internal surface of INS-PLC-SCAs. But the shuttle of release medium through scaffolds was impeded by PHBHHx matrix due to its strong hydrophobicity and thus the release of insulin was quite slow. In the other pathway, the loaded insulin was released following the degradation of PHBHHx matrix. The low degradation of PHBHHx (Fig. 5) is another major reason for the very slow insulin release. In order to regulate the release pattern of INS-PLC-SCAs, the hydrophilic molecule PEG-2000 was incorporated and a kind of lipase was present in the release medium. Disappointedly, neither of them had influence on the *in vitro* release pattern of INS-PLC-SCAs (Fig. 4). It was assumed that the affinity between PEG-2000 and PHBHHx was too low, or the PEG-2000 content was not sufficient to cause effects. Therefore, incorporation of an amphiphilic substance into the PHBHHx scaffolds may be able to adjust the release pattern. In the case of lipase, its low activity in the *in vitro* conditions may contribute to the useless effect on PHBHHx degradation. It was reported that the lipase had very small effect on the degradation of PHBHHx *in vitro* (35).

In addition, the extremely slow release rate of INS-PLC-SCAs occurring during the sustained release stage may contribute largely to the shorter term therapeutic profile *in vivo*, where the therapeutic effect of INS-PLC-SCAs in diabetic rats lasted for only several days (Fig. 6). In the first few days the blood glucose level was well controlled due largely to the initial relatively rapid release of insulin (Fig. 4) and the faster *in vivo* degradation of PHBHHx scaffolds (Fig. 5). In the following days, however, the sustained tiny amount release of insulin was not sufficient to control the blood glucose level. Moreover, the released insulin can not be accumulated in the blood since its plasma half-life is just around 5 min (36). Despite of the shorter term blood glucose control, the bioavailability of insulin was significantly enhanced by INS-PLC-SCAs compared to insulin solution (Table I), indicating that the insulin bioactivity was maintained.

This present work demonstrates the ability of PHBHHx scaffolds system in sustained delivery of proteins using insulin as a model protein. One of the potential applications of this system is in sustained delivery of growth factors for tissue repair. When growth factors are incorporated, it can serve as a bifunctional system that provides a porous skeleton for cells attachment and proliferation, as well as a matrix for long term release of the loaded growth factors. By use of this system, the instability and rapid clearance of growth factors may probably not be the problems any more. In addition, adjustment of the degradation speed of PHBHHx scaffolds and the release rate of loaded agents are possibly achieved by changing the monomer ratio and concentration of PHBHHx, and/or by incorporating some amphiphilic molecules.

CONCLUSIONS

A novel PHBHHx 3D scaffold based protein delivery system is developed in this present work. Enhancing the lipophilicity of the bioactive agent is helpful for its homogeneous dispersion throughout the PHBHHx scaffolds. It is demonstrated that INS-PLC-SCAs can not only maintain the bioactivity of insulin, but can also release insulin in a sustained manner, indicating that the PHBHHx based 3D scaffold is an effective system for sustained delivery of proteins. One of the great potential applications of this system is in sustained delivery of growth factors for tissue engineering. In addition, the degradation speed of PHBHHx scaffolds and the release rate of the loaded bioactive agents can be adjusted by changing the monomers ratio and concentration of PHBHHx, and/or by incorporating some amphiphilic molecules.

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