Preparation of Branched Dextran Microspheres of Soluble Interferon-alpha and its Activity *In Vitro* and *In Vivo*

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The study objective was to prepare biodegradable branched dextran microspheres encapsulated with His-tagged interferon-alpha (BDM-hIFN-α) and evaluate its activity *in vitro* and *in vivo*. The glycidyl methacrylate derivatized dextrans (Dex−GMA) as precursor was primarily synthesized by substituting hydroxyl groups of either the branched or linear type of dextran with GMA. Dex−GMA microspheres loaded with hIFN-α was then prepared by the water-in–water emulsion technique. *In vitro* release and Western blotting experiments demonstrated the retained activity of hIFN-α released from branched dextran microspheres at 24 h by inducing phosphorylation of signal transducer and activator transcription-1 (STAT-1), a down-stream effector of IFN-α, in HepG2 cells. Animal data further revealed a peak of plasma levels of IFN-α in rats injected intravenously with BDM-hIFN-α at 10 min post-injection, but a sharp decline at 2 h. High plasma levels of neopterin, a plasma protein induced by IFN-α, were also detected in rats injected with BDM-hIFN-α at 10 min post-injection. Notably, plasma levels of neopterin remained high at 4 h, but largely declined thereafter.

Keywords: Branched dextran microspheres, IFN-α, p-STAT-1, HepG2, neopterin

Interferon-alpha (IFN-α) is the first FDA-approved biotherapeutic cytokine with multifunctions, including antiviral, antiproliferative, and antitumorous activities [2]. Accumulating evidence, however, suggests that clinical use of IFN-α has many limitations, such as a short half-life (4–7 h) and rapid loss of biological activity, lack of liver-specific affinity, long-term frequent injection, a large fluctuation of plasma concentration, and side effects [16, 18, 26], which may eventually lead to its poor efficacy. Thus, a better delivery system for this cytokine that can not only improve its therapeutic efficacy but also prolong its plasma half-life is in high demanded.

A variety of administration systems of IFN-α have been utilized to improve both the therapeutic efficacy and plasma half-life of IFN-α, including pegylation of IFN-α [27], gelatin [29], poly(lactic-co-glycolic acid) (PLGA) microparticles [21], or polybutylcyanoacrylate nanospheres [15]. Among those, pegylated IFN-α (PEG-IFN-α) has been very successful since the early 1990s, and particularly PEG-IFN-α2β, owing to its sustained release over a 1-week period, was approved by the Food and Drug Administration [17]. PEG-IFN-α is now widely used in clinical practice alone or combined with other drugs, such as ribavirin, for the treatment of chronic hepatitis C [9, 24]. However, although PEG conjugation increases the apparent size of the polypeptide, reduces the renal filtration, and alters biodistribution [20], it is suggested that PEG, owing to its non-biodegradability, may be accumulated in the body, and PEG conjugates also may suppress protein immunogenicity and antigenicity [1], leading to generation of antipolymer, antiprotein, or anti-whole construct antibodies, which may reduce the conjugate residence time in the blood stream [5]. In the case of biodegradable PLGA, its degradation may cause the production and subsequent build-up of acidic molecules, and over time, the continued effects of degradation may lower the pH within the microsphere, leading to an acidic microclimate as low as pH 2.5 [7, 23]. Dextran is a natural biodegradable polysaccharide widely used in the pharmaceutical field [4, 12]. It has many attractive characteristics for drug delivery systems, including good biocompatibility and the degradation to non-toxic and readily excreted products [25]. Previously, we have addressed the production of a highly branched dextran with

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both α-1-6 and α-1-3 glucose linkages from *Leuconostoc* (*L.*) *mesenteroides* B-742 [28] and generation of soluble recombinant His-tagged IFN-α (hIFN-α) in *Escherichia coli* by controlling the inducible promoter-dependent transcription rate [11].

To gain insight to the use of branched dextran microspheres as a novel IFN-α delivery system, in this study, we prepared hIFN-α-encapsulated biodegradable branched dextran microspheres and evaluated their biological activities in cellular and animal systems.

MATERIALS AND METHODS

Materials

DMEM, penicillin, and streptomycin were from GIBCO-BRL (Grand Island, NY, USA). FBS was from Hyclone (Logan, UT, USA). Antibodies against anti-rabbit or mouse secondary horseradish peroxidase, and ECL Western detection reagents were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Bradford reagent was from Biorad (Hercules, CA, USA). Antibodies against phospho-ERK-1/2 (p-ERK-1/2) and total ERK-1/2 were purchased from Cell Signaling Tech (Beverly, MA, USA). Antibody against p-STAT-1 was from Santa Cruz Biotechnology (Delaware, CA, USA). Antibody against IFN-α was purchased from Invitrogen (USA). Dextran T-150 (MW 150,000), polyethylene glycol (PEG, MW 38,800), glycidyl methacrylate (GMA), triethylamine, 4-(*N,N*-dimethylamino)pyridine (DMAP), dimethyl sulfoxide (DMSO), *N*-methyl pyrrolidone (NMP), N,N,N^L,N^L-tetramethylethylenediamine (TEMED), and ammonium persulfate (APS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Branched dextran (B-742) was produced by *L. mesenteroides* B-742, and dextran hydrolyzate (MW 200,000) was obtained by HCl hydrolysis [28]. Native IFN-α (0.422 mg/ml) was kindly supplied from Dong-A Pharmaceutical Co. (Republic of Korea) as a recombinant human IFN-α. Other reagents were purchased from Sigma (St. Louis, MO, USA).

Synthesis of Dextran–Glycidyl Methacrylate (Dex–GMA)

The Dex–GMA precursor used for preparation of microspheres was synthesized according to a published procedure with some modifications [3]. B-742 dextran and T-150 (10 g each) were dissolved in 90 ml of DMSO and then 2 g of DMAP was added. After complete dissolution, 4 g of GMA was added to the dextran solution. The synthesis reaction was conducted at 50°C for 12 h under nitrogen gas. Dex–GMAs were recovered by precipitating the solution with cold isopropyl alcohol, washed several times, and then vacuum-dried.

Preparation of Dextran Microspheres Loaded with His-Tagged IFN-α

Linear and branched dextran microspheres were prepared by a water-in-water emulsion technique as described previously with some modifications [6]. Dex–MA [30% (w/v)] in 1 ml of phosphate buffer (pH 7.0) and 100 μl of His-tagged IFN-α (358 mg/ml) were added to 5 ml of PEG solution [30% (v/v)] to prepare water-in-water emulsion. The two-phase system was vigorously mixed with a vortex for 1 min. The resulting emulsion was allowed to stabilize for 30 min. Subsequently, 180 μl of APS [50 mg/ml (w/v)] and 100 μl of TEMED [20% (v/v)] were added. The mixture was incubated at

37°C for 30 min to polymerize the methacryloyl moieties bound to the dextran chains. The cross-linked dextran microspheres of His-tagged IFN-α were collected and washed by multiple centrifugation. The collected dextran microspheres of His-tagged IFN-α were freeze-dried and preserved in a desiccator.

In Vitro Release of His-Tagged IFN-α from Dextran Microspheres

About 0.4 g of dextran-microspheres-encapsulated His-tagged IFN-α were mixed with 1.0 ml of PBS in 1.5-ml microtubes. *In vitro* release test was done on a rotary shaker at room temperature. The amounts of released His-tagged IFN-α were measured by Bradford protein assay of the supernatant from the mixture of dextran microsphere and PBS. Two hundred and fifty microliters of PBS were added and withdrawn repeatedly, except that 1.0 ml of PBS was added initially. In particular, totally released His-tagged IFN-α was expressed as the cumulative amount [14]. Meanwhile, in order to investigate the instability of native IFN-α, 30-fold diluted native IFN-α was incubated at room temperature for 96 h in PBS.

Cell Culture and Preparation of Whole Cell Lysates

HepG2 human liver cancer cells were maintained at 37°C in a humidified condition of 95% air and 5% CO_2 in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For measuring the cellular activity of His-tagged IFN-α released from dextran microspheres at 0, 24, or 48 h, HepG2 cells were treated for 30 min with the His-tagged IFN-α (10,000 units/ml) released at respective time. After 30 min post-treatment, cells were washed twice with ice-cold PBS supplemented with 1 mM Na_3VO_4 and 1 mM NaF and exposed to cell lysis buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, and proteinase inhibitor cocktail (1×)] on ice for 10 min. Cells were then harvested and centrifuged for 20 min at 4°C at 12,000 rpm. The supernatant was saved and protein concentrations were determined with Bradford reagent.

Western Blot Analysis

Whole cell lysates (50 μg) were separated by SDS−PAGE (10%) and transferred onto nitrocellulose membranes (Millipore). The membranes were washed with TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (v/v) Tween 20 (TBST), followed by blocking with TBST containing 5% (w/v) non-fat dried milk. The membranes were incubated overnight with antibodies specific for p-STAT-1 (1:1,000) or ERK-1/2 (1:2,000). The membranes were then exposed to secondary antibodies coupled to horseradish peroxidase for 2 h and washed with TBST. Immunoreactivities were detected by ECL reagents. The expression level of ERK-1/2 was used as a protein loading control. Alternatively, the amounts of His-tagged IFN-α released from microspheres during 24, 48, or 72 h were detected by Western blotting as described in previous reports [10, 11, 13].

ELISA Determination of Plasma His-Tagged IFN-α (hIFN-α) and Neopterin

Rats (Sprague−Dawley, 12 week old) (Daehan Biolink Inc., Korea) were divided into two groups; control group [branched dextran microspheres (BDM) treated] and test group (BDM-IFN-α treated). For experiments, rats were primarily fasted the day before injection. They were then injected with 0.2 mg/kg of BDM or BDM-hIFN-α

in a 0.2-ml volume intravenously. Blood was collected at the time of 10 min, 2 h, 4 h, 8 h, 12 h, or 24 h post-injection. Plasma was obtained from blood after centrifugation. The amounts of plasma IFN-α were measured by IFN-α ELISA (Immuno-Biological Laboratories, MN, USA) following the manufacturer's instruction manual. Neopterin is a plasma protein induced by IFN-α [19]. The amounts of plasma neopterin were also determined by neopterin ELISA (Immuno-Biological Laboratories, MN, USA) following the manufacturer's instruction manual.

RESULTS AND DISCUSSION

Analysis of *In Vitro* Release of His-Tagged IFN-α from Linear or Branched Dextran Microspheres

We synthesized dextran-based microspheres with linear and highly branched structures using dextran produced by *L. mesenteroides* B-742. His-tagged IFN-α was loaded into those dextran-based microspheres. *In vitro* release of His-tagged IFN-α from linear or branched dextran-based microspheres was measured. As shown in Fig. 1, the total amount (cumulative) of released His-tagged IFN-α in branched dextran-based microspheres was lower than that in linear dextran-based microspheres. The encapsulated His-tagged IFN-α in linear or branched dextran microspheres was released in PBS during 96 h. In both linear and branched dextran microspheres, about 0.04 mg of His-tagged IFN-α was released into 1.0 ml of PBS, although the initial burst effect was observed. Interestingly, when
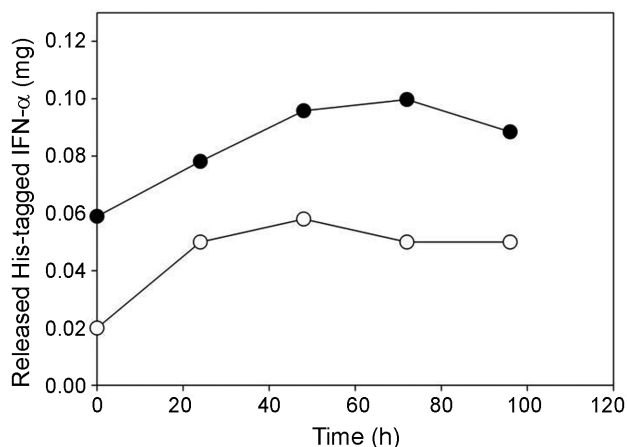

Fig. 1. *In vitro* release of His-tagged IFN-α from linear and branched dextran microspheres.

About 0.4 g of dextran microspheres encapsulated with His-tagged IFN-α was mixed with 1.0 ml of PBS in 1.5-ml microtubes. *In vitro* release test was then done on a rotary shaker at room temperature. Amounts of released His-tagged IFN-α were measured by Bradford protein assay of the supernatant from the mixture of dextran microsphere and PBS. Two hundred and fifty microliters of PBS was added and withdrawn repeatedly, except that 1.0 ml of PBS was added initially. Totally released His-tagged IFN-α was expressed as the cumulative amount [14]. Closed circle (●) and open circle (○), respectively, indicate branched dextran (B-742) and linear dextran (T-150, Sigma).

the branched dextran was used, the initial burst effect was less serious. This result might be because the branched dextran microspheres may have more cross-linking bridges between dextran molecules, compared with linear dextran microspheres. SEM micrographic data further indicated that the efficiency of encapsulation of His-tagged IFN-α into dextran-based microspheres showed a similar pattern to encapsulation of BSA protein (data not shown).

Maintenance of Stability of His-Tagged IFN-α Released from Linear or Branched Dextran Microspheres

The integrity of His-tagged IFN-α released from linear or branched dextran microspheres at 24, 48, 72, or 96 h was next evaluated. For this, an aliquot of His-tagged IFN-α released from linear or branched dextran microspheres at 24, 48, 72, or 96 h was analyzed by Western blotting using a specific IFN-α antibody. Results of Western blotting, as shown in Fig. 2A and 2B, demonstrated strong immunological activity of IFN-a protein released from both linear and branched dextran microspheres at 24, 48, and 72 h, respectively. On the other hand, His-tagged IFN-α released from linear or branched dextran microspheres at 96 h was not detected by Western blotting. This may be explained by that *in vitro* released His-tagged IFN-α at 96 h eventually lost immunological property by its instability or complete proteolytic degradation. This is based on the fact that the *in vitro* release test herein was performed under nonsterile conditions. To compare the stability, we further performed a control experiment with a native (non-encapsulated, soluble) IFN-α incubated under the same conditions. As shown in Fig. 2C, native IFN-α remained stable by 24 h but greatly lost its immunological activity by 48 or 72 h. The complete loss of its immunological activity was shown at 96 h. These results suggest that under these experimental conditions, the immunological property of the His-tagged IFN-α encapsulated in dextran microspheres may be better preserved than that of the native (non-encapsulated, soluble) IFN-α. The quantitative data of Fig. 2A−2C are shown in Fig. 2D−2F, respectively.

Measurement of *In Vitro* Activity of the Released His-Tagged IFN-α from Branched Dextran Microspheres

It has been shown that the cytokine IFN-α, by binding its cognate receptor, rapidly (within an hour) induces phosphorylation of several cellular proteins, including STAT-1 [8]. The ability of IFN-α to induce STAT-1 phosphorylation in cells was thus assessed as its *in vitro* activity. For this, HepG2 human liver cancer cells were treated for 30 min with 10,000 units/ml of His-tagged IFN-α released from branched dextran microspheres at the time of 0, 24, or 48 h, followed by measurement of STAT-1 phosphorylation in the conditioned cells. Herein, IFN-α released at 0 h means the IFN-α encapsulated in branched dextran microsphere (BDM) before release. As shown in
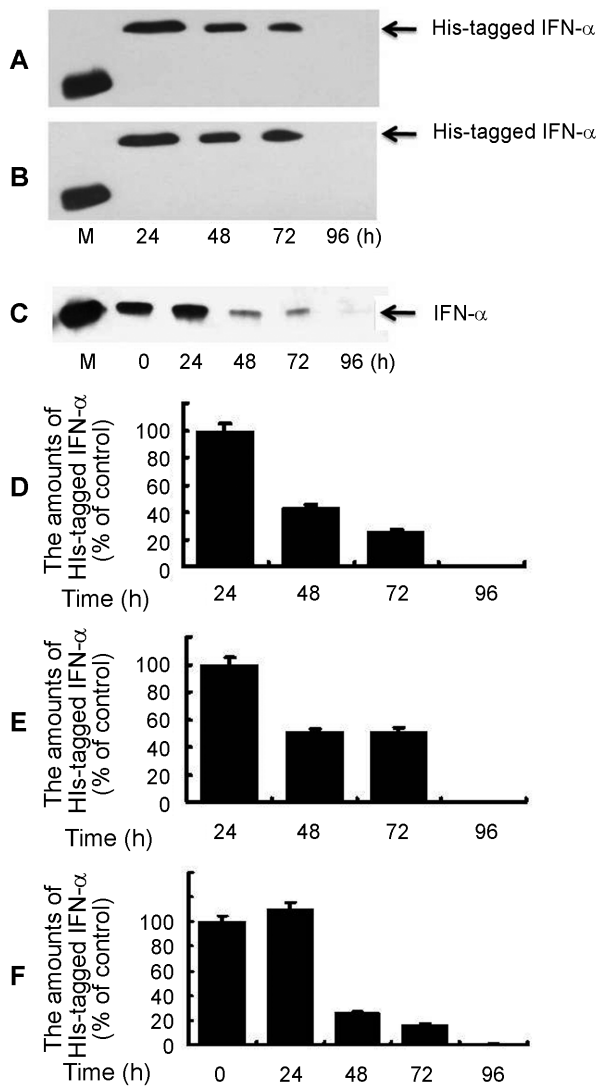
Fig. 2. Determination of the integrity of *in vitro* released His-tagged IFN-α from dextran microspheres.
An aliquot of the released His-tagged IFN-α obtained from the *in vitro* release test in Fig. 1 was run on a 10% PAGE gel. The immunological property of the His-tagged IFN-α released from microspheres during 24, 48, 72, or 96 h was detected by Western blotting using a specific IFN-α antibody, as described in previous reports [10, 11, 13]. As a control experiment, native IFN-α (non-encapsulated, soluble) was also incubated at room temperature in PBS for 24, 48, 72, or 96 h. **A**. His-tagged IFN-α released from linear dextran microspheres; **B**. His-tagged IFN-α released from branched dextran microspheres; **C**. Native IFN-α. M in (**A**), (**B**), and (**C**) indicates the native recombinant human IFN-α used as a positive control.

Fig. 3, treatment with His-tagged IFN-α encapsulated in branched dextran microspheres before release (0 h) resulted in strong phosphorylation of STAT-1 protein in HepG2 cells, suggesting that the His-tagged IFN-α in branched dextran microspheres has cellular activity. The quantitative result of Fig. 3A is shown in Fig. 3B. Notably, although mild, the His-tagged IFN-α released at 24 h still retained its ability to induce STAT-1 phosphorylation in HepG2
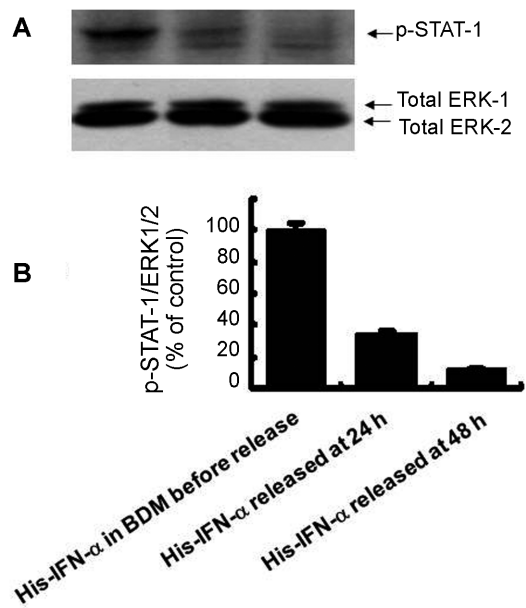

Fig. 3. Phosphorylation of STAT-1 by His-tagged IFN-α released at 24 h from dextran microspheres.
HepG2 cells were treated for 0.5 h without or with 10,000 units (U)/ml of His-tagged IFN-α (hIFN-α) released from branched dextran microspheres at the time of 0, 24, or 48 h. Whole cell lysates were prepared and subjected to immunoblot analysis with antibody specific for p-STAT-1 or total ERK-1/2. The expression level of total ERK-1/2 was used as the equal loading control of protein samples. The picture is a representative of three independent experiments. p-STAT-1, phospho-STAT-1.

cells. On the other hand, there was no stimulating effect by the His-tagged IFN-α released at 48 h on STAT-1 phosphorylation, indicating that the His-tagged IFN-α released at 48 h did not have its cellular activity. It is suggested that loss of the biological activity of His-tagged IFN-α released at 48 h may be associated with impaired (or altered) immunological property. Previously, it has been reported that the 6-histidine tagging into IFN-2α reduces activities of IFN-2α (antivirus and antiproliferation) and this is attributed to structural changes (influence of the N-terminal 6-histidine tag), which may alter the interaction between the IFN-2α-6-hisitidine tag and receptor [22]. It is therefore likely that lack of the cellular activity of His-tagged IFN-α released at 48 h may be due to structural change and/or lowered affinity between His-tagged IFN-α released at 48 h and its receptor during such a long-term release time (48 h).

Pharmacokinetics of His-Tagged IFN-α-Loaded Branched Dextran Microspheres in Rats
To next determine the *in vivo* release and activity of branched dextran microspheres of His-tagged IFN-α, rats were intravenously injected with 0.2 mg/kg of branched dextran microspheres loaded with His-tagged IFN-α (BDM-hIFN-α), branched dextran microspheres (BDM), or His-tagged IFN-α (hIFN-α). Blood was then withdrawn

from the conditioned rats at 10 min, 2 h, 4 h, 8 h, 12 h, and 24 h post-injection, respectively, followed by measurement of plasma levels of IFN-α by ELISA. Fig. 4 shows the blood IFN-α profiles after the administration of BDM-hIFN-α, BDM, or hIFN-α. About 245 pg/ml of plasma IFN-α was detected in hIFN-α-injected rats at 10 min post-injection. However, its plasma level declined sharply at 2 h. Of note, much higher levels of plasma IFN-α (475 pg/ml) were measured in BDM-hIFN-α-injected rats at 10 min post-injection. There were substantial levels of plasma IFN-α (about 100−110 pg/ml) in BDM-hIFN-α-injected rats at 2 or 4 h post-injection. However, there was no plasma IFN-α in BDM-hIFN-α-injected rats at 8 h post-injection. It has been previously demonstrated that plasma concentrations of IFN-2αb reached a peak at 7.5 h in rhesus monkeys injected with IFN-2αb-loaded PLGA microspheres, and the serum residence time of IFN-2αb in the PLGA microspheres was about 4 days [30]. The early peak (10 min) of plasma concentrations of IFN-α in BDM-IFN-α-injected rats seen in this study might be due to the fact that IFN-α was not completely encapsulated into BDM and/or it was merely contact on the surface of microspheres, thereby leading to such an early burst release. To improve this problem, it is necessary, in future, to establish proper experimental conditions that can prolong the release of IFN-α in the BDM. A notable thing in the present study is the observation of a 2-times higher plasma IFN-α level from rats injected with BDM-IFN-a compared with the plasma IFN-α level from rats injected with soluble IFN-α. It may be due to (i) a better protection of encapsulated IFN-α in BDM than intact hIFN-a without BDM encapsulation by plasma factors that may degrade IFN-α, (ii) poor accessibility of such serum factors to encapsulated IFN-α in BDM than hIFN-α without BDM encapsulation, and/or (iii) any miscalculated amount of each material injected.
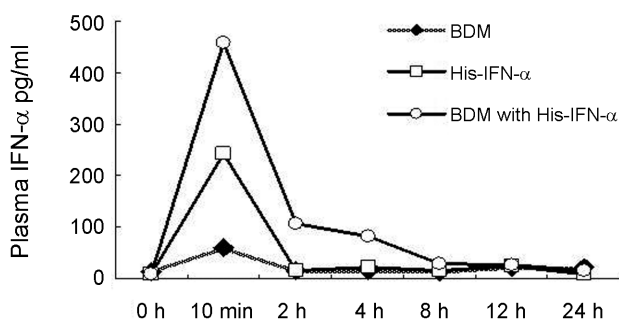
Pharmacodynamics of His-Tagged IFN-α Loaded Branched Dextran Microspheres in Rats

Neopterin is one of the serum immune factors and is highly secreted into the serum by macrophages after the exposure of IFNs such as IFN-α or IFN-γ, as evidenced by animal and human studies [19]. Plasma neopterin levels were therefore considered as the *in vivo* activity of plasma IFN-α herein. Fig. 5 shows the blood neopterin profiles after the administration of BDM-hIFN-α, BDM, or hIFN-α. In hIFN-α-injected rats, there was a peak (about 1.7 nM/ml) of plasma neopterin levels at 10 min post-injection, but its plasma levels declined sharply at 2 h. On the other hand, in BDM-hIFN-α-injected rats, although there was a peak (about 1.9 nM/ml) of plasma neopterin levels at 10 min post-injection, the high plasma levels of neopterin were sustained at 2 h. About 1.1 nM/ml of plasma neopterin was also detected at 4 h, but its plasma levels sharply declined thereafter. The sudden drop of the plasma neopterin seen at 8 h may be attributed to the sharp decrease in the plasma IFN-α at the same kinetics shown in Fig. 4 in this study.

The chemical structure of dextrans used in this work has been already identified by previous researchers. For example, it has been shown that *L. mesenteroides* B-512 produces a linear type of dextran (we called) with 95% α-1−6 linkages and 5% α-1−3 branched linkages, and this type of dextran is commercially available [31]. Mostly, this type of dextran has been used as a clinical dextran (blood expander) and for the synthesis of microspheres for drug delivery [32, 33]. On the other hand, it has been reported that *L. mesenteroides* B-742 produces a highly branched type of dextran with 50% α-1−6 linkages and 50% α-1−3 branch linkages of which the majority links single glucose units to the main chains [32], but this type of dextran is not commercial. Thus, one importance of the present study is the preparative approach for the encapsulation of IFN-α into branched types of dextrans produced by *L. mesenteroides* B-742. The biodegradability of dextran has been previously
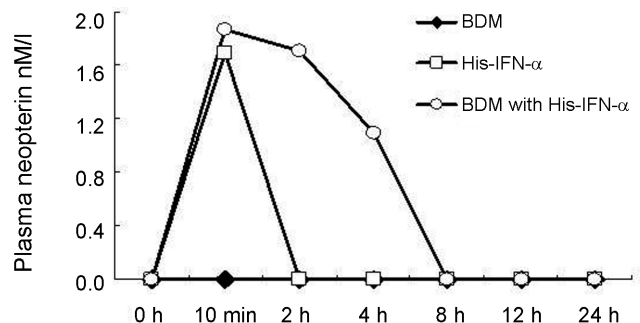
Fig. 4. Measurement of plasma IFN-α levels in rats after injection of dextran microspheres of His-tagged IFN-α.
Rats fasted the day before injection were intraveneously injected with 100 µg/kg body weight of branched dextran microspheres encapsulated with His-tagged IFN-α (BDM-hIFN-α), BDM, or hIFN-α, respectively. Plasma was then collected at the time of 10 min, 2 h, 4 h, 8 h, 12 h, or 24 h post-injection. The levels of plasma IFN-α were then quantified by IFN-α ELISA. Data are means ±SD (n=2).

Fig. 5. Determination of plasma neopterin levels in rats after injection of dextran microspheres of His-tagged IFN-α.
The samples used for the measurement of plasma IFN-α levels in Fig. 4 were also analyzed by neopterin (B) ELISA to determine plasma neopterin levels. Data are means ±SD (n=2).

reported [33]. Most results and information regarding the biodegradability of dextran in previous researches have been derived from use of the linear type of dextran. Thus, it will be interesting to investigate, in future, the biodegradability of *L. mesenteroides* B-742 dextran and its microspheres used herein.

In conclusion, we report the preparation of biodegradable branched dextran microspheres encapsulated with His-tagged IFN-α along with their *in vitro* and *in vivo* activities, which are evidenced with enhancement of STAT-1 phosphorylation in HepG2 cells and increased plasma neopterin levels in rats, respectively. Based on the findings presented herein, it is suggested that biodegradable branched dextran microspheres may be applied as a potential delivery vehicle for IFN-α.

Acknowledgment

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