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Injectable Gel Type Formulation of Hydrated Egg Phosphatidylcholine and Hyaluronate for Local Drug Delivery

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ABSTRACT–Injectable gel composed of egg phosphatidylcholine (egg PC), hyaluronate (HA) and water was formulated for local drug delivery. The lamellar liquid crystalline structure of the egg PC/water system did not change by adding HA in the formulation. However, egg PC/HA/water gel was more resistant to erosion than the egg PC/water gel. The egg PC/HA/water and egg PC/water gels containing model drugs, tetracycline and sudan IV were prepared to perform *in vitro* and *in vivo* drug release experiments. *In vitro* release of tetracycline was sustained in the gel type formulations. The release rate of hydrophobic sudan IV was extremely slow. More than 99% of sudan IV remained inside the gel after 5 days. *In vivo* release of drugs from the air pouch model in Balb/c mice shows that lipophilic sudan IV remained for more than 10 days whereas tetracycline remained for 1 day in the pouch. The compatibility of the gels was also examined by histopathology. The gels did not cause any adverse inflammatory effect in the air pouch.

Keywords–Injectable gel, Sustained release, Egg phosphatidylcholine, Hyaluronate

Introduction

Local delivery of a drug to a specific site is an attractive delivery route since it could minimize unwanted exposure of the drug to other parts of body.[1] Moreover, the amount of drug intake could be minimized by a local delivery. Due to modern surgical technologies, the local delivery to various tissue sites or different organs has become possible. Injectable gel is one of the formulations developed as local drug delivery systems.[2-4] Local anesthetic or NSAID delivery[5-9] has been used in a pain clinic or for the treatment of arthritis, respectively. Some of the main prerequisites to become local gel type formulations include biocompatibility and sustained drug release for a desired time period.[7,10-12] Since it is not easy to fulfill these important requirements simultaneously, it is a difficult task to formulate a suitable gel.

Local and sustained drug treatment is required especially for organ transplant patients, burn victims, arthritis patients or patients after surgery to prevent infection. Sustained drug delivery systems have additional benefits of delayed inactivation of drug. Recently, microparticles, gels and liposomes have been developed as local delivery systems.[13-16] Among them, the gel type formulation is a good candidate for a local drug delivery system (DDS) since it can stay at the site of the injection longer than the dispersion type systems. We have prepared gel type formulations composed of a phospholipid and hyaluronate. Phospholipid and hyaluronate are main components of cell membrane and extracellular matrix, respectively, and are expected to be biocompatible. Especially, hyaluronate is widely used as an injectable into the knee joint for viscosupplementation or in eye surgery.[17] We have prepared gel type formulations with egg phosphatidylcholine (egg PC), hyaluronate (HA) and water. The physical properties, *in vitro* and *in vivo* drug release characteristics, and biocompatibility of the gels have been evaluated in this paper.

Experimental

Materials

L-α-phosphatidylcholine from dried egg-yolk (egg PC, 60 % pure by TLC), hyaluronic acid sodium salt (hyaluronate, HA), tetracycline hydrochloride and sudan IV were purchased from Sigma Chemical Company (St. Louis, MO) and used

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without further purification. The major fatty acids in egg PC are palmitoyl (34% by weight), palmitoleyl (1%), stearyl (11%), oleyl (32%), linoleyl (18%) and others (3.3%), and the headgroup components are phosphatidylethanolamine (11.01% by weight), phosphatidylinositol (1.1%), phosphatidylcholine (72.7%), lysophosphatidylinositol (2.07%) and other insoluble neutral lipids (13.12%) (from Avanti Polar Lipids, Inc. Products Catalog Edition VI, http://www.avantilipids.com/). Water was purified by using a water purification system (Milli-Q Plus; Millipore Corp., Bedford, MA).

Preparation of gels

For *in vivo* experiments, all components in the gel were sterilized. Egg PC alone or egg PC containing drug was solubilized in excess ethanol. The ratio of egg PC to drug was 1: 0.08 by weight. Egg PC solution was put into a syringe and filtered through a sterile syringe-filter (PVDF, 0.45 μm pore size, Whatman Co. Clitton, NJ). Ethanol was completely evaporated by purging with air-filtered nitrogen gas inside a clean bench and subsequently by placing it in a sterilized vacuum chamber. Aqueous phase was autoclaved at 1.5 atm and 121°C for 15 min.

Mixing of the lipid and aqueous phases was accomplished by using a syringe assembly, which is modified from the lipid mixer of Cheng and co-workers.[18] Briefly, the lipid and water were inserted into two separate 3-ml syringes (disposable, Korean Vaccine Co., Korea) to prepare a gel containing egg PC and water with (egg PC/water/tetracycline or egg PC/water/sudan gel) or without (egg PC/water gel) drug. The weight ratio between the lipid and water was 1:0.5 for egg PC/water/tetracycline and egg PC/water gel and 1:0.75 for egg PC/water/sudan gel. The two syringes were connected by means of a three-way stop-cock (Discofix®, B. Braun, Emmenbrücke, Switzerland). Lipid and aqueous phases were mixed by pushing the plungers of the two syringes alternately until the mixture became homogeneous. To prepare gels containing hyaluronate, 0.2%(w/v) hyaluronate aqueous solution, was inserted instead of water into the syringe.

***In vitro* release of drugs**

For the release experiment, 100 mg of the gel containing tetracycline or sudan IV was injected into a dialysis bag (Spectra/Por® membranes, MWCO:3500, Spectrum Medical Industries, Inc., Los Angeles, CA). The bag was sealed and immersed in 10 ml phosphate buffered solution (PBS) at pH 7.4 at room temperature. The tubes were incubated in a shaking water bath (Vision Co., Ltd., Korea) at 37 °C at a shaking frequency of 150 rpm. The release medium was exchanged totally with fresh PBS solution of an equal volume when the concentration of the released drugs was determined. Concentration of tetracycline was determined by UV-VIS spectrophotometer (spectrometer UV/VIS Lamda 25, Perkin Elmer Co., Shelton, CT) at 280 nm where the absorbance represents the total concentration of tetracycline and its active degradation products.[19-20] The concentration of released sudan IV was determined also by UV-VIS spectrophotometer at 530 nm.

Murine air pouches

Air pouches were generated according to the methods of Sedgewick et al. in groups of 3 female Balb/c mice.[21] An area of the dorsal skin (2 cm^2) was cleaned with alcohol and shaved to provide the pouch site. Air (3 ml) was injected subcutaneously at a single site with 25-gauge needle and 3-ml syringe. The air pouches were injected with 2 ml of air on alternate days for 5 days to establish a definitive fluid filled pouch. On day 6, the pouches were injected with 100 mg of gels with or without drugs. Control pouches received 100 μl of sterile PBS (pH 7.4) alone. The mice were sacrificed 6 h to 20 days after the injection. The pouches were then dissected carefully from the surrounding tissue. The pouch was weighed and photographed under a UV lamp (tetracycline) or in normal settings (sudan IV). The pouch without drug was fixed in formalin for histological evaluation.

Histological analysis

Air pouches were embedded in OCT compound (Tissue-Tek®, Sakura, Japan) and frozen immediately in a deep freezer at –20°C. Tissue sections of 4~5 μm depth were cut using a cryostat at –25°C, mounted on silane-coated glass slides (Micro Slides Single Frosted 75×25 mm, Corning, N.Y) and dried at 60°C in a drying oven (Model 5831, Precision Scientific Co., Chicago, IL) for 30 min. Sections were stained with haematoxylin and eosin, and Mallory's azan (Muto pure chemicals Co., Ltd., Tokyo, Japan). The prepared tissue specimens were observed under a light microscope (Nikon optiphot-2, Japan).

***In vivo* release of drugs**

The pouches were weighed and homogenized (T-25-Ultra-Turrax, Janke & Kunkel GmbH &Co KG, Germany) in methanol or dimethylsulfoxide to extract the remaining tetracycline and sudan IV, respectively. The homogenized tissue was incubated for 3 h at 37°C to wait until the drug releases to the bulk solvent phase and centrifuged for 10 min at 1000 g. The supernatant was filtered selectively through a 0.22 μm

syringe filter (Millex®-GV, Millipore Co., Bedford, MA) for further analysis. The concentration of tetracycline was determined by high performance liquid chromatography (HPLC) as described elsewhere.[22] Briefly, HPLC system consisted of a SP8810 precision isocratic pump (Spectra-Physics Inc., San Jose, CA). Mobile phase consisted of 84%(v/v) of acetonitrile and 16%(v/v) PBS with a trace of hydrochloric acid to adjust pH to 2.5. The flow rate of the mobile phase was controlled to 1 ml/min. Waters μBondpack™ C_{18} Column (3.9 mm×300 mm, Waters Corp., Milford, MA) was used. The column effluent was monitored at 330 nm by using Spectra 100 variable wavelength detector (Spectra-Physics). The concentration of sudan IV was quantified at 330 nm by using a UV-VIS spectrophotometer.

X-Ray diffraction

Gels were sealed into 1.0 mm diameter quartz x-ray capillaries (Charles Supper Co., Natick, MA). Copper Kα x-rays were generated using an x-ray generator (FL CU 4 KE, Bruker, Karlsruhe, Germany). The instrument was operated at a tube voltage of 40 kV and an electron beam current of 45 mA. Focusing of the beam and detection of the small-angle x-ray diffraction pattern were achieved by using a General Area Detector Diffraction System (GADDS, Bruker, Karlsruhe, Germany). To avoid air scattering, the beam path was filled with helium. The sample-to-detector distance was 30 cm, and the exposure time was 3 h.

Differential scanning calorimetry

Measurements were made with a Mettler differential scanning calorimeter (DSC 821e/400, Mettler-Toledo GmbH, Switzerland) with the gel samples operated at heating or cooling rates of 5 °C/min. Egg PC/water and egg PC/HA/water samples consisted of the gels equivalent to 6.5 mg egg PC.

Polarized light microscopy

Measurements were made with a light microscope with cross polarizers (C-2000L, Olympus, Japan) with the gel samples in excess water.

Determination of the particle size in the dispersion

The average particle size in the dispersion was determined by quasielastic laser light scattering with a Malvern Zetasizer ® (Malvern Instruments Limited, England). The dispersion made by vortexing egg PC/water or egg PC/HA/water gel was diluted by 300 times before the measurement. The size determination was repeated 3 times/sample for at least three samples comprising an identical composition.

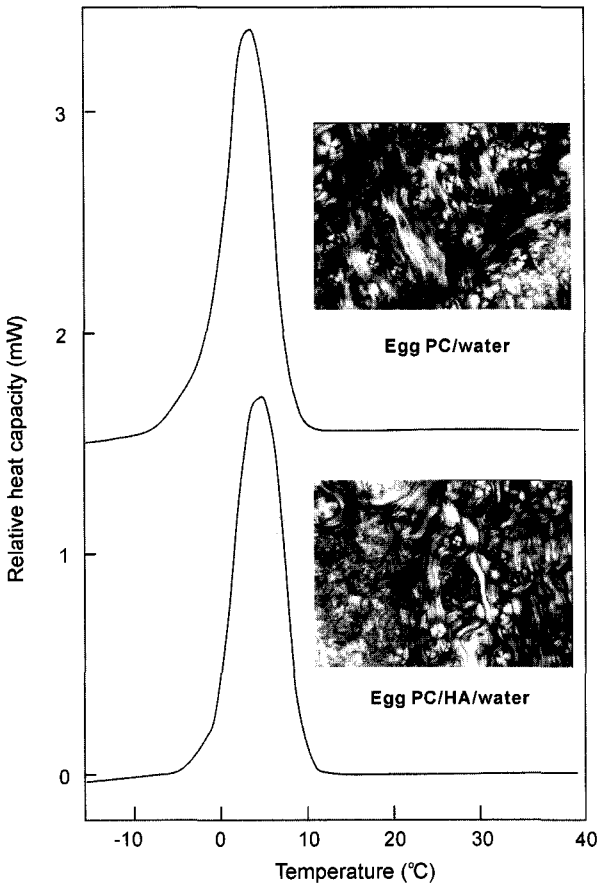

Figure 1–Heating thermograms of egg PC/water (1000:500 by weight) and egg PC/HA/water (1000:1:500 by weight) gels in excess water. The gels were thermally cycled between −20 and 40°C twice and cooled to −20°C before the measurement. The scan rate was 5°C/min, and the weight of egg PC in the samples is 6.5 mg. The photographs of the gel taken by using a polarized light microscope (×100) were shown as inserts in the Figure.

Results

Physical characterization of the gels

To find out how hyaluronate changes the thermal behavior of egg PC, the heating thermogram of egg PC/water (1000:500 by weight) and egg PC/HA/water (1000:1:500 by weight) were obtained (Figure 1). The samples were thermally cycled between −20 and 40°C twice and cooled to −20°C before obtaining the heating thermograms. The main phase transition temperature (T_m) from L_β to L_α phase was 2.6 °C similar to that reported in the literature.[23] Upon adding HA in the system, T_m increased to 1.0 °C without any changes in the transition enthalpy (ΔH=4.1±0.2 kcal/mole). To identify the phase above T_m, the gels were visualized under a polarized light microscope at 22±2°C (Figure 1). Furthermore, the small angle x-ray diffraction patterns were obtained at 25±4°C. Both egg PC/

water and egg PC/HA/water systems show typical textures of the L_α phase. Also, the x-ray diffraction shows that the lamellar repeat spacing of both systems was identical and was 50±0.5 Å. Due to technical limitations, the phase below T_m could not be identified.

Mechanical Stability of the gel

The formulations comprising egg PC/water (1000:500 by weight) or egg PC/HA/water (1000:1:500 by weight) were viscous gels. To test the mechanical strength of the gels, gels were injected into wells of a 6-well plate (Becton Dikinson Co., Franklin Lakes, NJ) containing 1 ml of PBS. The plate was vortexed for 30 s and 2 min for visual inspection (Figure 2). Immediately after injecting the egg PC/water gel, we could observe the erosion at the boundary of the gel (Figure 2A). In contrast, there was no noticeable change in case of the egg PC/HA/water gel. The egg PC/water gel broke into pieces after vortexing the plate for 30 s (Figure 2B). Only surface erosion was observed for egg PC/HA/water gel after vortexing it for 2 min (Figure 2C). In distinct contrast, most of the egg PC/water gels was dispersed in water. After a 2-min vortexing, the dispersion containing the egg PC/water gel was about 3 times more turbid than that with egg PC/HA/water gel. For both gels, the turbid dispersions contain multilamellar vesicles (MLV) with the average size (diameter) of about 350~400 nm and polydispersity of 0.2~0.6.

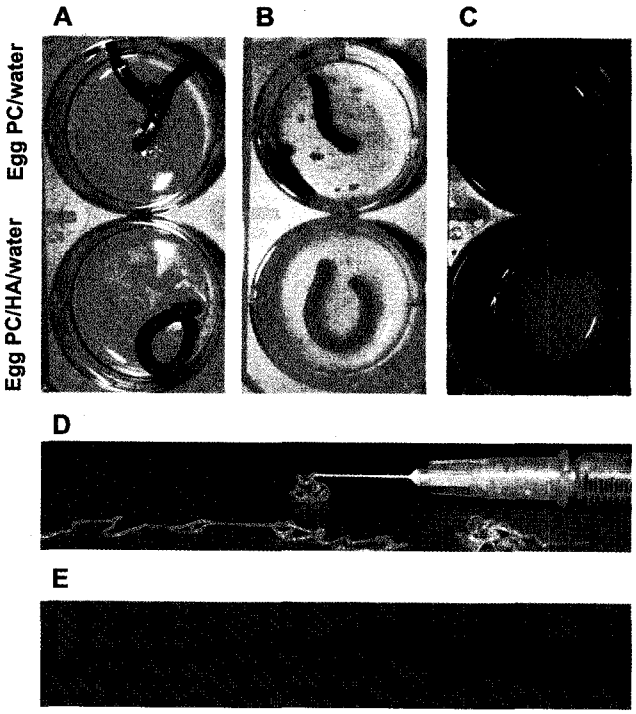

Figure 2–Photographs of the gels immersed in PBS (A, B, C) and injected from the syringes (D, E). The egg PC/water (1000:500 by weight, upper panels) and egg PC/HA/water (1000:1:500 by weight, lower panels) gels were immersed in excess PBS (A) and vortexed for 30 s (B) or 2 min (C) at 3000 rpm. The egg PC/HA/tetracycline/water (1000:1:75:500 by weight, D) and egg PC/HA/sudan IV/water (1000:1:75:750 by weight, E) were injected via 26 gauge needles.

To be a local gel-type drug delivery system, the gel must be injected through a needle without difficulties. Our egg PC based gel systems could be injected easily via 26 gauge needles (Figures 2D and 2E). Depending on the loaded drug, the viscosity of the gels changed considerably. It was possible to inject the gel containing tetracycline when the ratio between egg PC and water was lower than 1:0.5 by weight. In case of sudan IV, however, more water was required (egg PC: water= 1:0.75 by weight) to make the gels injectable (data not shown).

***In vitro* drug release from the gel**

A drug, tetracycline (50 mg/g gel) or sudan IV (43 mg/g gel) was loaded in the egg PC/water or egg PC/HA/water gels to investigate the *in vitro* release characteristics from the gels. Release of tetracycline dissolved in water (50 mg/ml) was also investigated as a control experiment. Due to extremely low solubility in water, the control experiment could not be carried out for sudan IV. The release of tetracycline was retarded considerably from the gels when compared to that from the aqueous environment (Figure 3A). The release rates from the egg PC/water and from the egg PC/HA/water were not different significantly. Approximately 50% of the drug was released in 200 h from both gels. Cumulative tetracycline release from the gels increased gradually as a function of time.

In case of an extremely hydrophobic sudan IV, the drug was

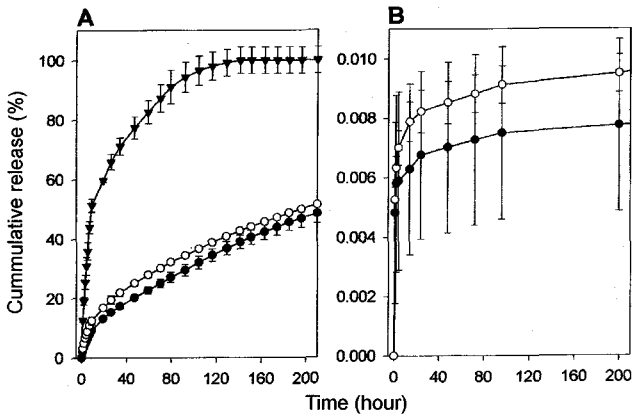

Figure 3–*In vitro* release of (A) tetracycline and sudan IV (B) from egg PC/water (●) and egg PC/HA/water (○) gels at 37°C. Release of tetracycline dissolved in water (▼) was also presented as a control. The concentration of tetracycline and sudan IV in the gels were 50 and 43 mg/ml, respectively.

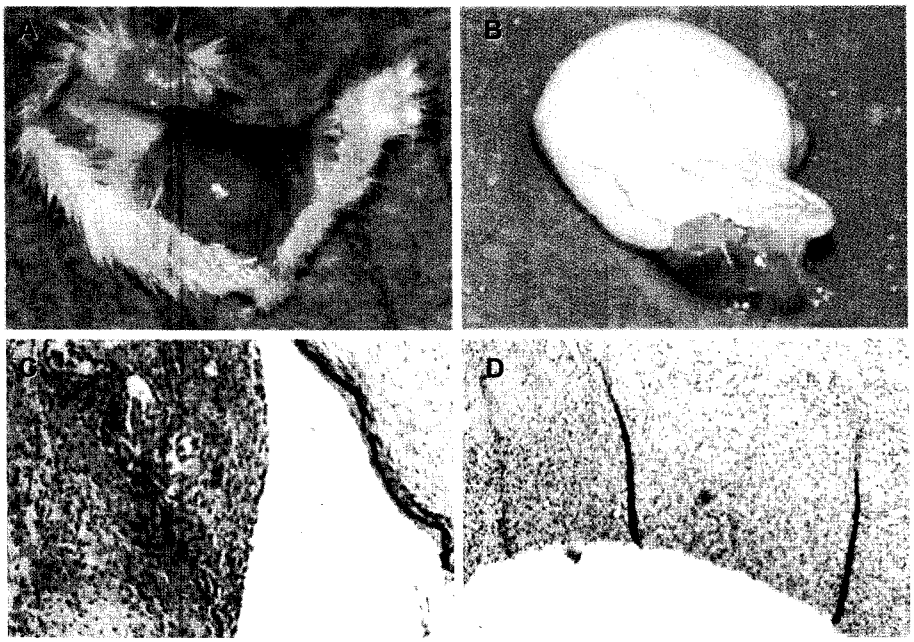

Figure 4–Photographs (A, B) and the histological appearance (C, D) of the air pouch. The partially(A) and fully (B) dissected air pouch obtained 1 day after injecting 100 mg of egg PC/HA/tetracycline/water (1000:1:75:500 by weight). The histological apparance of the control air pouch membrane (C) and the sample membrane obtained 1 day after injecting 100 mg of egg PC/HA/water (1000:1:500 by weight, D).

hardly released from the gels for the duration of the experiment (Figure 3B). Even after 200 h, more than 99.99% of the drug remained in the gels.

Biocompatibility of the gels in the air pouch membrane

The egg PC/HA/tetracycline/water gel (100 mg) was injected inside the air pouch (Figure 4A). Fully dissected air pouch was yellow when tetracycline loaded gel was injected (Figure 4B). The histological appearance of the air pouch membrane is shown in Figures 4C and 4D. The control membrane was characterized by an outer fibrous layer populated mainly by fibroblastic cells and an inner inflammatory layer predominantly comprised of macrophages (Figure 4C). The specimen injected with the egg PC/HA/water gel (Figure 4D) appears identical to the control in terms of the membrane thickness in pouches and the number of cells per unit area 2 weeks after the injection indicating that our gel type formulations do not evoke inflammation in the tissue and are biocompatible. The histological appearance of the pouch loaded with egg PC/water gel was also identical to the control.

***In vivo* drug release from the gels in the air pouch**

The gel containing tetracycline (50 mg/g gel) or sudan IV (43 mg/g gel) was injected into the air pouch for *in vivo* drug release experiment. Tetracycline solution in PBS (50 mg/ml) was also injected for control. Since we could not find a suitable biocompatible solvent that dissolves sudan IV, the control experiment could not be carried out for sudan IV. The remaining drug in the air pouch was quantified. Under a UV lamp or by eye, the remaining tetracycline or sudan IV, respectively, could be easily identified (Figure 5). The fluorescence of tetracycline was intense and located specifically inside the air pouch for up to 1 day. At day 2, the fluorescence was weakened considerably and could be seen in the surrounding tissues. The fluorescence was still visible, but extremely weak at days 3~5. In case of sudan IV, the intense red color locates mainly inside the air pouch for up to 10 days. The red color was still visible inside the pouch at day 20.

The remaining drug inside the air pouch was quantified to estimate the *in vivo* release rates (Figure 6A). The release rates of tetracycline from the egg PC/water and egg PC/HA/water gels were not different significantly. Approximately 50% of the drug was released completely in 10 h from both gels. In case of tetracycline solution, no drug was found in 6 hours. Therefore, we conclude that the gels retarded release of the drug considerably. The retardation of the drug release was more pronounced when a more lipophilic drug, sudan IV was used. Fifty percent of sudan IV remained in the gel in 10 or 15 days after injection in case of egg PC/water and egg PC/HA/water gels, respectively.

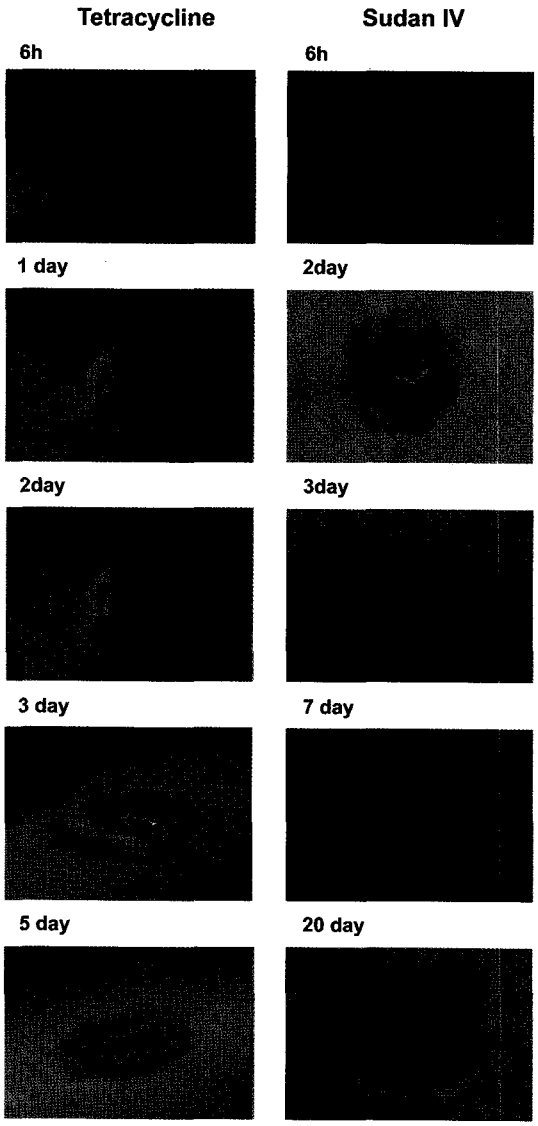

Figure 5–Photographs of partially dissected air pouch obtained at different time points after injecting 100 mg of egg PC/HA/tetracycline/water (1000:1:75:500 by weight, A) and egg PC/HA/sudan IV/water (1000:1:75:750 by weight, B). The air pouches containing tetracycline were viewed under a UV light to observe the fluorescence (A).

Discussion

Physical characteristics and stability of the gels

The gels comprising egg PC/water and egg PC/HA/water can easily be injected using a 26-gauge needle (Figure 2). The somewhat brittle egg PC/water gels was stabilized mechanically by adding hyaluronate in the system. The egg PC/HA/water gel was in the lamellar liquid crystalline (L_α) phase at temperatures above 10°C. By adding HA in the system, the gel acquired an added mechanical stability without changes in the phase. When HA was added, the gel doe not break easily upon vortexing. The surface erosion occurred from the egg PC/HA/water gels forming the solution containing *ca.* 350~400 nm particles. Considering the size of the particles and other physical characteristics from the x-ray diffraction, polarized light microscopy and DSC results, the particles seem to be multilamellar vesicles (MLV) in the L_α phase. Therefore, even after surface erosion, the drug could still be located inside the vesicle instead of in the bulk water phase. If the gel is confined inside tissue, e.g. air pouch, the release of the drug in the MLV could still be controlled.

Biocompatibility of the gels

It is known that phospholipid and hyaluronate are biocompatible.[24-25] Therefore, it is not surprising that the egg PC/HA/water gel does not cause inflammation in the air pouch. Since it is an important prerequisite for an injectable drug delivery system to be biocompatible, the egg PC/water or egg PC/HA/water gel could be used as an injectable gel.

Even if the gel is biocompatible, the eroded particles form the surface of the gels can be inflammatory in some cases.[26] The eroded particles of the egg PC/water or egg PC/HA/water gel, however, is a MLV that are also known to be biocompatible. Indeed we could not observe any adverse effects on the tissue for 2 weeks, which would be enough time for the gel to erode into MLV.

Drug release from the gels

For *in vitro* release experiment, the release rate of tetracycline was retarded considerably from the gels when

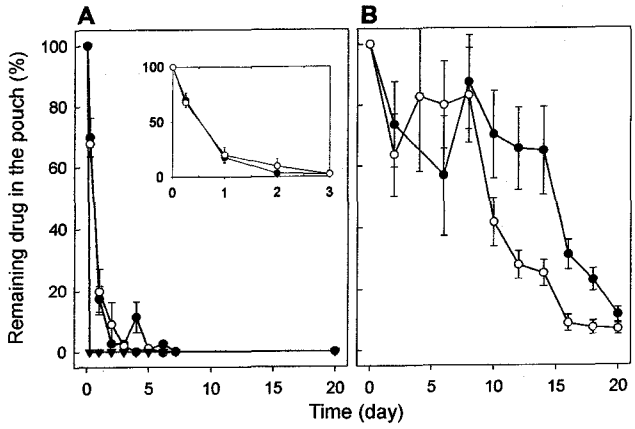

Figure 6–Remaining tetracycline (A) and sudan IV (B) inside the air pouch at different time points after injecting 100 mg of egg PC/water (●) and egg PC/HA/water (○) gels. Release of tetracycline dissolved in water (▼) was also presented as a control. The concentration of tetracycline and sudan IV in the gels were 50 and 43 mg/ml, respectively.

compared to that in aqueous environment (Figure 3). In case of sudan IV, most of the drug was contained inside the gel for 200 h. One of the possible explanations for the differences in the release rates is the disparity in the lipophilicity. While tetracycline is readily soluble, sudan IV does not dissolve in water. Therefore, while the tetracycline in the gel could diffuse out of the gel, sudan IV locates specifically inside the gel. Therefore, we could control the release rate of the drug by selecting a suitable drug with a desired release profile.

Similar pattern was observed in case of the *in vivo* experiment. The hydrophilic tetracycline disappeared from the air pouch in a few days after injection (Figure 6). More lipophilic sudan IV, however, remained inside the pouch for more than 10 days. It would be possible, therefore, to choose a drug from a spectrum of drugs to achieve a preferable release profile.

Conclusions

The egg PC/water and egg PC/HA/water formulations are biocompatible and injectable gels that can incorporate different types of drugs. Egg PC/HA/water gel is a mechanically more stable gel than egg PC/water gel while the liquid crystalline phase characteristics of both gels are virtually identical. Depending on the symptoms to treat, the required time span for the drug to remain in the tissue could be different. Since the release rate of the drugs can be governed by the physico-chemical properties of the loaded drug, we could choose a suitable one for the treatment.

Acknowledgements

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