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Sustained Release of Transgenic Human Factor IX: Preparation, Characterization, and in Vivo Efficacy

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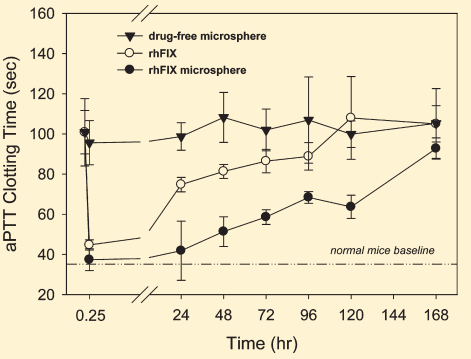
ABSTRACT: The current regimen of factor IX (FIX) injection is of an episodic format, which leads to limited efficacy. A sustained release dosage form is beneficial in terms of reducing the injection frequency and improving the therapeutic effectiveness. The aim of this study was to formulate a new microsphere form of a FIX-containing preparation to diminish these shortcomings. Using the water-in-oil-in-water (W/O/W) double emulsion technique, injectable long-acting FIX microspheres were prepared with transgenic recombinant human FIX (rhFIX) and poly(lactic-*co*-glycolic acid) (PLGA) polymer. The rhFIX microspheres prepared had diameters ranging between 25−350 μm and easily passed through a small-gauge-number needle for subcutaneous injection. In *in vitro* release testing, the microspheres had a sustained release profile featuring an initial burst and sustained release spanning a 5-day period. In *in vivo* pharmacodynamic testing, normalization of the bleeding of hemophilic mice was maintained for 5 days with microsphere injection as compared with 2 days with native rhFIX. Taken together, these results indicated that long-acting FIX microspheres were successfully prepared for potential use in hemophilic prophylaxis.



KEYWORDS: hemophilia B, prophylaxis, transgenic factor IX, long-acting preparation, microsphere

1. INTRODUCTION

Factor IX (FIX) is one of the serine proteases of the coagulation system in the body. It functions to maintain the hemostatic clotting activity of the host defense system. An inherited defect in this protein causes a bleeding disorder known as hemophilia B or Christmas disease, which is an X chromosome-linked disorder with an incidence of 1/25,000 male births.[1] The standard treatment for hemophilia B, so-called replacement therapy, involves the infusion of FIX concentrates at the time of bleeding (on-demand therapy) and in a prophylactic schedule to prevent bleeding episodes. Due to the limited supply of FIX, approximately 75% of hemophilic patients worldwide have inadequate treatment or no access to this therapy.[2]

Gene therapy is the sole approach that can lead to a cure for the congenital disease, but it is yet to be developed to the point of an accepted treatment.[3] Prospective approaches such as ectopical expression of FIX in platelets to increase *in vivo* storage,[4] fusion of FIX with albumin or immunoglobulin to improve the pharmacokinetic properties,[5,6] and bioengineering restructure of FIX to increase its catalytic activity[7] are in the preclinical or clinical stage. Hence, a more practical approach capable of swiftly lifting the burden on hemophilic patients is required. Although recent improvements in replacement therapy including sustained release of FIX, more potent bypassing agents, and mass production of FIX using transgenic animals are envisioned as new therapeutic tools in the near future,[8] the development of a long-acting FIX product is of the greatest priority. The advantages of a long-acting FIX product include (1) improvement in quality of life by reducing the injection frequency; (2) on-demand therapy with fewer venipunctures; and (3) the ability to treat patients with prophylactic therapy, the success of which has been demonstrated in a recent randomized clinical trial.[9]

Previous developments of long-acting FIX products have been conservative. Ongoing research including into a FIX-immunoglobulin fusion protein[5] and a FIX variant prepared by chemical modification of recombinant FIX with hydrophilic polymer-polyethylene glycol (PEG)[10,11] are in the initial phases of clinical trials. As the structure and conformation of both FIX variants are changed permanently, concerns and debates regarding their efficacy and safety persist. Indeed, it has been reported that the amine chemistry of PEGylation used to conjugate protein therapeutics often leads to loss of coagulation activity.[12] Therefore, in order to lend a more practical direction to the development path, we proposed to prepare a long-acting FIX product without changing the sequence and conformation of FIX. The method is based on the physical encapsulation of FIX into a biocompatible polymer to prepare drug delivery microspheres with a sustained clotting activity.

Long-acting pharmaceuticals using biodegradable microspheres have been under development for the past few decades.

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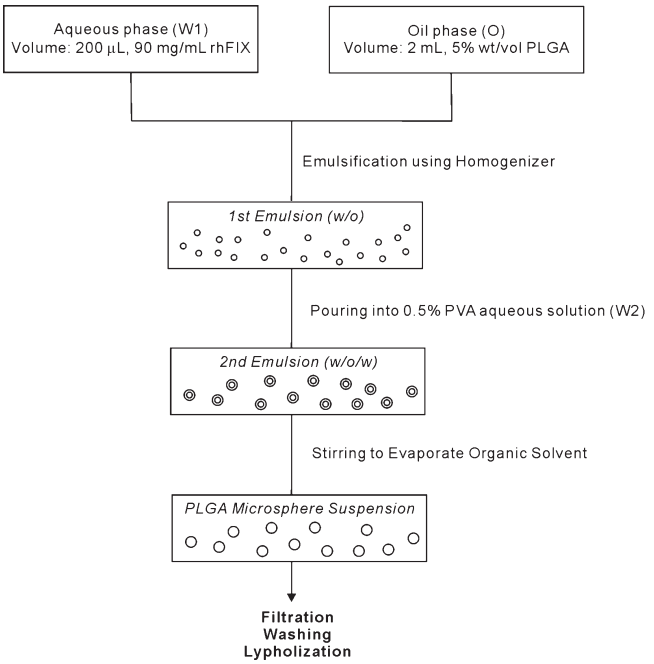

Figure 1. Schematic description of the preparation of the long-acting FIX microspheres.

The advantages of the microsphere delivery system are well-documented,[13,14] and include a long duration of action, ease of administration through regular intramuscular or subcutaneous injections, and biodegradability. The mechanism of the sustained release of the system involves drug release from the biodegradable polymer over a period of time, and prominent macromolecular drugs include leuprolide, triptorelin, and goserelin. Poly(lactic-*co*-glycolic acid) (PLGA) is an FDA-approved polymer for use in drug delivery, diagnostics, and other basic and clinical research; therefore, it was chosen for evaluating the feasibility of the proposed method of formulation of a long-acting FIX microsphere preparation. Similarly, the first recombinant human FIX (rhFIX) from transgenic pigs of the Animal Technology Institute Taiwan (ATIT)[15,16] was used as the model FIX, as the production of protein therapeutics from transgenic animals is viewed as an up-and-coming industry for the production of complex bioactive proteins.[17]

The aim of this work was to investigate the formulation conditions suitable for the preparation of rhFIX-containing PLGA microspheres of a size suitable for subcutaneous injection. We report herein the development and characterization of a rhFIX microsphere preparation, which showed an improved pharmacodynamic and pharmacokinetic efficacy in hemophilic mice at a FIX dose equivalent to that administered in its native form.

2. MATERIALS AND METHODS

2.1. Materials. Poly(lactic-*co*-glycolic acid) (PLGA) with lactide-to-glycolide (LA/GA) ratios of 75/25 and 85/15 was purchased from Bio Invigor Co., Taiwan. Recombinant human factor IX (rhFIX) from the milk of transgenic pigs with a specific activity of 52 IU/mg was supplied as a lyophilized powder by the Animal Technology Institute Taiwan (ATIT), Taiwan. FIX-deficient plasma and activated partial thromboplastin time (aPTT) reagent were obtained from Dade Behring Inc., Germany. Polyvinyl alcohol (PVA, M_w: 9−10K, 80% hydrolyzed), dichloromethane (DCM), and calcium chloride were purchased from Sigma-Aldrich Co., USA. Hemophilia B mice, R333Q-hFIX strain,[18] were acquired as a gift from Dr. Darrel W. Stafford of the University of North Carolina, USA.

2.2. Preparation of PLGA Microspheres. The water-in-oil-in-water (W/O/W) emulsification/solvent evaporation method was used to prepare the sustained release microspheres (Figure 1). In brief, suitable amounts of rhFIX and PLGA were dissolved in distilled water and DCM, respectively, to prepare the primary emulsion bases. rhFIX aqueous solution (W1) was added to 2 mL of polymer solution (O) and ultrasonicated at 75 W for 2−3 min to obtain the primary emulsion. The primary emulsion was then poured into stirred 0.5% PVA aqueous solution (W2) in a ratio of 1:30 (v:v) to obtain the secondary emulsion. The emulsion solution was stirred continuously at room temperature for 3−4 h to evaporate the organic solvent and to allow the droplets to harden into microspheres. The PVA solution was removed by filtration through a 0.45 μm cellulose nitrate membrane. The resulting microspheres were collected, washed, and lyophilized to produce a homogeneous powder preparation. The filtrate and cleaning liquid from this process were collected for determination of the encapsulation efficiency and drug loading.

2.3. Particle Size, Distribution, and Morphology. The particle size and distribution of the microspheres were determined by optical microscopy (BX 40, Olympus, Japan). For each batch of microspheres, at least 500 particles in deionized water were examined using a graticule slide to calculate the average diameter and size distribution. The surface morphology was examined using scanning electron microscopy (SEM) (Tescan, USA). Samples for SEM were freeze-dried, mounted on metal stubs, and coated with gold under vacuum.

2.4. Drug Loading, Encapsulation Efficiency, and Yield. The average drug content was indirectly determined by measuring the residual unentrapped protein in the outer water phase using a Noninterfering Protein Assay kit (Geno Technology Inc., USA). The percentages of drug loading, encapsulation efficiency, and yield were calculated based on the following equations:

$$\textit{drug loading}(\%) = \text{weight of drug/weight of microspheres} \times 100$$

$$\textit{encapsulation efficiency}\ (\%) = \text{actual drug content/theoretical drug content} \times 100$$

$$\textit{yield}\ (\%) = M/M_o \times 100$$

$$(M = \text{weight of microspheres}, M_o = \text{total weight of drug and polymer})$$

2.5. Assay for rhFIX. An activated partial thromboplastin time (aPTT) assay[19] was used to detect the FIX activity released from the long-acting microspheres *in vitro*, and also to quantify its efficacy in restoring the coagulation function in hemophilic mice. In brief, 25 μL of FIX standards (0.05−10 IU/mL) or test samples was mixed with 25 μL of a PTT reagent and 50 μL of human FIX-deficient plasma to compose the reaction mixture, which was incubated at 37 °C for 1 min. Subsequently, 50 μL of $CaCl_2$ (0.025 M) was added to initiate the analysis, which involved recording the time to clot for 4 min using an automated blood coagulation analyzer (TECO Coatron M4). The bioactivity

of rhFIX in the samples was calculated based on a calibration curve of clotting time versus activity unit concentration (in IU/mL).

2.6. *In Vitro* Drug Release. The *in vitro* drug release of the microspheres was measured under simulated physiological conditions (0.5% Tween 20, PBS, pH 7.4). Approximately 20 mg of microspheres was weighed into a 15 mL centrifuge tube with 10 mL of the simulated medium. Then, the capped tube was sealed with Parafilm and placed on an orbital shaker operating at 100 rpm at 37^{o}C. At various time points, centrifugation was performed before sampling and 1 mL of releasing solution was collected, replacing with an equal volume of fresh and preheated releasing medium. The solution samples were filtered using a 0.45 μm syringe tip filter, and the FIX activity was determined by aPTT assay. For each microsphere batch, the data were calculated from three runs.

2.7. *In Vivo* Bioactivity of Microspheres in Hemophilia B Mice. The animal study was conducted in compliance with the approved protocol of the Institutional Animal Care and Use Committee at ITRI. In brief, FIX-deficient mice weighing between 20 and 25 g were randomly divided into two groups, with six mice at each time point in each group. A single dose of native rhFIX or rhFIX microspheres in 0.9% saline solution was then administered subcutaneously to the abdominal site of mice at a dose of 50 IU/kg body weight using an 18-gauge needle. The mice were maintained *ad libitum* during the study period, and blood samples were obtained at 0.25, 24, 48, 72, 96, 120, and 168 h after administration by cardiac puncture after humane killing by CO_2. Citrated plasma was prepared from blood for determination of the clotting activity by aPTT assay. Statistical evaluation of the *in vivo* efficacy of the microspheres was based on independent *t*-testing with a significance level of 0.05.

3. RESULTS

3.1. Preparation and Characteristics of the Microspheres. Preparation of the FIX-containing microspheres was carried out by a 2-step emulsification process. The first step involved the formation of hydrophobic droplets in the first emulsion and removal of solvent from the droplets; the second step included precipitation of the polymer and solidification of the core of the microspheres. The detailed procedure was as illustrated in Figure 1, and the factors affecting the preparation are discussed below.

The volume ratio of the inner water phase to the oil phase (W1:O) apparently played a critical role in the microsphere morphology. When the ratio was smaller than or equal to 1:10, the microspheres had a uniform spherical shape, a narrow particle size distribution, and a high encapsulation efficiency. When the ratio was greater than 1:5, the primary emulsion likely became unstable, and microspheres formed under these conditions had an irregular shape. In addition, these irregular microspheres tended to aggregate, collapse, and leak. On the other hand, when the volume ratio of the primary emulsion to the external aqueous phase (E1:W2) was increased or the stirring rate of W2 phase was decreased, the microspheres were of a larger size.

Variation of the rhFIX concentration in the internal aqueous phase had no influence on the particle size and morphology. Therefore, this factor was important in drug loading. In the study, 10 mg/mL of rhFIX aqueous solution was used to prepare the microspheres, with a drug loading of around 4 IU/mg microsphere. As the quality of the primary emulsion had a great impact on the success of the preparation, the concentration of PLGA in DCM, which typically affected the viscosity of the organic phase, was also important. A slightly higher concentration thickened the organic phase and reduced efflux of the internal rhFIX solution into the external aqueous phase, consequently increasing the drug loading. In contrast, very high or low PLGA concentrations detrimentally affected the preparation. For the preparation of the rhFIX microspheres with a desired particle morphology, the suitable PLGA concentration was around 90 mg/mL.

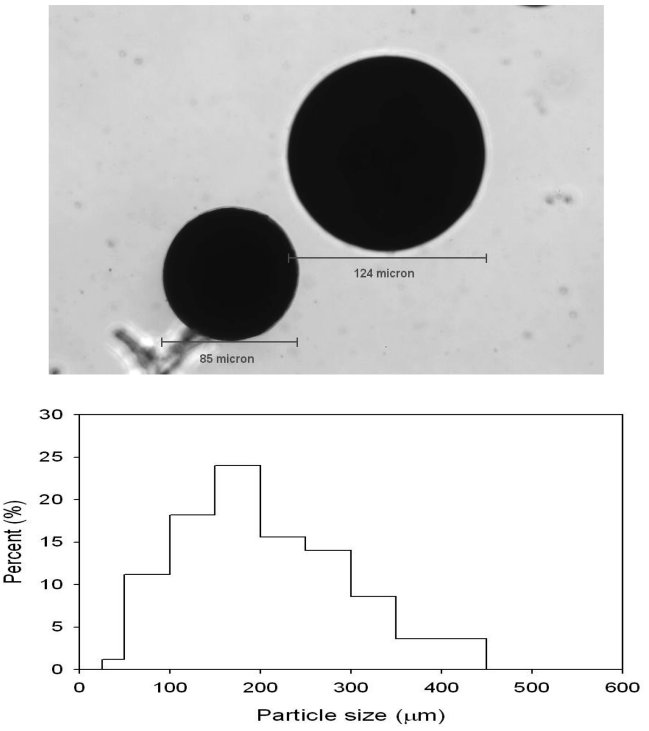

Figure 2. Optical microscopy of the rhFIX PLGA microspheres and their size distribution.

Figure 2 shows the particle size and size distribution of the long-acting rhFIX microspheres. Using the W/O/W method, the microspheres obtained had a smooth round morphology and the particle size ranged from 25 to 350 μm in diameter. For a general batch, about 70% of the microspheres were in the size range of 50−250 μm, and thus could easily pass through an 18-gauge needle for injection. Table 1 shows the product yield, drug loading, and encapsulation efficiency for four batches of the PLGA-based microspheres prepared using the described method. In general, these batches prepared with optimal mechanistic settings (i.e., homogenizer power and stirring rate) had an encapsulation efficiency and yield of around 84−97% and 80−89%, respectively. Due to these being small batch-size preparations, a maximum approximate 15% variation was obtained for the encapsulation efficacy from batch to batch.

3.2. *In Vitro* Protein Release. Four batches of microspheres varying in drug loading and lactide-to-glycolide ratio of PLGA were used to characterize the long-acting FIX preparation in terms of the drug release pattern *in vitro*. As the biological activity is a critical element in detecting the success of protein delivery via a novel dosage form, *in vitro* assessment of drug release from the rhFIX microspheres was conducted using an aPTT clotting assay. As seen in Figure 3, the release of rhFIX from the microspheres to the outer buffer was similar and was a function of time: there was an initial burst, followed by a plateau, then a significant decay for a period of 5 days. For the high-drug-loading batches, the release

Table 1. Encapsulation Efficiency, Yield and Drug Loading of Long-Acting FIX Microspheres

formulation (LA/GA)	encapsulation efficiency (%)	yield (%)	drug loading (IU/mg microsphere)
PLGA 75/25 (batch 1)	97	87	4.6 ± 0.3
PLGA 85/15 (batch 2)	96	80	4.2 ± 0.1
PLGA 75/25 (batch 3)	95	89	1.1 ± 0.3
PLGA 85/15 (batch 4)	84	86	1.0 ± 0.2

of FIX activity was larger than that of the low-drug-loading batches, and had a maximum peak of about 30% of the theoretical value based on the total rhFIX released from the microspheres. A release burst was apparent in the first 6 h for all batches of microspheres. After the burst release, an immediate activity drop was observed for the high lactide-to-glycolide ratio preparations, but there was little fluctuation throughout the test for the low lactide-to-glycolide preparations.

In the study, a regular release profile for the PLGA microspheres covering at least a one-month period was unobtainable. It was found that there was no measurable FIX activity present in the samples beyond the experimental period of 5 days. Since aPTT assay focused on activity measurement, the problem was later ascertained to be caused by the *in vitro* fragility of FIX.[20] Accordingly, the ongoing release of protein components from the microspheres was confirmed by a quantity assay (i.e., using a noninterfering protein assay kit, Geno Technology Inc., USA; data not shown).

In addition, the process of sustained release of rhFIX from the microspheres was characterized qualitatively by SEM imaging. Figure 4 shows exemplary images, in which it can be seen that, as the releasing time increased (from day 0 to 7), the originally smooth surface of the microspheres gradually featured marks demonstrating surface erosion and pore inclusion. The releasing mechanism of the rhFIX-containing microspheres thus may be based on erosion, the typical mechanism for PLGA-based polymeric drug delivery systems.

3.3. *In Vivo* Protein Release in Hemophilic Mice. To demonstrate the *in vivo* efficacy of the rhFIX microspheres, the biological activity of rhFIX in its native form was first evaluated by comparison with approved FIX products, including BeneFIX (i.e., recombinant FIX from cell bioreactors) and Immunine (i.e., plasma-derived FIX), in hemophilia B mice. As seen in Figure 5, the aPTT of the plasma in the FIX-deficient mice decreased from 101 s prior to the dosing of each FIX preparation to an average of 52 s for rhFIX and 55 s for both BeneFIX and Immunine, indicating that the efficacy of the transgenic rhFIX in restoring the coagulation activity was statistically equivalent to that of approved FIX products in FIX-deficient mice.

Then, the ability of rhFIX to shorten the blood coagulation time in its native form and in the long-acting preparation was explored using a single dose regimen in the same FIX-deficient mice. As seen in Figure 6A, rhFIX in its native form or in the long-acting preparation both rapidly corrected the blood aPTT time to normal (i.e., ~35 s, C57BL/6J mice) fifteen minutes after administration in FIX-deficient mice. While correction of the defective coagulation of hemophilic mice was statistically retained for 48 h (2 days) and then returned to the nonclotting baseline by 72 h (3 days) for the mice treated with native rhFIX, blood from the mice treated with the long-acting preparation clotted for at least 120 h (5 days), becoming insignificant by

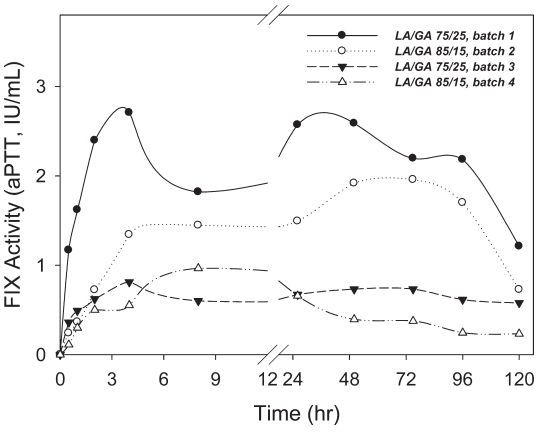

Figure 3. *In vitro* release of rhFIX from microspheres in a physiological buffer (0.5% Tween 20, PBS, pH 7.4) at 37 °C examined by aPTT assay. Each data point represents the average of three measurements.

168 h (7 days) after administration. Compared to the native rhFIX-treated mice, the clotting activity in the blood was maintained for about 2.5-fold longer in the mice treated with the FIX-containing microspheres. Given the prominent efficacy of the rhFIX microspheres, control mice receiving the empty microspheres on the contrary did not exhibit significant aPTT deviation from the nonclotting baseline of the FIX-deficient mice, although subcutaneous injection of microspheres *per se* seemed to trigger trivial coagulation, as the average aPTT changed from 101 to 96 s 15 min after administration.

Owing to the activity-based experiment having limitations in detailing the chemical features of protein delivery, a counterpart study focused on complementing the above aPTT data with related plasma FIX antigen levels was also performed. As seen in Figure 6B, the delivery characteristics of rhFIX from the microspheres at the abdominal subcutis were apparently dissimilar, as the plasma rhFIX activity determined by different assays showed different patterns. For the circulating FIX level as determined by the activity-based aPTT assay, the defective clotting activity of hemophilic mice was quickly corrected by the rhFIX microspheres, then gradually returned to the baseline along the time course of the experiment. For the circulating FIX level as determined by the quantity-based ELISA assay, the circulating antigen level of FIX however remained steady one day after microsphere administration at the levels of 0.07−0.09 IU/mL, suggesting that rhFIX might still be released into blood circulation from the injection site. Together, it was apparent that the microsphere preparation lost its clotting activity faster than expected.

4. DISCUSSION

Severe hemophilia B (FIX < 1 IU/dL) patients have repeated bleeding episodes resulting in hemophilic anthropathy and other sequelae.[9] FIX products such as BeneFIX used to treat bleeding episodes function by maintaining the minimum coagulation activity for host defense. Owing to the relatively short half-lives of current FIX products,[17] pursuit of a prophylactic regimen recommended by the National Hemophilia Foundation requires 2 to 3 injections per week of these products at a dosage level of 40 to 100 IU/kg body weight.[21] Therefore, patients undergoing prophylaxis unavoidably have to be subjected to repeated venous access, which is particularly difficult in the pediatric population.

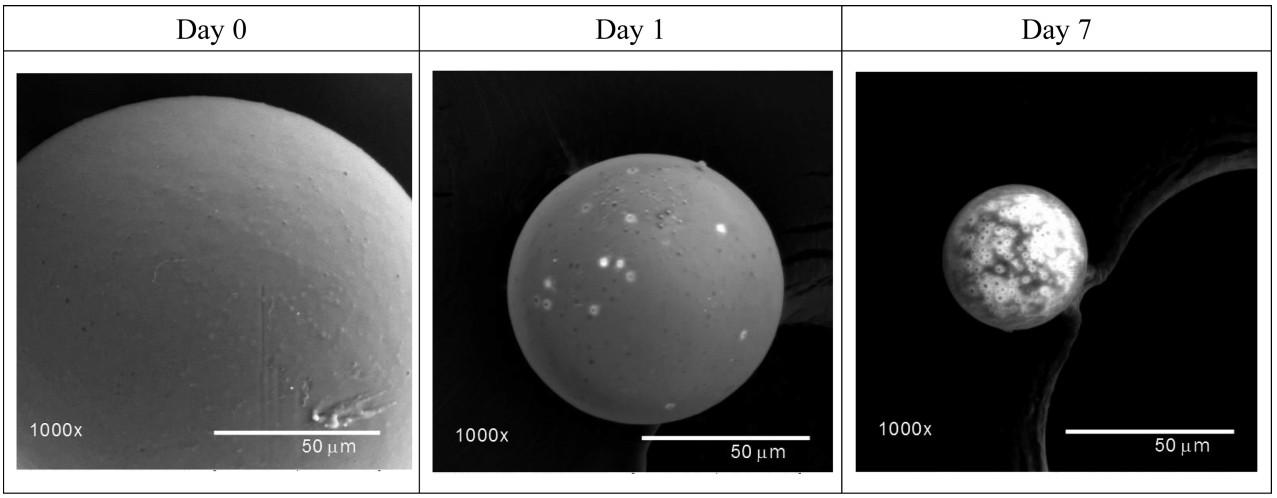

Figure 4. SEM of the rhFIX microspheres at different states of degradation: before release, after 1 day, and after 5 days in releasing medium at 37 °C.

Following numerous efforts to alleviate this problem, this is the first study to show that a microsphere format FIX-containing preparation could be used to reduce the dosing frequency of FIX therapy.

The low concentration in human plasma and limited production by cell bioreactor along with difficulty in harvesting functional proteins due to molecular ramifications have resulted in a scarcity of FIX for several decades. Developing new avenues to produce FIX in large quantities continues to be a central focus of many related studies. The production of protein therapeutics from transgenic animals is viewed as an up-and-coming industry, but so far only the transgenic antithrombin III developed by GTC Biotherapeutics has been approved for clinical use.[22] In this study, it was demonstrated for the first time that transgenic rhFIX from pig milk can function effectively in its native form or in a microsphere preparation in FIX-deficient mice. At a production cost approximately equivalent to 4.4% of its market price, the positive data presented herein represent a major step in terms of advancing transgenic FIX with a strong influence on the optimization of hemophilic care.

The transgenic rhFIX used here has been ensured by various analyses to possess the requisite structure and functionality.[15,16] In the substitution of γ-carboxylation of glutamate residues in the N-terminal, which is essential for biological activity, rhFIX possesses 11.8−12.0 Gla per molecule, representing 12 Gla of plasma-derived FIX. Also, rhFIX has a clotting activity equal to that of other commercial FIX products such as BeneFIX and Immunine in hemophilic mice and follows a linear dose−response relationship in Sprague−Dawley rats, with no signs of toxicity or disturbance to the cardiovascular, respiratory and nerve systems at dosages up to 500 IU/kg.[16] Because the molecular characteristics of rhFIX and native human FIX are almost identical, the encapsulation strategy used in this study should be able to be easily translated to other FIXs or FIX derivatives in order to prepare a long-acting FIX formulation.

The *in vivo* pharmacodynamic data presented in Figure 6 show that the microsphere preparation possessed a prolonged coagulation function in comparison with native rhFIX. The efficacy magnitude was further compared by utilizing two estimated noncompartmental pharmacokinetic parameters, including the area under the curve (AUC) and clearance (CL). As seen in Table 2, under the same experimental settings, the AUC and CL

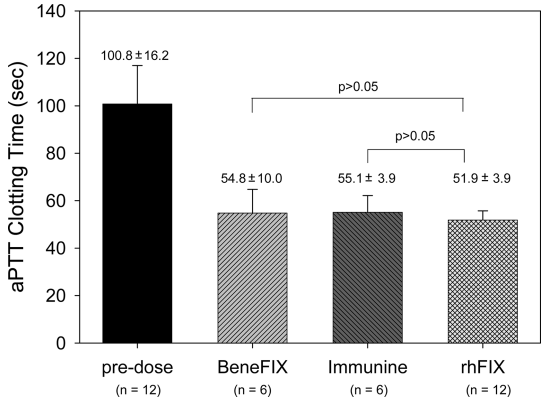

Figure 5. Functional comparison of rhFIX with approved FIXs in FIX-deficient mice. FIX-deficient mice were administered intravenously with a single dose of rhFIX, BeneFIX, or Immunine at a dose of 50 IU/kg via the tail vein. Then, plasma samples were collected five minutes after dosing and analyzed for clotting activity by aPTT assay. Statistical evaluation was performed by the independent t test, and $p < 0.05$ was considered to indicate statistical significance.

for the microsphere preparation were 9.3 times higher and 5.3 times slower than those for free rhFIX, respectively, indicating that rhFIX encapsulated in microspheres could result in a greater drug exposure and a lower total clearance than free rhFIX. Also, greater bioavailability for the microsphere preparation was suggested from these two parameters, in accord with pharmacodynamic observations described in Results. On the other hand, it was noted that based on the aPTT prolongation the elimination rate of rhFIX between 24 and 96 h was faster in the microsphere-injected mice, indicating that rhFIX might experience rapid activity decay during the overall release process. It is outlandish that a sustained release formulation exhibits rapid activity decay. As the phenomenon might be rationalized by the fact that it was not obtained from a real pharmacokinetic study in which the FIX concentration is determined by a protein quantitative method (i.e., ELISA) rather than by a biological assay (i.e., aPTT), the change of rhFIX activity during the release process might also play an important role in diminishing the function of the rhFIX microspheres. Thus, while the activity performance of rhFIX in an extended-release (ER) format out-surpasses that of an

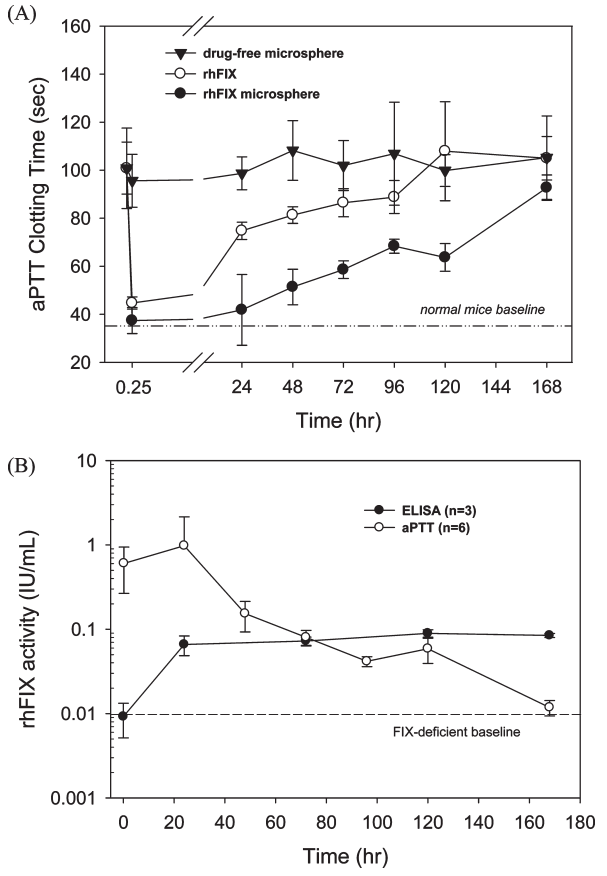

Figure 6. (A) Bioactivity of FIX after a single subcutaneous injection of native rhFIX and rhFIX microspheres at a dose of 50 IU/kg, and use of drug-free microspheres as the negative control in FIX-deficient mice. Each point represents the mean ± SD of six animals. The baseline aPTT clotting times were 100.8 ± 16.2 and 35.3 ± 6.9 s for the FIX-deficient and wild-type mice, respectively. (B) Measured clotting activity versus antigen level for evaluation of the bioactivity of the rhFIX microspheres in FIX-deficient mice. FIX-deficient mice were administered rhFIX microspheres at a dose of 50 IU/kg body weight. Then, plasma samples were collected at predefined time points, and the clotting activity was analyzed by aPTT and the antigen level by ELISA against human FIX. The measured clotting time and antigen level were converted to the specific activity of IU/mL for comparison purposes on the basis of 52 IU/mg rhFIX.

Table 2. Pharmacokinetic Parameters of rhFIX and rhFIX-Encapsulated Microspheres Estimated by Noncompartmental Analysis of the Activity Time Profile[a]

	rhFIX	
	free	microsphere
AUC_{0-120}, IU/mL/h	7.95 ± 0.79	73.97 ± 63.23
CL_{0-120}, mL/h	0.16 ± 0.02	0.03 ± 0.02

[a] Hemophilic mice weighing between 20 and 25 g were administered a single dose of native rhFIX and rhFIX-encapsulated microspheres (50 IU/kg). Blood samples were collected at various time points between 0.25 h and 168 h. Plasma was prepared for analysis of the clotting time by aPTT assay. Data were converted from clotting time to specific aPTT activity for analysis of noncompartmental pharmacokinetic parameters. These parameters were determined by the following methods: the area under the time concentration curve (AUC) was calculated using the linear trapezoidal rule; and clearance (CL) was calculated as dose/AUC.

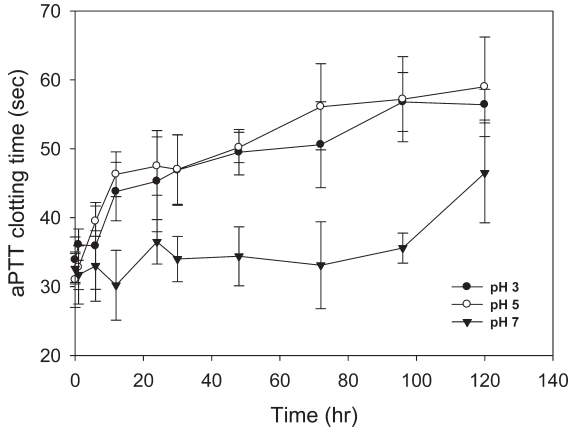

Figure 7. Stability of rhFIX in PBST buffer (0.1% v/v Tween 20 in PBS) at varying pHs. Lyophilized rhFIX was reconstituted at 10 IU/mL and stored in a serum bottle at 37 °C. Aliquots were removed at each time point, then mixed with 25 μL of FIX-deficient plasma and subjected to aPTT assay. Each data point represents the average of three measurements.

immediate-release (IR) format, a sturdy movement to develop the microsphere preparation with the priority on improving the overall stability of rhFIX is recommended.

Polymeric drug delivery systems have been widely used for the long-term delivery of therapeutic agents. Pharmaceutical advantages of the system include the following: (1) drugs can be delivered to tissue in a sustained and continuous fashion; (2) drugs are well-protected; and (3) site-specific delivery can be achieved. To adopt this system to prepare a long-acting FIX microsphere formulation, a W/O/W encapsulation method was used in this study based on the solubility of rhFIX. The burst release of rhFIX is attributed to the rapid diffusion of the drug located at the surface of the microparticles. The mechanism for the long-acting function is ascribed to the encapsulation of rhFIX within the PLGA polymer followed by release of the protected rhFIX from the PLGA particle in a prolonged manner. The driving force for this release is erosion, and the *in vivo* polymer erosion rate is critical to its sustained effect. In this study, we demonstrated that the PLGA-based system has prominent advantages over the conventional formulation. Not only could rhFIX disclose its coagulation activity in a sustained manner, but also better pharmacodynamic and pharmacokinetic properties were ascribed to the parent protein after preparation. Together, these results suggest that the PLGA-based FIX formulation could have a great chance following successful precedents such as the advance to clinical use of Lupron Depot and Zoladex.

There also exist quite a few disadvantages associated with PLGA-based systems. Although PLGA is widely used in clinical circumstances, such an application for hemophiliacs that requires frequent FIX infusions for life however has not been studied. To evaluate the safety of the rhFIX microsphere system is therefore something that out to thoroughly assess the biocompatible and antigenic nature of PLGA. On the other hand, one of the most prominent problems is the acid-catalyzed nature of PLGA. It is known that the pH inside the aqueous pores of the PLGA matrix can become very acidic due to acidic polymer impurities and the hydrolysis of the PLGA polyester to form acidic monomers and

oligomers during erosion.[23,24] As a consequence, encapsulated drugs that are sensitive to pH change may be degraded during the release process. In this regard, a stability study of rhFIX under different pH conditions was performed to assess whether the microenvironmental pH influences delivery of the rhFIX microspheres. As seen in Figure 7, reconstituted rhFIX stored at pH 7 retained most of its clotting activity for at least 4 days, the activity then slightly decreasing, but rhFIX stored at either pH 3 or 5 however gradually lost its activity after buffer reconstitution. In this study, it was observed both *in vitro* and *in vivo* that the biological activity of the encapsulated rhFIX rapidly vanished approximately 7 days after beginning the experiment, but whether or not the impotence of rhFIX is related to the phenomenon is not certain, as the PLGA degradation rate was not measured. In this regard, as human factor IX has been reported to be more stable in the presence of calcium ions,[25] further study with the aim of improving the duration of action of the microsphere preparation is underway to examine the effects of adding stabilizing ions to the formula.

In this study, the microsphere preparation exhibited an improved efficacy in prolonging the biological activity of rhFIX. Therefore, it is interesting to compare it with another long-acting FIX preparation, 40K PEG-rFIX,[10,11,26] where 40 kDa polyethylene glycol (PEG) is selectively attached to *N*-glycans of the activation peptide. Compared with the only available recombinant FIX product, BeneFIX, 40K PEG-rFIX showed no differences in efficacy and potency, but had a longer half-life of nearly 2.5- and 5-fold in FIX-deficient mice and minipigs, respectively. In terms of the duration of action, 40K PEG-rFIX maintained normalization of bleeding for 3 days in FIX-deficient mice, while bleeding increased in mice dosed with BeneFIX at day 1. At a glance, our microsphere preparation is likely to have an equivalent or superior *in vivo* performance to 40K PEG-rFIX, while different experimental conditions may lead to some deviations. Although PEGylation is widely used to increase the half-life and reduce the immunogenicity of protein pharmaceutics, early studies utilizing this technique have indicated that this method may still be complicated by several limitations with regard to the biological functions of the proteins. Indeed, 40K PEG-rFIX was observed to possess reduced activation activity with tissue factor (TF).[26] From the above information, it may be concluded that the PLGA encapsulation method could be more advantageous than other techniques for the development of long-acting preparations.

Free and PLGA-encapsulated rhFIX exhibited a similar activity immediately after injection. Administration of the long-acting preparation did not lead to thrombotic complications resulting from excessive FIX activity. Also, the formulation of FIX with PLGA does not result in decreased coagulation activity, as has been shown for PEGylated FIX derivatives.[26] Unlike PEGylated FIX, the interaction between FIX and PLGA is noncovalent and does not involve modification of the FIX amino acid sequence, chemical reaction or enzymatic reaction. Therefore, it is unlikely that changes in the conformation of FIX occur. Taken together, our results suggested that the encapsulation approach is safe and effective for the formulation of a long-acting FIX preparation.

We have demonstrated in this study that a preparation of FIX-containing microspheres has several advantages over native FIX and PEGylated FIX preparations. These advantages include retained FIX activity after encapsulation and a prolonged coagulation activity. These results suggested that further development of the FIX microsphere formulation may lead to a safe and effective long-acting FIX preparation that will improve the care of hemophilia B patients.

In conclusion, our results demonstrated that sustained release of factor IX could be achieved by encapsulating factor IX in a biodegradable polymer. Using these microspheres, the clotting activity was corrected to normal in factor IX-deficient mice for 5 days. Compared to other prolonged preparations of PEGylated factor IX, the method used and the excipient have an outstanding safety record and are widely accepted worldwide in the pharmaceutical field, therefore representing an excellent way to create a sustained release factor IX preparation. Research is now underway to evaluate the potential of escalating doses of factor IX in animal models with a view to testing the proposed microsphere preparation in humans.

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