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Research Paper

Composite Hydrogel Formulations of Stratifin to Control MMP-1 Expression in Dermal Fibroblasts

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Purpose. Stratifin is a potent anti-fibrogenic factor that stimulates the expression of matrix metalloproteinase-1 (MMP-1) in dermal fibroblasts. The propose of this work was to develop a controlled release delivery system for stratifin that can be applied at the time of wound closure to release stratifin and stimulate the expression of MMP-1 in a sustained manner over the late stages of wound healing (after 3 days).
Methods. Stratifin was complexed to chitosan particles, which were then encapsulated in PLGA microspheres and blended into crosslinked hyaluronic acid films. *In vitro* release was assessed using fluorescent-tagged stratifin, cytotoxity by MTT assay and bioactivity by measuring the levels of MMP-1 expression in cultured fibroblasts.
Results. The release of stratifin was delayed for 3 days and then controlled for 30 days so that 60% of the total stratifin loaded was released. The released protein significantly stimulated the expression of MMP-1 in cultured fibroblasts without compromising cell viability. By complexing to chitosan, the initial burst release was reduced, so that only 5% of stratifin was released in 3 days.
Conclusion. This stratifin delivery sytem has the potential to be used as an anti-fibrogenic factor-associated wound insert for improving post-surgical scarring in closed wound.

KEY WORDS: hydrogel; hypertrophic scarring; matrix metalloproteinase; microspheres; stratifin.

INTRODUCTION

Collagen deposition is an essential component of normal wound healing, but if unregulated or excessively prolonged, contributes to the development of fibroproliferative disorders such as hypertrophic scarring and keloid induction (1). Currently, there are no effective treatment modalities for these conditions (2).

It is well known that when keratinocytes infiltrate and epithelialize on the wound area within 2-3 weeks, only one third of the anatomically matched wounds become hypertrophic. However, the incidence of hypertrophic wounds increases to 78% when a wound is epithelialized later than 21 days (3). These findings suggest that upon epithelialization, some of the keratinocyte releasable factors may function as wound healing termination factors at the late stage of the wound healing process. In fact, recent studies indicate that keratinocytes synthesize and release a strong collagenase stimulating factor for dermal fibroblasts which was identified to be stratifin or 14-3-3 σ protein (4).

These findings suggest that stratifin, whose anti-fibrogenic effect has previously been established, is an ideal natural factor for improving and/or preventing excessive scarring. However, topical application of such a protein of this size is not feasible because the epidermal barrier prevents penetration (5). The objective of this study was to develop a controlled release delivery system for stratifin that can be applied at the time of wound closure. This formulation should optimally release stratifin for extended periods after granulation tissue is formed beginning at 3 to 5 days post operation. Intra-wound application of stratifin may be complicated because the biological half-life of most proteins is short. To overcome this problem, stratifin should be encapsulated in a controlled release formulation that may be retained at the wound site.

A suitable biomaterial for the controlled delivery of proteins is the biocompatible biodegradable polymer poly (lactic-co-glycolic acid) (PLGA). The main potential problems with using PLGA for this purpose are protein aggregation during the loading process and a high burst phase of proteins (6,7). Some studies have shown that polymer microspheres containing both hydrophobic and hydrophilic regions successfully encapsulated proteins like BSA, bFGF and superoxide dismutase (SOD) showed controlled release properties and maintained the bioactivity of the proteins (8,9). Geng *et al.* showed that erythropoietin could be successfully encapsulated in polysaccharide particles achieving sustained release of the protein while preserving bioactivity (10). Other studies have shown that a reduction in the burst

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phase of protein release may be achieved by embedding microspheres in hydrogel scaffolds that further restrict water movement and protein dissolution or diffusion (11–14).

In the present study, stratifin was encapsulated in PLGA microspheres, which were embedded in a crosslinked hydrogel scaffold. Since stratifin is a negatively charged protein, it was electrostatically complexed to the positively charged polysaccharide, chitosan, before encapsulation in PLGA microspheres. Chitosan is a hydrophilic polymer that is both biocompatible and biodegradable (15). Furthermore, it has been used to enhance the delivery of plasmid DNA by condensing the DNA to protect it from nuclease degradation (16–18). Chitosan was also found to protect the activity of lysozyme during encapsulation in PLGA polymer (19).

In this study, microparticulate chitosan (approximately 40 μm diameter) was soaked in a stratifin solution, dried and the particles were then encapsulated in large (approximately 300 μm) PLGA microspheres. These microspheres were homogenously distributed in crosslinked hyaluronic acid films. Hyaluronic acid is a glycoaminoglycan component of extracellular matrix with unique wound healing and anti-adhesion properties and it has been widely used as the base material for artificial skin, as an injectable tissue expansion solution and biodegradable wound dressing (20–22).

The ideal performance characteristics of this delivery system were, firstly, to create a lag phase and, secondly, to control the release of stratifin from the matrix while maintaining the biological activity of the protein. This multi-component formulation of stratifin in chitosan, encapsulated in PLGA microspheres and dispersed in a hyaluronic acid film successfully achieved these objectives. The biological activity of sustained released stratifin was evaluated by measuring the level of MMP-1 expression in cultured dermal fibroblasts.

MATERIALS AND METHODS

Materials

PGEX-6P-1 expression vector, PreScission protease and glutathione sepharose-4B beads were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Protein expressing bacteria, BL-21 (DE3) was purchased from Novagene (Madison, WI). Anti human MMP-1 IgG_1 (R&D Systems Inc., Minneapolis, MN), mouse anti- ß-actin IgG_1 (Sigma, MO, USA), secondary horseradish peroxidase conjugated anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA), western blotting detection reagent (Santa Cruz Biotechnoloy, Santa Cruz, California), 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT; Sigma, MO, USA), EZ-Label™ FITC protein labeling kit (Pierce Biotechnology Inc., Rockford, IL) and D-Salt™ dextran desalting columns (Pierce Biotechnology Inc., Rockford, IL) were all used as received.

Chitosan (ULTRASAN™) (MW.226000, degree of deacetylation=74.2%) (Biosyntech Inc., Québec Canada), PLGA (85/15, IV=0.61 dl/g) (Birmingham Polymers, Birmingham, AL), Poly (vinyl alcohol) (PVA) (98% hydrolyzed, MW. 25000) (Polysciences, Warrington, PA) and medical grade sodium hyaluronate (HA) (MW. 1,000,000) (Lifecore Scientific, Chaska, NJ, USA) were used as supplied. Ethyl-3- (dimethylamino) carbodiimide (EDAC) was obtained from Sigma (St. Louis, MO, USA). The diblock copolymer, methoxypolyethylene glycol-block-poly (D, L-lactide) (MePEG-b-PDLLA) (60:40 W/W), was synthesized as previously described (23) . The MePEG molecular weight was 2000 Da and the molecular weight of the diblock (measured by GPC) was 5240 Da (23).

Expression of Recombinant Stratifin

Human recombinant stratifin (SFN) protein was prepared as previously described with slight modification (4). Briefly, the cDNA of stratifin from human keratinocytes was cloned into a pGEX-6P-1 expression vector and transformed into protein expressing bacteria, BL-21 (DE3). A single positive clone was grown in 100 ml of LB medium containing 50 μg/ml of ampicillin for 4–6 h at 37°C until an $OD_{600\,nm}$ of 0.4–0.6 was reached. Bacteria were then diluted to 1:10 with LB medium plus 0.1 mM IPTG for 12 h. To purify the protein, bacteria were centrifuged and lysed by sonication using short cut bursts (30 s intervals). Glutathion-S-transferase (GST)-fused stratifin was purified by adding to glutathione sepharose-4B beads and subsequently digested using PreScission protease according to the manufacturer's procedure. It was previously shown that the recombinant stratifin protein produced in bacteria is more than 95% pure (4).

Conjugation of Stratifin with FITC

For detection and quantification purposes, stratifin was conjugated with Fluorescein isothiocyanate. Fluorescent probe conjugation of stratifin was done by EZ-Label™ FITC Protein Labeling Kit. The excess fluorescent dye was removed using dextran desalting column (D-Salt™). Analytical quantification of FITC-conjugated stratifin was done in a Fluoroscan spectrofluorimeter and the fluorescence was measured at the maximum of the emission band, 520 nm (excitation at 485 nm). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight of FITC-conjugated stratifin.

Preparation of Stratifin-loaded PLGA Microspheres Incorporated in Hyaluronic Acid Films

The schematic procedure of preparation of stratifin-loaded PLGA microspheres incorporated in hyaluronic acid films is presented in Fig. 1. Stratifin was electrostatically complexed within chitosan particles as follows. 5 mg chitosan particles, smaller than 45 μm, were swelled in 30 μl solution of SFN-FITC in phosphate buffer pH 5.5 (SFN-FITC: chitosan, 1 μg:1 mg) with the molar ratio of 7.5:1.0. The suspension was vortexed and incubated at room temperature for 1 h in order to form ionic complexes. In order to remove the free/uncomplexed stratifin from the complex, 500 μl PBS 5.5 was added to the microtubes. The suspension was gently vortexed and centrifuged at 1000 rpm for 3 min. After removing the supernatant, the complexes were dried under nitrogen gas and the particles were broken up into a fine powder using a mortar and pestle. These particles were passed through a sieve with a 40 μm pore size and only particles smaller than 40 μm diameter were encapsulated in PLGA using a suspension-solvent evaporation technique described below. Stratifin bound chitosan particles were dispersed in a 12.5% solution of PLGA in dichloromethane (Chitosan: PLGA, 1:12.5 (w/w)). The mixture was vortexed for 30 s. It was slowly pipeted

into 100 ml aqueous solution of PVA 1% (w/v) and stirred at 400 rpm. After 2 h the microspheres were separated by gravity, washed 3 times with distilled water and dried under nitrogen gas for 1 h. The same procedure was used to make PLGA microspheres loaded with 15% MePEG-b-PDLLA diblock. Crosslinked hyaluronic acid (HA) films were made according to the method previously described with some modification (22). Briefly, 3.2 g of the 1% HA solution (+ 20% glycerol) containing 8 mM EDAC was pipetted into 2.5 cm diameter plastic Petri dishes and dried for 24 h at 60°C. Microspheres were weighed and uniformly distributed between two layers of dry hydrogel films. The films were sealed by placing the compositions in a humid chamber for 5 min. To evaluate the *in vitro* release rate of stratifin from different preparations, FITC conjugated stratifin was used. The loading procedures and release studies were performed under dark conditions to preserve FITC fluorescence.

Microscopic Images and Particle Size of Microspheres

The morphology of empty and protein loaded PLGA microspheres was examined using a scanning electron microscope (FESEM, HITACHI S-4700) after coating the samples with a thin layer of gold under vacuum. Fluorescence microscope images were taken over a period of 30 days for particles loaded with SFN-FITC. Unencapsulated chitosan particles and encapsulated particles either in PLGA or PLGA/ diblock microspheres were photographed at day 1 and 30 using a fluorescence microscope (Motic AE-31) and compared simultaneously.

The particle size analysis was performed using an optical microscope and Motic images software. In all measurements at least 50 particles in four different fields were examined. This procedure was performed on four various preparations of microspheres.

Evaluation of Stratifin Binding Efficiency of Chitosan Particles and Encapsulation Efficiency of PLGA Microspheres

The amount of stratifin complexed to chitosan particles was calculated by two methods. The first method was the calculation of the difference between the total amount of SFN-FITC used and the amount of free unbound protein remaining dissolved in aqueous suspending medium. To measure unbound protein, 5 mg dried chitosan particles loaded with 5 μg SFN-FITC were suspended in 1 ml phosphate buffer, pH 7.4 and the suspension was gently vortexed. The suspension was then centrifuged and the supernatant assayed for protein concentration by spectrofluorometer. The stratifin binding efficiency of chitosan particles was calculated as indicated below:

$$\textit{Entrapment efficiency}: \quad \frac{(\textit{Total amount of SFN} - \textit{FITC loading} - \textit{free SFN} - \textit{FITC in supernatant})}{\textit{Total amountof SFN} - \textit{FITC loading}} \times 100$$

The second method for evaluation of stratifin bound to chitosan particles was an estimation of the protein directly bound to particles. For this method, 5 mg dry chitosan particles loaded with SFN-FITC (SFN-FITC: chitosan, 1 μg:1 mg) were suspended in 1 ml phosphate buffer, pH 11 and incubated for 30 min. Since both stratifin (pI 4.7) and chitosan (pKa 6.7) are negatively charged at pH 11, they will be separated under this pH. The suspension was sonicated for 15 s and then centrifuged. The concentration of SFN-FITC in supernatant was assayed and considered as the total bound amount.

The encapsulation efficiency of stratifin in PLGA microspheres was then calculated by measuring the difference between the total amount of SFN-FITC bound to chitosan particles (used in the microencapsualtion procedure) and the amount of free SFN-FITC remaining in the PVA 1% (w/v) suspending medium at the end of encapsulation procedure. The encapsulation efficiency of PLGA microspheres was calculated as indicated below:

$$\textit{Encapsulation efficiency}: \quad \frac{(\textit{Total amount of SFN} - \textit{FITC bound to chitosan} - \textit{free SFN} - \textit{FITC in PVA 1\%})}{\textit{Total amount of SFN} - \textit{FITC bound to chitosan}} \times 100$$

***In Vitro* Release Study**

The *in vitro* release of stratifin was obtained from chitosan particles alone, chitosan particles encapsulated into PLGA or PLGA / diblock microspheres and hyaluronic acid films containing stratifin, stratifin bound chitosan particles and PLGA microspheres. Under these experimental conditions, stratifin was released within the sink condition determined. SFN-FITC was used to prepare formulations and it was analyzed by spectroflurometry.

Five milligram of unencapsulated chitosan particles or 45 mg of microspheres were weighed into a microcentrifuge tube containing 1 ml of PBS, pH 7.4 and kept in an incubator at 37°C. Samples were taken at 1 h, 1, 3, 5, 10, 15, 20, 30 days and at each sampling time, the release medium was collected after centrifugation and replaced by the same amount of fresh buffer. The samples were analyzed using spectrofluorometry with an exication and emission wavelength of 480 nm and 520 nm respectively.

Hyaluronic acid films containing either 7.5 μg SFN-FITC or 10 mg SFN-FITC bound chitosan particles or 100 mg PLGA microspheres were assessed for *in vitro* release studies. These films were put on top of the Millicell cell culture inserts (PTFE, 0.4 μm) (Millipore, Billerica, MA 01821 USA) and then placed into 6 well cell culture plates containing 1.5 ml PBS, pH 7.4 containing 0.1% BSA. The release media contained 0.1% BSA as a carrier to reduce protein adsorption to the surface of the plates. In fact 0.1% BSA did not interfere with fluorescence measurement of FITC under these conditions. The plates were kept in an incubator at 37°C and samples were taken at 1 h, 1, 3, 5, 10, 15, 20, 30 days. At each sampling time, the release medium was removed from the bottom chamber and replaced by the same amount of fresh

buffer. The samples were analyzed using spectrofluorometry and percent cumulative release was determined for each time point. The experiments were repeated three times using $n=4$ samples.

Fibroblast Cell Culture

Neonatal foreskin pieces were used as the source of fibroblasts and the procedure was done based on the approval of Ethics Committee of the University of British Columbia (UBC). Cultures of human foreskin fibroblasts were established as described previously (24). In brief, punch biopsy samples were prepared from human foreskins. The tissue was collected in Dulbecco's Modified Eagle Medium (DMEM; GIBCO, Grand Island, NY) with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY), minced into small pieces of<0.5 mm in any dimension, washed with sterile medium six times, and distributed into 60×15 mm Petri culture dishes (Corning Inc., Corning, NY), with four pieces per dish. A sterile glass coverslip was attached to the dish with a drop of sterile silicone grease to immobilize the tissue fragment. DMEM containing antibiotics (penicillin G sodium 100 U/mL, streptomycin sulfate 100 mg/mL, and amphotericin B 0.25 mg/mL; 3 mL, GIBCO) with 10% FBS was added to each dish and incubated at 37° C in a water-jacked humidified incubator in an atmosphere of 5% CO2. The medium was replaced twice weekly. After 4 weeks of incubation, cells were released from dishes using 0.1%trypsin (Life technologies Inc., Gaithersburg, MD) and 0.02% EDTA (Sigma, St. Louis, MO) in PBS, pH 7.4 and transferred to 75 Cm^2 culture flasks (Corning Inc., Corning, NY). Cells were then subcultured 1:6 and fibroblasts from passages 4–7 were used for this study.

In Vitro Bioactivity Assay

The bioactivity of stratifin released from the microspheres and hyaluronic acid films containing PLGA microspheres was evaluated by measuring its ability to stimulate expression of MMP-1 in cultured dermal fibroblasts using a procedure described previously (4). The fibroblasts were seeded onto 6-well plates at density of 2.5×10^5 cells per well in DMEM with 10% FBS. After cell attachment, fibroblasts were treated for 24 h as described bellow.

Before preparing the formulations containing stratifin, the biological activity of recombinant stratifin and FITC-conjugated stratifin was determined. In this experiment, fibroblasts were treated with either 2.5 μg/ml recombinant stratifin or an equivalent amount of SFN-FITC. Untreated fibroblasts were considered as negative control.

Stability and biological activity of recombinant stratifin in aqueous media at 37°C were determined and fibroblasts were treated with either fresh stratifin (day 0) or stratifin incubated in DMEM at 37°C for periods of 7, 14, 21 and 28 days. Untreated fibroblasts were considered as negative control.

To evaluate the biological activity of stratifin following encapsulation in PLGA, 150 mg of microspheres (recombinant stratifin was complexed to chitosan particles and then encapsulated into PLGA microspheres under aseptic conditions) were placed in microcentrifuge tube containing 1 ml DMEM and incubated in 37°C for 24 h. The supernatant was collected after centrifugation and added to the cultured fibroblasts in 6 well plates. The fibroblasts treated with 2.5 μg/ml stratifin were considered as positive control for MMP-1 expression and the untreated fibroblasts considered as negative control. The cells treated with the media released from empty microspheres served as a control for MMP-1 stimulatory effect of vehicle.

The long term bioactivity of stratifin released from PLGA microspheres was assessed at 3 and 30 days. 100 mg PLGA microspheres were placed in a microcentrifuge tube containing 1 ml DMEM and kept at 37°C. The tubes were centrifuged and the supernatant was removed after 3 days and kept at −20°C. The microspheres were resuspended in 1 ml fresh DMEM and incubated at 37°C up to 30 days. The fibroblasts were treated with the release medium of 3 and 30 days at the same time.

The stimulatory effect of stratifin released from hyaluronic acid films containing PLGA microspheres after 3 and 30 day was also determined. Hyaluronic acid films containing 100 mg PLGA microspheres were placed on top of Millicell cell culture inserts and then they were placed into 6 well cell culture plates containing 1 ml DMEM containing 0.1% BSA. The plates were incubated at 37°C and samples were taken at 3 and 30 days. The release medium was removed from the bottom chamber and replaced by the same amount of fresh DMEM. Samples were kept at −20°C and used for treating fibroblasts at the same time. Hyaluronic acid films containing empty PLGA microspheres were considered as a control for MMP-1 expression due to the vehicle. The experiments were performed with three different preparations for each set ($n=3$).

After harvesting the cells, the level of MMP-1 expression was evaluated by western blot analysis. Western blots were performed by loading 20 μg denatured protein into each lane and run on 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractionated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA 01821 USA). Immunoblotting was performed using monoclonal mouse anti-human MMP-1 antibody (1:250 dilution). The membranes were then incubated with the appropriate secondary horseradish peroxidase conjugated anti-mouse IgG antibody (1:2,500 dilution). Immunoreactive proteins were then visualized using the

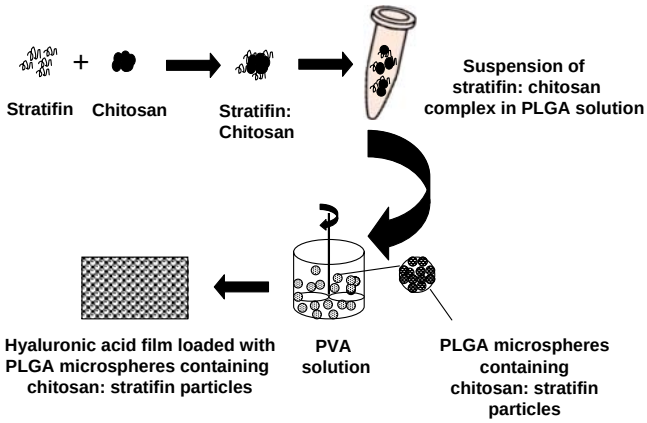

Fig. 1. Schematic drawing of the preparation of stratifin-loaded PLGA microspheres incorporated in hyaluronic acid films.

western blotting detection reagent. The protein bands were detected by exposure to x-ray film. The membrane was then stripped and reprobed with ß-actin antibody and used as a control for protein loading. Quantitative analysis of autoradiograms was accomplished by measuring density of bands using Scion Image, digital image processing software.

In Vitro Cytotoxicity Assay

The cytotoxicity of stratifin released from hyaluronic acid films containing PLGA microspheres was quantitatively assessed by MTT assay which measures the metabolic reduction of 3-(4, 5-dimethylthiazol-2yl)-2, 5, diphenyl tetrazolium bromide to a colored formazan by viable cells. MTT was prepared by dissolving in sterile PBS, pH 7.4 at a concentration of 5 mg/ml and then it was filtered through a 0.22 μm filter. Fibroblasts were cultured in 12 well cell culture plates at a concentration of 1×10^{5} cells per well in DMEM supplemented with 10% FBS and were incubated at 37°C, 5% CO_2 atmosphere for 24 h. After 24 h, the culture medium was removed from the wells and 1 ml of the experimental materials was added into each well. Toxicity was assessed on stratifin released from hyaluronic acid films containing PLGA microspheres in DMEM at 3 and 30 days. Hyaluronic acid

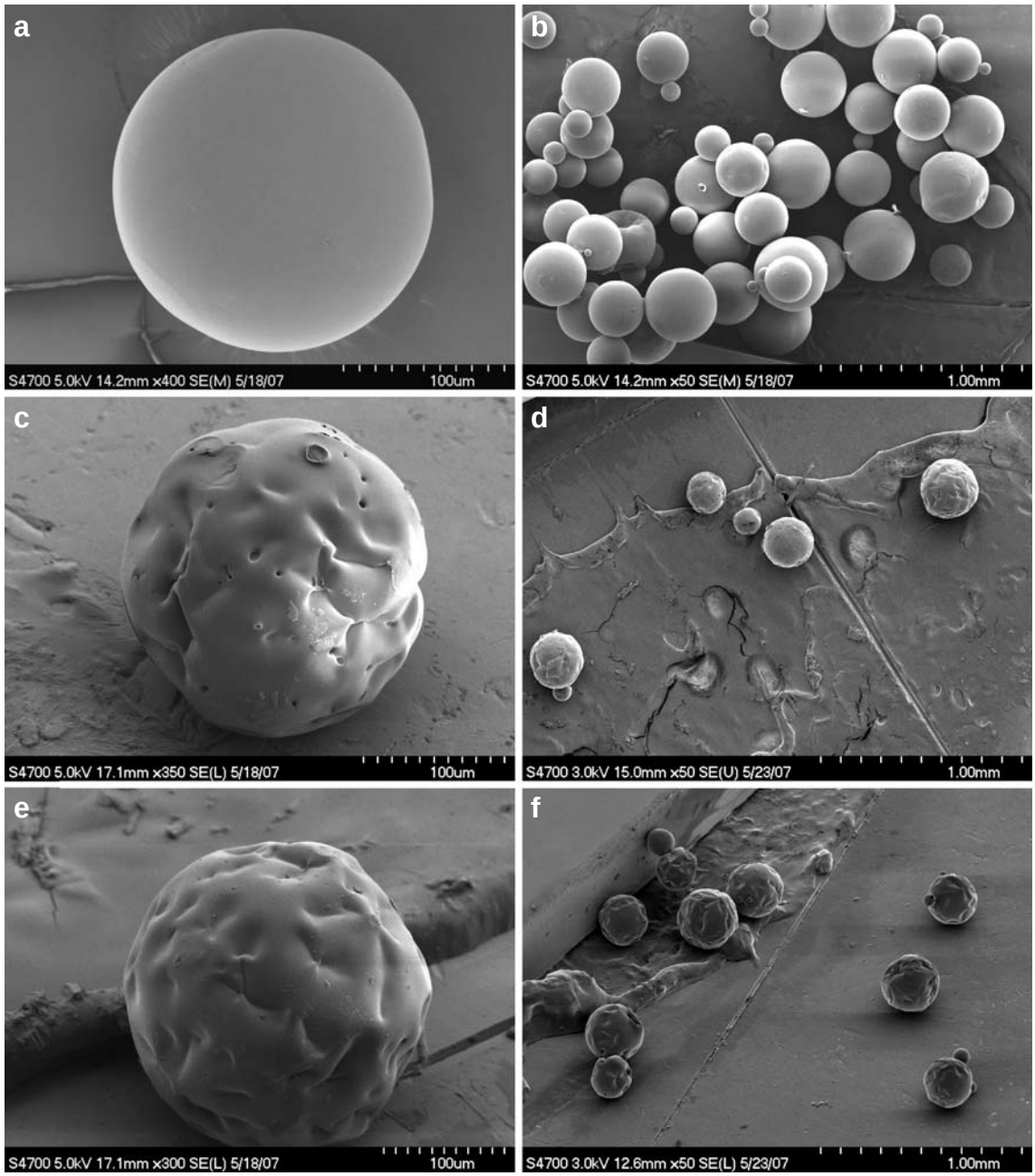

Fig. 2. Scanning electron microscopic images of PLGA microspheres. Empty PLGA microspheres are spherical (**a** and **b**) whereas PLGA microspheres encapsulating chitosan particles (**c** and **d**) or stratifin: chitosan complexed particles (**e** and **f**) are dimpled.

films containing 100 mg PLGA microspheres were placed on top of Millicell cell culture inserts and then they were placed into 6 well cell culture plates containing 1 ml DMEM containing 0.1% BSA. The plates were kept in an incubator at 37°C and samples were taken at 3 and 30 days. The release medium was removed from the bottom chamber and replaced by the same amount of fresh DMEM. Samples were kept at −20°C until used to assess for cytotoxicity. In control (untreated) wells, 1 ml of DMEM was added. Cells incubated with the release media of hyaluronic acid films containing 100 mg empty microspheres served as a control for vehicle and cells incubated with 2.5 μg/ml stratifin served as a control for toxicity of soluble stratifin. Plates were kept for 24 h at 37°C in 5% CO_2 atmosphere. After 24 h, the treatment media was replaced with 500 μl of MTT solution. Plates were wrapped with aluminum foil and incubated at 37° C for 5 h. After removing the reagent solution and rinsing with PBS, 1 ml of dimethyl sulfoxide (DMSO; Sigma, MO, USA) was added to each well and plates were shaken for 20 min to dissolve crystals of formazan. The absorbance of the resulting solution in each well was recorded immediately at 570 nm using an ultra micro plate reader (ELX 808, Bio-Tek Instruments, and Winooski, VT, USA). MTT assays were performed in triplicate.

Statistical Analysis

All data are given as mean ± standard deviation for $n=4$ (release studies) and $n=3$ (biological activity and cytotoxicity studies) and compared with a student's t test or Kruskal-Wallis Test (Nonparametric ANOVA).

RESULTS

FITC-conjugated Stratifin

Stratifin was conjugated with FITC, a fluorescent label for quantification purposes. The excess fluorescent dye was removed using dextran desalting columns. The SDS-PAGE results showed that FITC-conjugated stratifin (SFN-FITC) had an apparent molecular weight of 30 KDa similar to that of unconjugated stratifin (data not shown).

Morphology and Particle Size of Microspheres

Scanning electron microscopy of microspheres shows that empty PLGA microspheres (Fig. 2a, b) are smooth and spherical whereas PLGA microspheres encapsulating either chitosan alone or stratifin-complexed chitosan particles are dimpled (Fig. 2c–f).

The average size of PLGA microspheres encapsulating chitosan particles was 317±20 μm in diameter measured by optical microscopy.

Fluorescence microscopic images of SFN-FITC complexed chitosan particles and PLGA and PLGA/diblock microspheres showed that chitosan particles were encapsulated in both PLGA and PLGA/diblock microspheres (Fig. 3a–c, upper line). After a 30 day incubation in PBS pH, 7.4 the presence of SFN-FITC is still visible in PLGA microspheres (Fig. 3c, bottom) whereas it is barely visible in PLGA/diblock microspheres (Fig. 3b, bottom) and absent in unencapsulated chitosan particles (Fig. 3a, bottom).

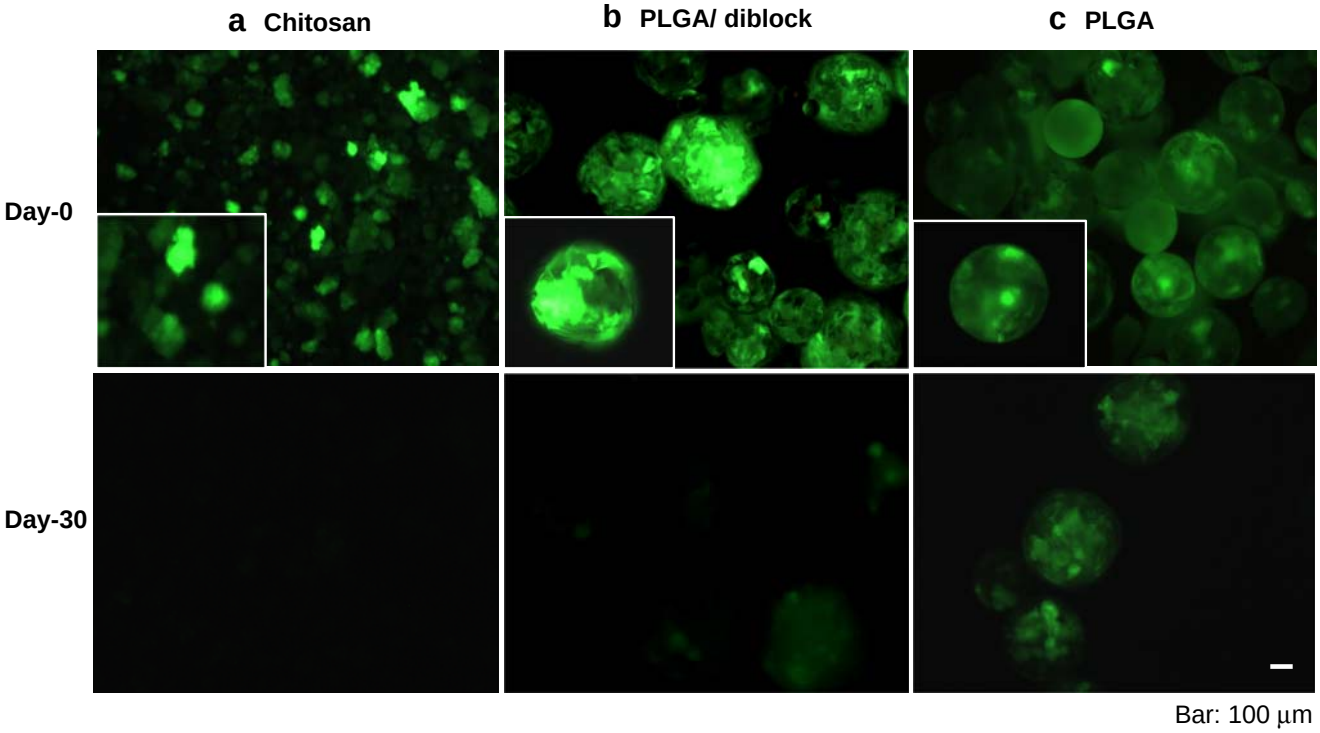

Fig. 3. Fluorescence microscopic images of SFN-FITC complexed chitosan particles alone (**a**) or loaded in PLGA/diblock (**b**) and PLGA microspheres (**c**). At day 0, images are shown as upper line. After 30 days incubation in PBS, SFN-FITC is noticeably visible in PLGA microspheres (*right bottom*) whereas it is barely visible for PLGA/diblock microspheres (*middle bottom*) and absent for unencapsulated chitosan particles (*left bottom*). *Scale bar*: 100 μm.

Stratifin Binding Efficiency of Chitosan Particles and Encapsulation Efficiency of PLGA Microspheres

Chitosan particles (1 mg) loaded with different amounts of SFN-FITC (0.5–3 μg) was tested for binding efficiency and release kinetics (data not shown). The average binding efficiency of stratifin with chitosan particles was calculated by measuring the free unbound SFN-FITC remaining dissolved at aqueous medium, pH 5.5. Under this condition, the maximum binding efficiency was seen for the complex with the ratio of stratifin:chitosan, 1 μg:1 mg which was 74±3%. This value was in good agreement with the result obtained by releasing the total bound SFN-FITC at pH 11, which was 72±3%. Evaluating the release of SFN-FITC from chitosan, the complex with the ratio of stratifin:chitosan, 1 μg:1 mg showed a suitable release profile, (low burst release and steady protein release afterwards). At this ratio and at pH 5.5, stratifin and chitosan particles interacted with a charge ratio of approximately 1:10.

When chitosan particles were encapsulated in PLGA microspheres, the encapsulation efficiency was 78±2.5%, determined by measuring the free SFN-FITC left in suspending medium following encapsulation.

In Vitro Release of Stratifin

The *in vitro* release of stratifin from different formulations was obtained over a period of 30 days using SFN-FITC. When SFN-FITC was directly encapsulated in PLGA microspheres, the encapsulation efficiency was lower than 50% (in agreement with previous publications demonstrating low encapsulation efficiency for proteins in polymeric microspheres) (8,9).

In panel a of Fig. 4 the SFN-FITC release from chitosan particles is compared with the release from PLGA and PLGA/ diblock microspheres. There was a high initial burst release of stratifin from chitosan particles in the first day followed by a steady phase up to 30 days (Fig. 4, panel a). About 57±4% of total SFN-FITC loaded was released in 1 h and that increased to 73±6% by 24 h. By encapsulating chitosan particles into PLGA microspheres, this initial burst release was reduced to 9±1% in 1 h and 18±3% in 24 h. PLGA microspheres prolonged the release of SFN-FITC and as a result, only 54±6% of total loaded protein was released by 30 days. Adding diblock copolymer to PLGA resulted in almost complete release of SFN-FITC (86±6%) in 30 days.

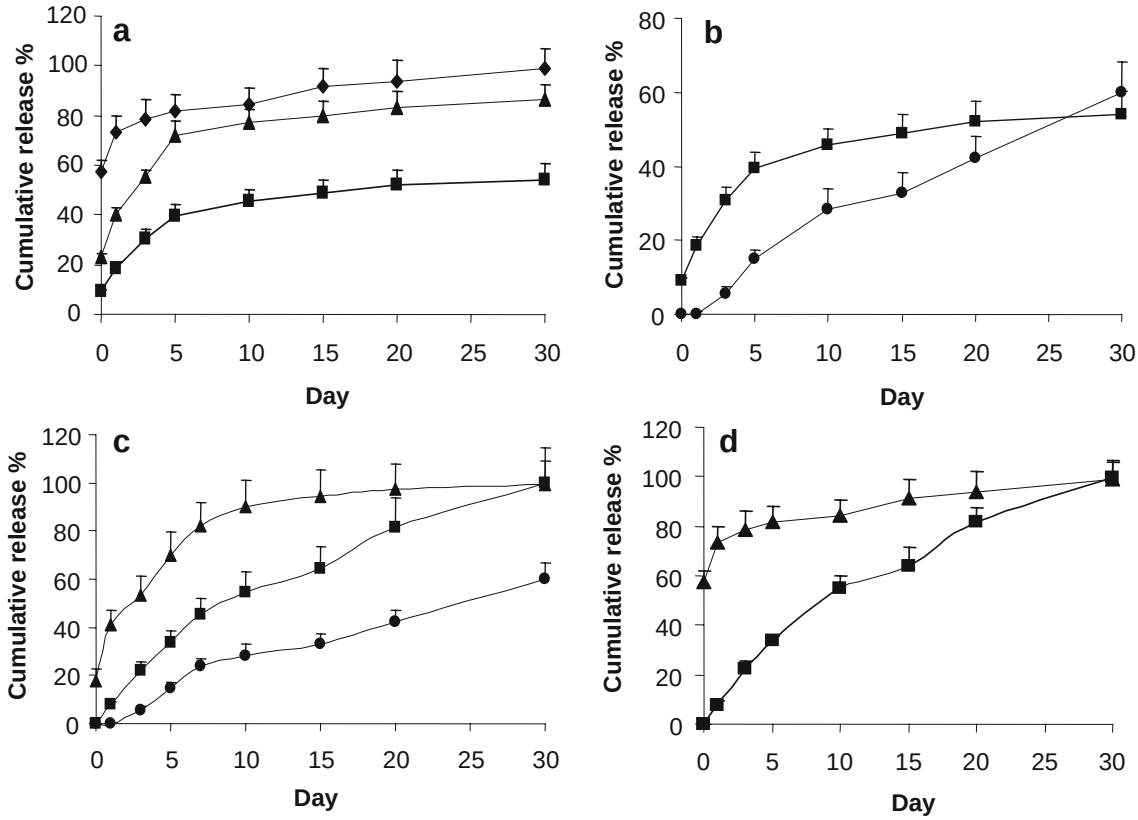

Fig. 4. *In vitro* release profiles of SFN-FITC from either SFN-FITC: chitosan particles, PLGA or PLGA/diblock microspheres containing SFN-FITC: chitosan particles or different formulations of hyaluronic acid films loaded with particles and microspheres. *Panel **a***: SFN-FITC release from either chitosan particles (—◆—), PLGA microspheres containing chitosan particles (—■—) or PLGA / diblock microspheres containing chitosan particles (—▲—). *Panel **b***: SFN-FITC release from either PLGA microspheres containing chitosan particles loaded in hyaluronic acid films (—●—) or PLGA microspheres (no film) containing chitosan particles (—■—). *Panel **c***: SFN-FITC release kinetic from either hyaluronic acid films loaded with soluble SFN-FITC (—▲—), SFN-FITC: chitosan particles (—■—) or PLGA microspheres containing chitosan particles (—◆—). *Panel **d***: SFN-FITC release from either SFN-FITC: chitosan particles (—▲—) or SFN-FITC: chitosan particles blended into hyaluronic acid films. (—■—). *Error bars* represent the mean ± SD for n=4.

To further reduce the initial burst release of stratifin from PLGA microspheres, they were embedded in hyaluronic acid films. During the first week, water absorption and swelling were seen in all preparations. The burst release was almost eliminated and a lag time in the release of stratifin was observed for 24 h (Fig. 4, panel b). Following that, the release gradually increased to 5.38±2% in 3 days. After 3 days, a constant release phase was observed with about 60% of total protein released in 30 days.

Panel c of Fig. 4 describes the release profiles SFN-FITC from hyaluronic acid films loaded with soluble SFN-FITC, SFN-FITC complexed chitosan particles and PLGA microspheres encapsulating SFN-FITC complexed chitosan particles. Films containing soluble SFN-FITC exhibited a rapid release profile with a high burst release of 41±6% by 24 h. It was followed by almost linear release for 10 days, which resulted in the release of 89±11% of SFN-FITC within 10 days. The release rate of soluble SFN-FITC from hyaluronic acid films was reduced when the protein was complexed to chitosan particles and reduced even further when chitosan particles were encapsulated in PLGA microspheres (Fig. 4, panel c). Fig. 4d shows that Hyaluronic acid films significantly reduced the initial burst release of SFN-FITC from chitosan particles and as a result, a lag time of 1 h in the release of protein was observed followed by only 8±2% release by 24 h.

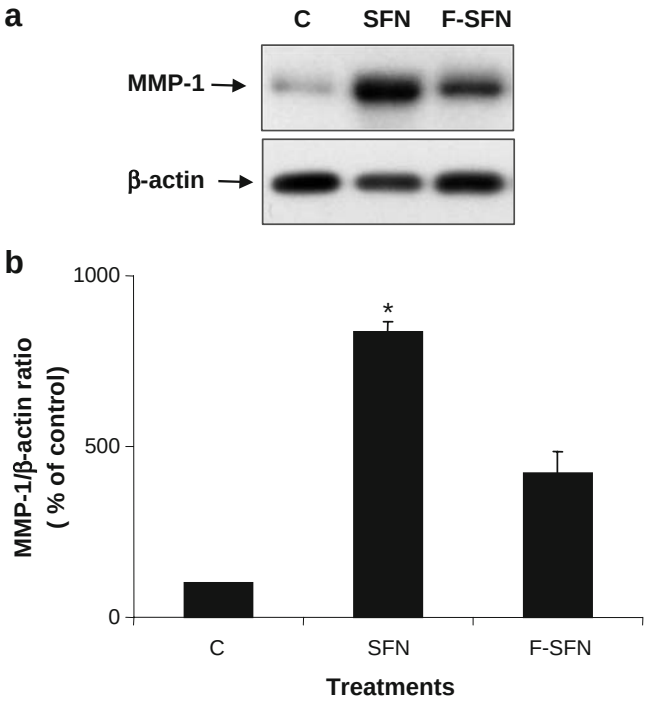

Fig. 5. Bioactivity of recombinant and FITC conjugated stratifin. *Panel **a***: Western blot analysis of MMP-1 expression in fibroblasts treated with either recombinant stratifin (SFN) or FITC conjugated stratifin (F-SFN). Untreated fibroblasts were used as negative control (C) and protein loadings were compared using ß-actin bands. *Panel **b***: Semi-quantitative level of MMP-1 expression for each treatment group was analyzed by densitometry of the corresponding bands and represented as the ratio of MMP-1 expression to ß-actin. *Error bars* represent the mean ± SD for $n=3$.

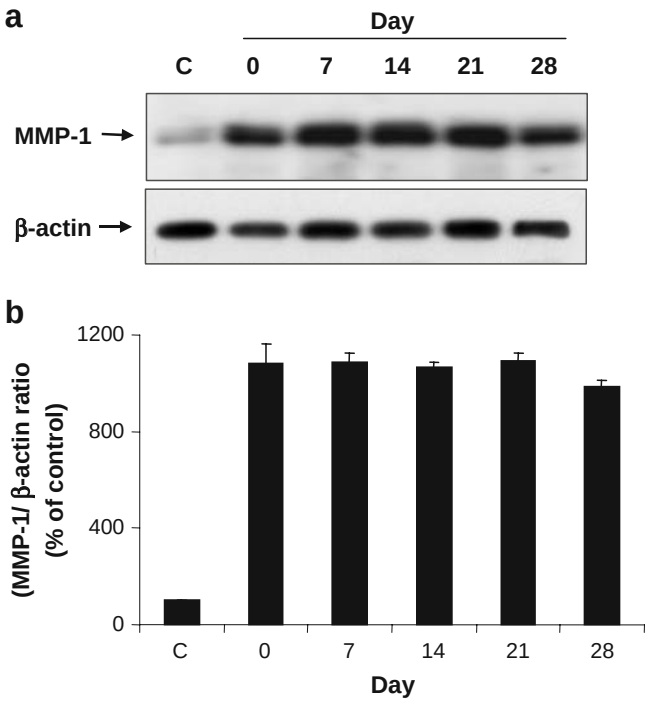

Fig. 6. Stability and biological activity of stratifin in aqueous media at 37°C. *Panel **a***: Western blot analysis of MMP-1 expression in fibroblasts treated with either fresh stratifin (day 0) or stratifin incubated at 37°C for 7, 14, 21 and 28 days. Protein loadings were compared using ß-actin bands. *Panel **b***: Semi-quantitative level of MMP-1 expression for each treatment group was analyzed by densitometry of the corresponding bands and represented as the ratio of MMP-1 expression to ß-actin. *Error bars* represent the mean ± SD for $n=3$.

In Vitro Bioactivity of Stratifin

To demonstrate the *in vitro* bioactivity of stratifin, the level of MMP-1 expression was evaluated in dermal fibroblasts using western blot analysis. As expected, the level of MMP-1 expression in fibroblasts treated with recombinant stratifin was elevated. However, this effect was less pronounced for FITC conjugated stratifin (Fig. 5, panel a, MMP-1). To determine the total protein loadings, the same blot was probed with ß-actin antibody and similar loadings were found (Fig. 5, panel a, ß-actin). When the corresponding autoradiograms were quantified by densitometry, the level of MMP-1 expression in cells treated with recombinant stratifin was found to be significantly higher than control (837±27%, $p<0.05$, $n=3$) (Fig. 5, panel b). In subsequent experiments addressing the biological activity of stratifin, unconjugated stratifin was used.

To address the stability and biological activity of stratifin in aqueous media, the protein was incubated in DMEM at 37°C up to 28 days. Fig. 6 shows that the stimulatory effect of stratifin on the expression of MMP-1 was not compromised up to 28 days of incubation in aqueous media at 37°C (Fig. 6, panel a, MMP-1). ß-actin bands show the similar protein loadings (Fig. 6, panel a, ß-actin). The results of densitometry show that the relative levels of MMP-1 expression to ß-actin for the treatments of 7, 14, 21 and 28 days were equivalent to the fresh stratifin ($p>0.05$, $n=3$) (Fig. 6, panel b). This data confirm that stratifin was stable and biologically active at 37°C for at least 28 days in aqueous media.

The bioactivity of stratifin following encapsulation was evaluated by measuring the MMP-1 expression stimulatory effect of stratifin released from PLGA microspheres in 24 h and it was compared to the bioactivity of the equivalent amount of naked stratifin. The naked stratifin encapsulated in PLGA microspheres did not show any MMP-1 stimulatory effect (data not shown). Therefore, in this study to increase the stability of stratifin during microencapsulation procedure, it was complexed to chitosan particles and then encapsulated in PLGA microspheres. It was observed that the stratifin released from PLGA microspheres (first, stratifin was complexed to chitosan particles and then encapsulated into PLGA microspheres) significantly increased the level of MMP-1 expression in fibroblasts (Fig. 7, panel a, MMP-1). This stimulatory effect was comparable to the effect of an equivalent amount of naked stratifin. The empty PLGA microspheres (containing unloaded chitosan particles) were tested to evaluate the stimulatory effect of polymeric vehicles on expression of MMP-1. The media released from empty microspheres did not show any stimulatory effect. Related ß-actin bands ensure the similar protein loadings (Fig. 7, panel a, ß-actin). The densitometry results indicated the equivalent ratio of MMP-1/ ß-actin for fibroblasts treated with stratifin released from PLGA microspheres and fibroblasts treated with the naked stratifin (1398±88% *vs.* 1240±46%, $p>0.05$, $n=3$) (Panel b, Fig. 7). This data indicates that neither complexing stratifin with chitosan particles, nor the process of microencapsulating in PLGA reduce the biological activity and stimulatory effect of stratifin on expression of MMP-1 in cultured fibroblasts.

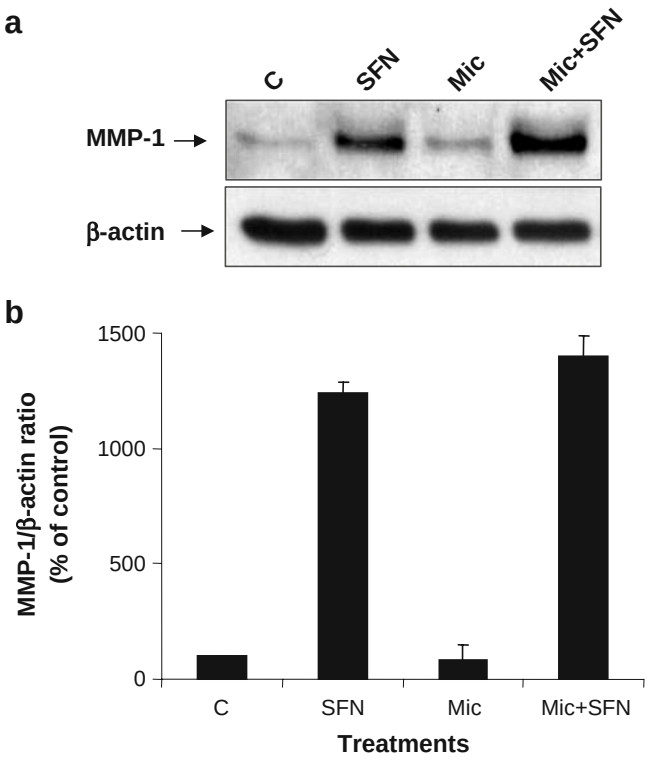

Fig. 7. Bioactivity of stratifin following encapsulation in PLGA microspheres containing chitosan particles. *Panel* ***a***: MMP-1 expression in fibroblasts treated with stratifin released from PLGA microspheres containing stratifin complexed chitosan particles for 24 h (SFN+Mic), equivalent amount of naked stratifin (SFN) and PLGA microspheres containing unbound chitosan particles (Mic). Protein loadings were compared using ß-actin bands. *Panel* ***b***: Semi-quantitative level of MMP-1 expression for each treatment group was analyzed by densitometry of the corresponding bands and represented as the ratio of MMP-1 expression to ß-actin. *Error bars* represent the mean ± SD for $n=3$.

The biological activity of stratifin released from PLGA microspheres and hyaluronic acid films containing PLGA microspheres was evaluated at 3 and 30 days (Fig. 8). As expected the level of MMP-1 expression in fibroblasts treated with 3 day stratifin released from hyaluronic acid films containing PLGA microspheres was very low whereas it was highly pronounced in fibroblasts treated with 3 day stratifin released from PLGA microspheres alone (Fig. 8, panel a, MMP-1). ß-actin bands ensure the similar protein loadings (Fig. 8, panel a, ß-actin). By measuring the density of the related bands and calculating the relative level of MMP-1 expression to ß-actin, this difference was found significant (74± 11% *vs.* 1265±44%, $p<0.05$, $n=3$) (Fig. 8, panel b). This data agrees with the result of the *in vitro* release of SFN-FITC, which revealed a reduced burst release of stratifin upon incorporating the PLGA microspheres in hyaluronic acid films.

The stratifin released from 3 to 30 days from both preparations showed a high stimulatory effect on expression of MMP-1 (Fig. 8, panel a, MMP-1). The relative level of MMP-1 expression to ß-actin for these treatments was greater for stratifin released from hyaluronic acid films containing PLGA microspheres than stratifin released from PLGA microspheres (1063±74% *vs.* 667±60%, $p<0.05$, $n=3$) (Fig. 8, panel b). The incubation media obtained from both mentioned vehicles from 3 to 30 days did not show any stimulatory effect on expression of MMP-1 in fibroblasts. In contrast, controlled release of stratifin from PLGA microspheres was biologically active and had the ability to elevate expression of MMP-1 in cultured fibroblasts for at least 30 days. By incorporating these microspheres in hyaluronic acid films, the burst release of stratifin was reduced and a lag phase occurred.

In Vitro Cytotoxicity

The cytotoxicity of release media from hyaluronic acid films containing stratifin loaded or empty PLGA microspheres was tested on human dermal fibroblasts using MTT assay. The number of viable cells was assessed after 24 h incubation with the release media. Statistical analysis showed no significant reduction in viable cells following incubation with samples from either day 3 or day 30 (Fig. 9) ($p>0.05$, $n=3$).

DISCUSSION

Collagen deposition is an essential component of normal wound healing, but if unregulated or excessively prolonged it may contribute to the development of fibroproliferative disorders (1). Such problems might be preventable by prolonged expression and release of matrix metalloproteinases to degrade the excessive mass of collagen at the late stage of healing process. We have previously found that keratinocyte releasable stratifin markedly stimulates the expression of matrix metalloproteinase-1 (MMP-1) in dermal fibroblasts suggesting that this protein might be useful in the

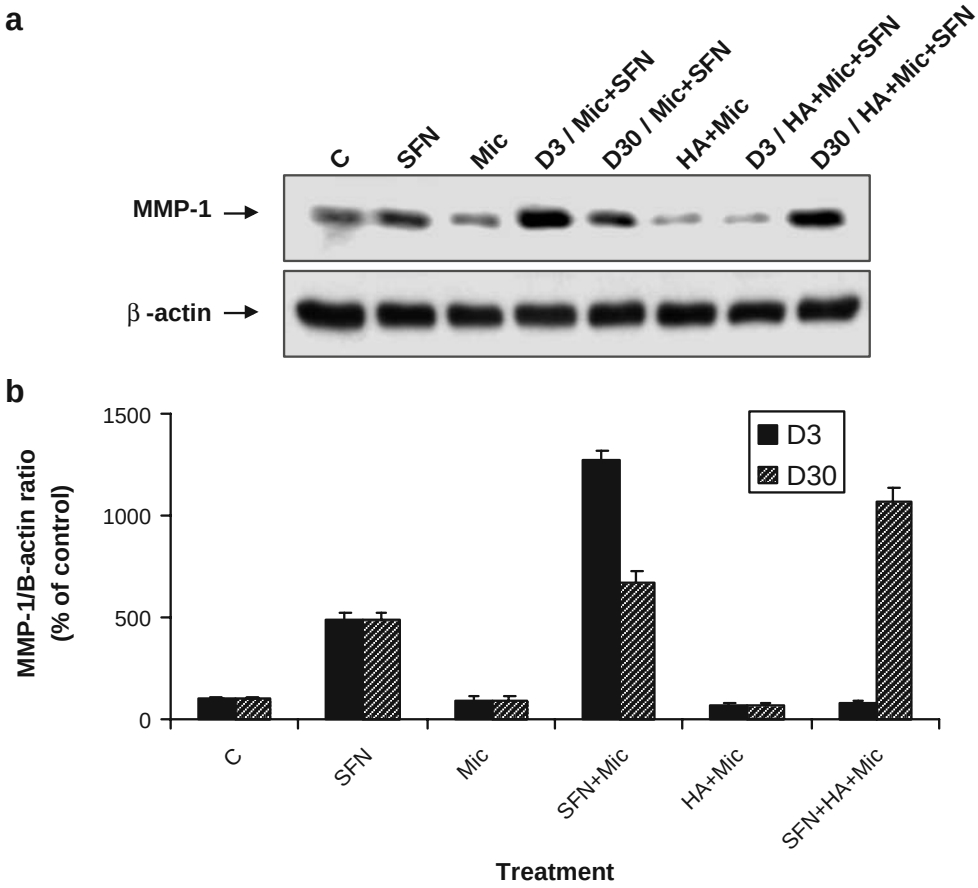

Fig. 8. Long term bioactivity and burst phase of stratifin released from chitosan particles encapsulated in PLGA microspheres alone or loaded in hyaluronic acid films. *Panel **a***: MMP-1expression in fibroblasts treated with stratifin released at 3 and 30 days from PLGA microspheres containing stratifin complexed chitosan particles (D3/Mic+SFN and D30/Mic+SFN) and hyaluronic acid films loaded with PLGA microspheres containing stratifin complexed chitosan particles (D3/HA+Mic+SFN, D30/HA+Mic+SFN). MMP-1 expression in fibroblasts treated with the media released from PLGA microspheres alone or in films, containing control (no protein) chitosan particles at 30 days served as control for polymeric vehicles. Protein loadings were compared using ß-actin bands. *Panel **b***: Semi-quantitative level of MMP-1 expression analyzed by densitometry of the corresponding bands. The relative level of MMP-1 expression to ß-actin quantified by densitometry of the corresponding bands. *Error bars* represent the mean ± SD for $n=3$.

treatment of collagen associated healing disorders. However efficacy might depend on the presence of therapeutic concentrations of stratifin at later stages of wound healing and the absence of this protein during the early healing process. The objective of this study was to formulate a controlled release formulation for stratifin, with minimum burst release (and ideally an initial lag phase) followed by a sustained release to improve and /or prevent formation of keloid and hypertrophic scarring frequently seen in post surgical procedures. This study successfully complexed stratifin to chitosan particles which were encapsulated in PLGA microspheres and finally blended in hyaluronic acid films. The release of bioactive stratifin was negligible for up to 3 days and this was followed by controlled release.

The use of polymeric microspheres for sustaining the release of bioactive proteins has been previously described (8,9), but typically still produced a burst release of protein. For example, gelatin nanoparticles containing bFGF loaded in PLGA microspheres produced 30% release of bFGF in the first 24 h (8). Using a composite of alginate-chitosan in PLGA microspheres, Zheng *et al*. reported a reduction in the burst phase of BSA release from 53% to 39% (9). To further reduce early protein release, some groups have blended protein loaded microspheres in hydrogel films (11–14). Defail *et al*. reported the controlled release of TGF-ß1 from PLGA microspheres incorporated into PEG-genipin hydrogels (12). Within 1 day, the microspheres demonstrated a high burst release of protein (69±29%) while the PEG-genipin scaffold delayed the burst release up to 17 days (72±11%).

In this study, to avoid protein denaturation during the process of encapsulation and to reduce the burst release effect, stratifin was complexed with chitosan particles. These complexes were then encapsulated into PLGA and the microspheres were blended into crosslinked hyaluronic acid films. SEM images showed that PLGA and PLGA/diblock microspheres were dimpled as a result of encapsulating chitosan particles (Fig. 2) and fluorescent microscopy showed significant amounts of chitosan and protein embedded in the micrsopheres (Fig. 3).

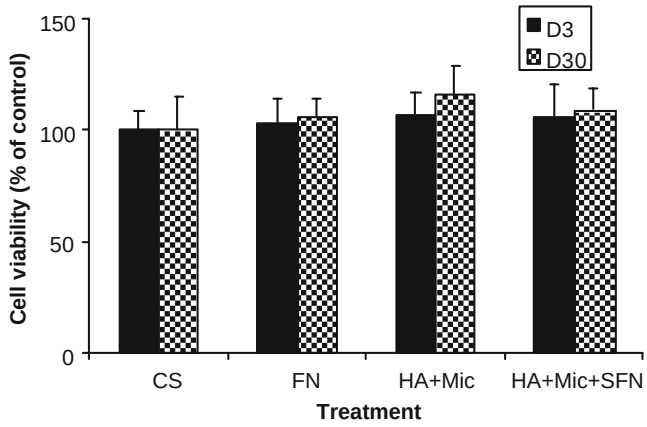

Fig. 9. Effect of stratifin and polymeric vehicles on cell viability. The fibroblasts were either untreated or treated with 2.5 μg stratifin (SFN), release media from hyaluronic acid films containing empty PLGA microspheres (HA+Mic) at 3 and 30 days or hyaluronic acid films containing stratifin complexed chitosan particles (SFN+HA+Mic) at 3 and 30 days. The result is represented as a percentage of the untreated group. *Error bars* represent the mean ± SD for n=3.

The encapsulation of chitosan particles in PLGA microspheres significantly reduced the burst release of stratifin from 73% to 18% in 24 h. It was shown that PLGA microspheres sustained the release of stratifin from chitosan particles so that 54% of the total loaded protein was released in 30 days. In some experiments a diblock copolymer was incorporated into the PLGA microspheres at a ratio of 15% diblock to 85% PLGA. Previous studies have shown that this strategy allowed for increased drug release from PLGA microspheres because the diblock copolymer slowly partitions out of the PLGA matrix allowing for increased microsphere porosity (25). In this study microspheres made of 15% diblock polymer and 85% PLGA released stratifin almost completely in 30 days (Fig. 4). Although no significant burst phase of protein release was observed using this method, there was a steady release over the first 10 days without the preferred initial delay of release observed with PLGA-only microspheres.

Fluorescence microscopy of SFN-FITC demonstrated the presence of the protein in chitosan, PLGA and PLGA/diblcok microspheres (Fig. 3, Day 0). After 30 days there was little SFN-FITC in the chitosan or PLGA/diblock microspheres but evidence of residual protein in the PLGA-only microspheres (Fig. 3, Day 30) in agreement with protein release profiles (Fig. 4). These findings demonstrated the success of the chitosan/PLGA encapsulation process in allowing the sustained release of the protein.

By blending PLGA microspheres into hyaluronic acid films the initial burst release of stratifin was significantly reduced so that only 5% of the protein was released in 3 days. As a result, most of the stratifin was released at later time points thereby achieving the preferred release profile of an initial lag phase followed by controlled release. It was likely that the movement of water through the swollen, cross linked hyaluronic acid film was restricted so that the efflux of protein from the microsphere surface and subsequent diffusion processes were reduced.

Both stratifin and the FITC conjugated stratifin were shown to be effective in stimulating MMP-1 expression in fibroblasts (Fig. 5) demonstrating the bioactivity of both forms of protein. Furthermore, following incubation in aqueous media, there was little change in the bioactivity of the stratifin after 28 days demonstrating the resistance of this protein to hydrolytic degradation processes (Fig. 6). To study the bioactivity of stratifin released from polymeric formulations, the aqueous release media from *in vitro* protein release studies was used as incubation media with fibroblasts, cells treated with the release media of the PLGA microspheres containing stratifin bound chitosan showed equivalent increases in MMP-1 expression to that induced by protein alone (Fig. 7). This study showed that the encapsulation and release processes did not degrade the protein and full bioactivity was maintained. More detailed bioactivity studies showed that the protein released from both types of formulations (i.e. chitosan in PLGA microspheres either free or blended in hyaluronic acid films) at day 3 or day 30 of the release study was non degraded and effective at inducing MMP-1 expression in fibroblasts (Fig. 8). Interestingly, for the hyaluronic acid formulation, the *in vitro* protein release study showed a marked delay in protein release at 3 days and this was reflected in the detailed bioactivity study whereby the aqueous media from this study showed a reduced ability to increase MMP-1 expression. This scenario of a reduced expression of MMP-1 at day 3 followed by sustained increases in expression after that time satisfies a preferred therapeutic release profile for use in wound healing settings.

None of the release media from stratifin bearing formulations had any cytotoxic effect on fibroblasts *in vitro* (Fig. 9). The release media from formulations incubated in media for 3 or 30 days was incubated with fibroblasts with no observed change in cell proliferation rates. This study suggests that any observed increases in MMP-1 expression caused by stratifin were not the result of cell stress induced by polymeric or protein materials.

Overall, these studies support the use of a hyaluronic based microparticulate wound dressing for the prevention of abnormal wound healing processes. The compatibility of hydrocolloid dressings is well established and materials such as carboxymethylcellulose (CMC), gelatin and hyaluronic acid are commonly used as wound dressings. Commercially available hydrocolloid dressings include Granuflex™ and Aquacel™(Conva Tec, Hounslow, UK), Comfeel™ (Coloplast, Peterborough, UK) and Tegasorb™ (3 M Healthcare, Loughborough, UK). They are manufactured in the form of thin films and sheets and they have been used to topically administer some of the growth factors to wound sites. These include hydrogel dressings for delivering transforming growth factor-β1 (TGF-β1) (26), or collagen film for delivering PDGF (27) and human growth hormone, alginate dressings (28) in the form of beads used to deliver endothelial growth factor (29), polyurethane and collagen film dressings for delivery of EGF (30). Although all these previous studies support the application of thin sheets of hydrocolloid dressings to wound sites, none of these formulations have been developed for the delivery of an antifibrogenic agent which might require delayed drug release properties. Drug release from hydrogel wound dressings is mostly controlled by physical processes. For example, hydration resulting from wound exudate, permits diffusion of drug through the swollen gel and eventual erosion of polymer (31). Therefore, factors such as rapid erosion of the polymer matrix following water

diffusion and swelling are known to be the main reasons for immediate release of factors from these wound dressings. However, the microspheres in hyaluronic acid film formulation described in this study is the first report of a delayed and controlled release system prepared from biocompatible materials suitable for application to a wound site.

These *in vitro* studies established that we were successful in producing a lag phase followed by a controlled release phase of bioactive stratifin. We are currently investigating the antifibrogenic efficacy of this delivery system through *in vivo* studies using animal model.

CONCLUSIONS

Hyaluronic acid embedded PLGA microspheres were developed to delay and then control the release of an antifibrogenic factor, stratifin. This delivery system might be inserted into an incision at the time of wound closure to release stratifin after granulation tissue is formed within 3 to 5 days post operation. In these studies, stratifin bioactivity was preserved during the encapsulation process and the release of the protein was successfully delayed for 3 days and then slowly released over 30 days. The stratifin released during these *in vitro* studies increased the level of MMP-1 expression in cultured fibroblasts at later time points without causing any cytotoxicity.

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