Abstract

Injectable spherical porous hydroxyapatite microparticles (SP-HAp), with an average diameter of about 5,,m and an approximate porosity of 58%, prepared by a spray-dry method were applied to the delivery of bioactive proteins and lipophilic drugs. SP-HAp loaded with interferon alpha (IFN,,), testosterone enanthate (TE) or ciclospolin A (CiA) were investigated in the drugs release *in vitro* and *in vivo*. IFN,, release from IFN,,-adsorbed SP-HAp without and with reinforcement substances, human serum albumin and zinc, was investigated *in vitro* by incubating with PBS containing 10% mouse serum, and *in vivo* by injecting them subcutaneously to mice. In the both experiments, IFN,, release was slower and longer by adding the reinforcements. SP-HAp filled with TE or CiA in their pores were administrated subcutaneously or intravenously to the mice with a 27-gage needle, that is very small gage, and the release of drugs in the mice was confirmed. The facts indicate that it is possible to administrate intravenously lipophilic drugs and also that the subcutaneous injection of these drugs can be done less painfully, using SP-HAp as a carrier. The speed of biodegradation of SP-HAp was regulated by choosing the calcined temperature. The SP-HAp may be useful for the delivery of protein and lipophilic drugs in humans.

1. Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAp) is the main mineral component in bone and tooth, and has been widely used in orthopedic and dental fields as various forms of pastes, granules and porous blocks for implants [1-2]. On the other hand, it is utilized as an adsorbent of chromatographic supports for purification and separation of many molecules because of its diverse adsorption to them [3-5]. Porous HAp blocks and ceramics have also been investigated as sustained release system for therapeutic agents in drug delivery system (DDS) [6-10]. In these studies, HAp loaded with antibiotics and anticancer drugs were implanted in animals and these drugs were reported to release slowly [8-10]. However, an injectable HAp for the drug delivery has not been reported.

In this study, we fabricated spherical porous HAp microparticles (SP-HAp) for an injective preparation. SP-HAp was fabricated by a spray-dry method, and then adsorbed with bioactive proteins or filled to their pores with lipophilic drugs. We made experiments of drug release from the drug-loaded SP-HAp *in vitro* and *in vivo*. Highly lipophilic drugs are usually unable to be injected intravenously.

2. Materials and Methods

2.1 Animals and drugs

Male and female ddY mice, 8 weeks of age, and male Wistar rats, 13 weeks of age, were obtained from SLC Experimental Animals (Shizuoka, Japan). The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of Jikei University School of Medicine. Interferon alpha (IFN,,) was a kind gift from Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan). Testosterone enanthate (TE), ciclospolin A (CiA), human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). An oil preparation of TE (Enarmon Depot®) was obtained from Teikoku Hormone Co., Ltd.(Tokyo, Japan).

2. 2 Preparation of spherical porous hydroxyapatite microparticles (SP-HAp)

A 100 g of $CaCO_3$ powder (Wako pure chemical industry Ltd; Alkaline analysis grade) was heated at 1050°C for 3h, and the resultant CaO powder was hydrated with one third quantity of distilled water in order to produce $Ca(OH)_2$. A 1000 ml of 0.6M H_3PO_4 was dropped into 2000 ml of 0.5M $Ca(OH)_2$

suspension, and then stirred vigorously at room temperature. The obtained suspension was aged overnight. The pH of the suspension was adjusted to ca. 7.5. The SP-HAp was fabricated by a spray-drying method: the suspension was atomized under a pressure of 1.5 MPa with a flow rate of 500 ml/h, and inlet and outlet temperatures of a nozzle were adjusted to 180°C and 80°C. The SP-HAp was calcined at 400°C for 0.5h.

The prepared SP-HAp was analyzed by powder X-ray diffractometry (XRD, Philips PW1700) and Fourier transformed infrared spectrometer (FT-IR, Perkin-Elmer SPECTRUM-2000). The morphology of the SP-HAp coated with Pt was observed by using a scanning electron microscopy (SEM, JEOL-5600LV) with 20 kV of an accelerating voltage. The interior structure of the SP-HAp was observed by using a transmission electron microscopy (TEM, JEOL JEM-1010). The samples for the TEM observation were prepared to be 100 nm in thickness by embedding the microparticles in a photo setting resin, degassing in a vacuum and cutting with an ultramicrotomy diamond knife. The surface areas and total volumes of the particles synthesized were determined by Brunauer-Emmett-Teller method after degassed at 200°C for 2 h (Beckman-coulter, SA-3100). The porosity was calculated using a theoretical density of HAp (3.156 g/cm^3). The particle size distributions were measured by a laser diffraction method (Shimadzu Salada-2100, Kyoto, Japan).

2.3 Biodegradation of SP-HAp in the subcutaneous tissue of rats

Three sorts of HAp suspension were prepared; 1) lyophilized, 2) spray-dried and heated at maximum 180°C and 3) spray-dried and then calcined at 400°C. A 24mg of three sorts of SP-HAp were suspended in 6ml of 5% mannitol, and were mixed for 1 min, and then injected in the dorsal area of Wistar rats at a volume of 0.5ml. Animals were sacrificed 4,7,11,14,18 and 21 days after the injection and the amount of SP-HAp in the subcutaneous space was roughly estimated subjectively by three observers, and an average values were recorded.

2.4 Preparation of INFα-loaded SP-HAp

A 200mg of SP-HAp calcined at 400°C was added into 0.86ml of 93.5μg/ml solution of IFN,, and 0.14ml of distilled water. The mixed suspension was stirred for 10 min with a vortex mixer. This sample was used for *in vitro* and *in vivo* study. When the suspension was centrifuged, no INF,, was measured in the supernatant, that means all of INF,, used was adsorbed to the SP-HAp. In the experiment of reinforcement, 0.28ml of 35.73mg/ml solution of HSA was further added to the SP-HAp suspension and stirred, and then centrifuged. The precipitated SP-HAp was dried up in an evacuator

and 0.02ml of 1M zinc acetate and 0.98ml of distilled water was mixed, and was centrifuged. The precipitate was resuspended in 1ml water. Thus, we obtained two samples, INF„-loaded SP-HAp without and with the reinforcements.

2.5 Release of IFNα from INFα-loaded SP-HAp in vitro

Each 1ml of 2 samples without and with reinforcement substance was added to 5ml of PBS containing 10% mouse serum (PBS-MS) and shaken gently for 10 min at room temperature, then centrifuged at 3000 rpm for 5 min at 4°C. The supernatants were withdrawn and replaced with an equal volume of PBS-MS. This procedure was repeated 5 times. The amount of IFN„ released in the supernatants was determined by an ELISA kit (Biosource, USA). After the final centrifugation, the precipitate was mixed with PBS at pH4.5 containing 1% BSA and was shaken overnight in order to dissolve SP-HAp. The amount of INF„ in the solution was shown as that in the residue.

2.6 Release of IFN„ from INF„-loaded SP-HAp in vivo

Each 1ml of the two samples prepared in the section 2.4 was added to 1ml of 10% mannitol solution

to prevent the aggregation of SP-HAp. As a control, IFN„ solution was prepared by mixing 0.85 ml of 225 μg/ml IFN„ and 1.2 ml of 2% HSA, 0.5 ml of 20% mannitol and 0.65 ml of distilled water (final IFN„ concentration was ca. 60 μ g /ml). The two suspension formulations of IFN„-loaded SP-HAp without and with the reinforcements and control IFN„ solution were administered subcutaneously in male mice at a dose of 20„g/head. Blood samples were taken from the inferior ophthalmic vein on 0.6, 1, 2, 3, 4, 5, 6 and 10 days, and IFN„ concentration in plasma was determined with the ELISA kit.

2.7 Preparation of SP-HAp filled with lipophilic drugs

Fifty mg of SP-HAp calcined at 400°C was mixed with 250 mg of testosterone enanthate dissolved with 200 μl of acetone. In another experiment, 10 mg of SP-HAp were mixed with 57 mg of ciclospolin A (CiA) dissolved in 200 μl of ethanol. The two suspensions were stirred vigorously and centrifuged at 3500 rpm for 5 min to fill TE or CiA in the pores of SP-HAp. Supernatant was discarded and residual acetone or ethanol in the precipitates was removed in desiccation under vacuum. These samples were used for *in vivo* stadies. The amount of TE or CiA in the SP-HAp was determined by HPLC after the extraction of TE or CiA from the SP-HAp by a large amount of acetone. It was observed that approximately 47 mg of TE or 36.5mg of CiA were loaded in 50 mg of SP-HAp.

2.8 Release of TE or CiA from Drug-filled SP-HAp in vivo in mice

Two samples obtained above were suspended in 0.3% carboxymethyl-cellulose (CMC) solution, respectively. It was confirmed that 0.3% CMC almost prevented the release of TE and CiA from the SP-HAp. The suspensions and an oil preparation of TE as a control were administered subcutaneously in female mice. SP-HAp filling CiA was also injected subcutaneously in male mice. Blood samples were taken from the inferior ophthalmic vein for the determination of TE and CiA concentration with an ELISA kit (Oxford Biomedcal Research Inc., MI). In the experiments with intravenous injection only the SP-HAp formulations were used, since the oil formulations of both drugs were impossible to be injected intravenously with a small gaze needle. Time when the blood samples were taken, and doses used were described in the Legend of Figure.

3. Results

3.1. Characterization of spherical porous hydroxyapatite microparticles (SP-HAp)

Crystal phases of HAp particles prepared were confirmed by using XRD and FT-IR. The SP-HAp,

SP-HAp calcined at 400°C and lyophilized HAp were a single phase of HAp. It means that other phases of calcium hydroxide ($Ca(OH)_2$), calcium oxide (CaO), tricalcium phosphate ($Ca_3(PO_4)_2$) and octacalcium phosphate were not detected by XRD patterns. FT-IR spectra indicated that the absorption bands assigned to the CO_3 group were detected at 1458 and 1422cm^{-1}, which means that carbonate ions were substituted for the phosphate site in the crystal structure of HAp. The carbonate ions in the air substituted for phosphate site were not decomposed by heating it at 400°C. Fig. 1-A shows a SEM image of SP-HAp. The morphology was spherical with rough surface, because the nano-pores were interconnected within the inside of spherical microparticles. Fig. 1-B shows a TEM image of cross-section of SP-HAp. microparticle. The HAp crystallites are quite small, 25 nm in length and 5 nm in breadth, and their aggregation forms many interstitial pores of nano size. The surface area, total volumes and porosities were 87.9m^2/g, 0.435ml/g and 57.8% for SP-HAp and 77.4m^2/g, 0.413ml/g and 57.5% for SP-HAp400, respectively. The average particle size was 5.8μm for SP-HAp and 5.2 μm for SP-HAp400. The heating treatment at 400°C was not dramatically changed for the surface area and particle sizes.

3.2 Biodegradability of SP-HAp in rat subcutaneous tissue

The biodegradation of SP-HAp was dependent on the calcined temperature as shown in Fig. 2. SP-HAp calcined at 400°C decreased in amount most slowly and took 21 days to disappear completely. Thus, we can regulate the speed of biodegradation of SP-HAp.

3.3 Sustained release of IFNα from IFNα-loaded SP-HAp in vitro

The amounts of IFN„ released from the IFN„-loaded SP-HAp without and with the reinforcements in PBS-MS were plotted in Fig. 3. The amount of IFN„ released from the IFN„-loaded SP-HAp without or with the reinforcements gradually increased with the times of replacing the PBS-MS as shown in Fig3. Adding the reinforcements, the release of IFN„ was slightly slower than that without the reinforcements. When the amount of IFN„ in the residue was added, over 90 percent of IFN„ used was recovered in this experiment.

3.4 Plasma IFNα concentration after subcutaneous injection of IFNα-loaded SP-HAp in mice

Plasma IFN„ concentration after the subcutaneous injection of IFN„-loaded SP-HAp was measured in mice (Fig. 4). Following the injection of IFN„-solution, plasma IFN„ concentration was initially very high, and then decreased steeply and disappeared within 3d. The IFN„-loaded SP-HAp preparation without reinforcement showed some sustained release. On the contrary, in the case of IFN„-loaded SP-HAp with the reinforcements, a low but remarkably long lasting plasma concentration of IFN„ was observed until day 10. Abnormal signs were not detected in clinical observation.

3.5 Subcutaneous and intravenous injection of SP-HAp filled with lipophilic drugs in mice

Plasma testosterone concentration after the subcutaneous injection of TE-filled SP-HAp was high at 3h, and then rapidly decreased to a similar level to that of oil preparation (Fig. 5A).

After the intravenous administration of TE-filled SP-HAp plasma testosterone concentration was maintained in high levels as compared with that after the subcutaneous injection (Fig.5B).

As shown in fig.6, CiA-filled SP-HAp was able to be injected both subcutaneously and intravenously and obtained an adequate blood concentration. Abnormal systemic symptoms were not observed in

animals injected SP-HAp filled with TE and CiA.

3.6 Easiness in injection

At least a 22-gage needle was required for the injection of the oil preparation of TE. While, SP-HAp filling TE and CiA were able to be injected smoothly with a much smaller gage needle of 27.

4. Discussion

As reported many researchers, HAp adsorbs reversibly many chemicals and proteins [3-5], and is biodegradable and safe. Therefore, HAp will be a suitable material for DDS. However, it has not been used as injectable particles. We fabricated spherical porous HAp microparticle (SP-HAp) by a spray dry method, the average diameter of which was about 5μm. The SP-HAp thus prepared could be injected subcutaneously and intravenously as a suspension formulation with a very small size of needle and the porous particle has a very large area for adsorbing drugs. Kandari et al. observed a marked adsorption of immunoglobulin onto carious HAp particles [11]. We found also brain derived neurotropic factor (BDNF), superoxide dismutase (SOD), granulocyte colony stimulating factor

(G-CSF) were adsorbed well onto SP-HAp (data were not shown). The release of proteins from SP-HAp will be influenced by the ionic strength and protein concentration of the solution in which SP-HAp are suspended, since the main binding sites of SP-HAp with proteins are considered to be calcium cation and phosphate anion. We used PBS containing 10% mouse serum, and obtained a slow release of IFN$_{\alpha}$ from the SP-HAp preparation *in vitro*. Addition of HSA and Zn as possible reinforcement substances caused some increase in the slow release *in vitro*. The sustained release of IFN$_{\alpha}$ from the SP-HAp with the reinforcements was not adequate *in vitro* experiments. However, the reinforcements caused a very improvement in sustained release *in vivo*. Queiroz et al. used glass for a reinforcement of porous HAp implanted for the local treatment with ampicillin [12].

Oil or organic solvent preparations containing lipo-philic drugs such as TE and CiA usually cannot be injected intravenously. This study is probably first one showing that a large amount of lipophilic drugs dissolved in oil or organic solvents, which have been evaporated before use, were filled into nano-scale pores of microparticles and that a high blood concentration of the drugs was obtained with the intravenous as well as subcutaneous injection of the microparticle formulations.

5. Conclusion

In this study, we showed that SP-HAp were excellent biodegradable carriers for some bioactive proteins and lipophilic drugs for subcutaneous and intravenous injections. Using the SP-HAp without and with reinforcement substances we can expect a sustained release of bioactive proteins *in vivo*.

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Figure Legends

Fig. 1. SEM and TEM images of spherical porous HAp microparticle (SP-HAp).

A: SP-HAp are approximately 5μm in diameter and have a rough surface.

B: HAp crystallites prepared by a spray-dried method are 25 x 10 nm in size, and their aggregation forms many interstitial pores of approximately 100 nm in size.

Fig. 2. Biodegradation of spherical porous HAp microparticles (SP-HAp) in subcutaneous tissues of rats.

Three sorts of HAp were prepared; lyophilized, spray-dried and heated at maximum 180°C and spray-dried and then calcined at 400°C. These SP-HAp suspensions in volume of 0.5 ml were injected subcutaneously in dorsal area of rats. Disappearance of SP-HAp was estimated subjectively. (n=3)

Fig. 3. Released IFNα from IFNα-loaded spherical porous HAp microparticles (SP-HAp) in PBS containing 10% mouse serum (PBS-MS). n=3

IFNα-loaded SP-HAp were suspended in 5 ml of PBS-MS and shaken. After centrifugation, PBS-MS was replaced 5 times and IFNα concentration in the supernatant was measured with an ELISA

kit.

Fig. 4. Plasma IFNα concentration after subcutaneous injection of IFNα-loaded spherical porous HAp microparticles (SP-HAp) in mice.

Bolus doses of IFNα in SP-HAp and in solution were 20 μg/head. Each point and vertical bar represents the mean ± SEM of 3 experiments. Blood samples were obtained 4h and 1, 2, 3, 4, 5, 6 and 10 d after the administration.

Fig. 5. Plasma testosterone concentration after injection of TE-filled spherical porous HAp microparticles (SP-HAp) in mice.

A: Bolus doses of TE in SP-HAp and in oil preparation (Enarmon Depot®) were 23.8 mg/head and 29.9 mg/head, respectively. Each TE preparation was administered subcutaneously in mice, in a volume of 0.25 ml. Blood samples were obtained 1, 3, 24, 48, 96, and 120 h after the administration.

B: A bolus dose of TE in SP-HAp was 1.9 mg/head. TE-filled SP-HAp was administered intravenously in mice, in a volume of 0.3 ml. Blood samples were obtained before and 1, 24, and 48 hr after the administration.

Each point and vertical bar represent the mean ± SEM of 3 experiments in A and B.

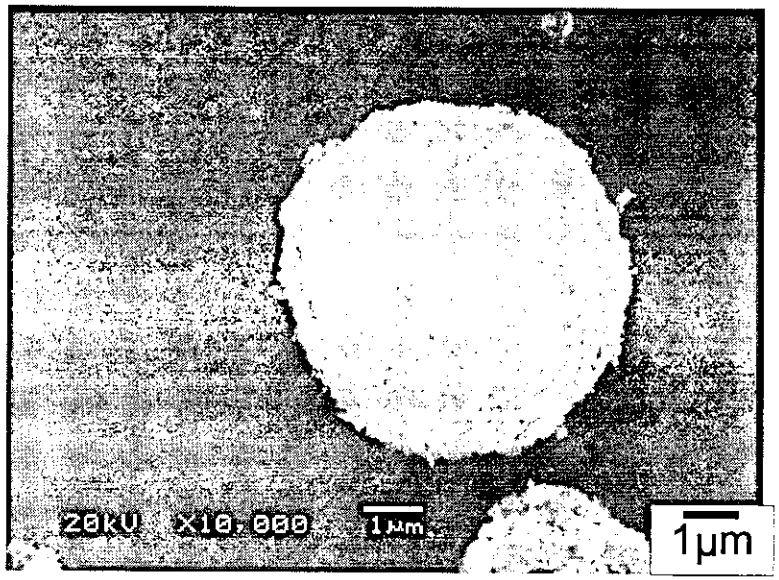
Fig. 6. Plasma CiA concentration after injection of CiA-filled spherical porous HAp microparticles (SP-HAp) in mice.

A: A bolus dose of CiA in SP-HAp was 1.64 mg/head. CiA-filled SP-HAp was administered subcutaneouly (A) or intravenously (B) in mice, in a volume of 0.2 ml. Blood samples were obtained before and 2, 24, 48 and (72) h after the administration.

Each point and vertical bar represents the mean ± SEM of 3 experiments.

Fig. 1. Mizushima

A



B

Fig.2. Mizushima

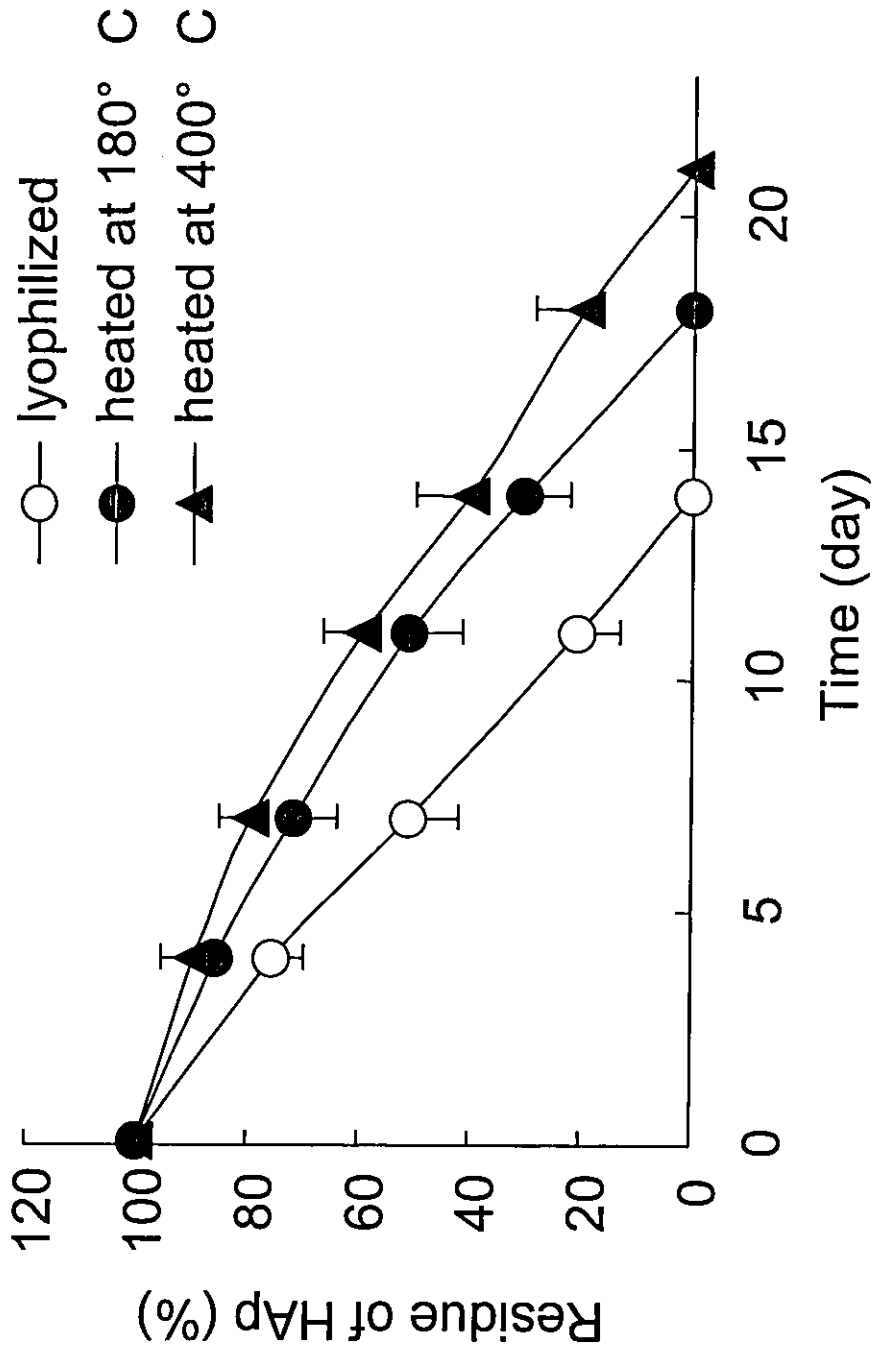


Fig. 3. Mizushima