Wound Dressings Containing bFGF-Impregnated Microspheres: Preparation, Characterization, *In Vitro* and *In Vivo* Studies

Sha Huang,[1] Yan Jin,[1] TianzhengDeng,[1] Hong Wu[2]

[1]*Department of Oral Histology and Pathology, College of Stomatology, Fourth Military Medical University, Xi'an, Shaanxi, 710032, People's Republic of China*
[2]*Department of Chemistry, Faculty of Preclinical Medicine, Fourth Military Medical University, Xi'an, Shaanxi, 710032, People's Republic of China*

Received 31 May 2005; accepted 28 September 2005
DOI 10.1002/app.23319
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: The purpose of this study was to synthesize a novel wound dressing containing bFGF-loaded microspheres for promoting healing and tissue regeneration. Gelatin was chosen as the underlying layer and was prepared in porous sponge. As the external layer, elastomeric polyurethane membranes were used. bFGF was loaded in microspheres to achieve prolonged release for higher efficiency. The microspheres were characterized for particle size, *in vitro* protein release, and bioactivity. The dressings were tested in *in vivo* experiments on skin defects created on pigs. At certain intervals, wound areas were measured and tissues from wound areas were biopsied for histological examinations. Average size of the microspheres was 14.36 ± 3.56 μm and the network sponges were characterized with an average pore size of 80–160 μm. Both the release efficiency and the protein bioactivity revealed that bFGF was released in a controlled manner and was biologically active, as assessed by its ability to induce the proliferation of fibroblasts. The rate of wound-area decrease was much faster and the quality of the newly-formed dermis was almost as good as the normal skin. The application of this novel bilayer wound dressing provided an optimum healing milieu for the proliferating cells and regenerating tissues. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 4772–4781, 2006

Key words: basic fibroblast growth factor; wound dressing; sustained-release; microsphere; tissue regeneration

INTRODUCTION

The primary objective in wound care is the promotion of rapid wound healing with the best functional and cosmetic results.[1,2] In case of bulk loss of tissue or nonhealing wounds, such as burns, trauma, and diabetic, decubitus, and venous stasis ulcers, a proper wound dressing is needed to cover the wound area. The dressing achieves the functions of the natural skin by protecting the area from the loss of fluid and proteins, preventing infection through bacterial invasion, and subsequent tissue damage. In some cases, it improves healing by providing a support for the proliferating cells.[3] Wound healing may be considered as a specific biological process related to the general phenomenon of growth and regeneration. It is a dynamic process in which a variety of cellular and matrix components act in concert to reestablish the integrity of injured tissue.[4,5] Therefore, an ideal wound dressing may play an important role in this process. In the last 20 years, there has been an intense scientific activity in this area. For an ideal wound dressing, materials should have flexibility, durability, adherence, a capability of absorbing wound exudates, and protecting the lesion from dehydration. From an engineering viewpoint, it should also be easy to handle and to apply, comfortable when in place, and cost-effective. More than anything else, wound dressing materials should be nontoxic, nonantigenic, and stable (insoluble, nonresorbable) during the few days of contact with the wound.[6]

Currently, a large number of research groups are working on the synthesis and modification of new biocompatible materials to obtain a material that would be applicable as wound dressing, activate tissue regeneration, inhibit wound contraction, and protect the wound site against unwanted external effects. In recent years, researchers have focused on drug-loaded wound dressings.[7–10] The use of microspheres for the sustained release in the drug delivery system has been of increasing interest. The advantages of this system include biocompatibility, controlled biodegradability, absorbability and nontoxicity of degradation products, potential for controlled release, and ease of administration. The principal function of the novel wound dressing developed to contain drugs is

Correspondence to: Y. Jin (yanjin@fmmu.edu.cn).
Contract grant sponsor: Hi-tech Research and Development Program of China.

to provide an optimum healing milieu for natural healing and tissue regeneration. Growth factors play an important role in cell growth and in the process of wound healing.[11–16] Among growth factors, basic fibroblast growth factor (bFGF) plays a key role in development and remodeling, and it has the ability to regulate proliferation of endothelial cells and development of angiogenesis.[11,12] The potent angiogenic activity of bFGF has suggested that it can be used to stimulate wound healing regeneration and augment collateral artery development to relieve myocardial and peripheral ischemia.[14] bFGF also contributes to normal wound healing by improving wound strength and the quality of the scar.[15] Therefore, bFGF was selected in anticipation of promoted tissue regeneration in the gelatin sponge. However, since the *in vivo* half-life of bFGF is very short, bFGF injected in soluble form rapidly diffuses away from the site of injection and is also denatured and degraded enzymatically.[11] It is known that many proteases are activated in the injured tissue, and they easily decompose bFGF in the wounded or burned site of the skin. Various systems for the topical delivery of bFGF to the injured tissues have been examined and described. These include direct injection or application of the soaked gauze to the wound, covering the wound with a gel, cream or ointment containing bFGF, spraying bFGF over the wounded site[17–22].But the more possible way to enhance the *in vivo* efficacy of bFGF is to achieve sustained-release with incorporation or encapsulation of bFGF into a polymer matrix over an extended time period. Therefore, the sustained-release system of biologically active bFGF by the use of gelatin microspheres were designed.[23] In this system, bFGF is released from the microspheres as a result of matrix degradation. Biodegradable gelatin sponge has also been used to deliver bFGF,[13] and acidic gelatin has been shown to form a polyanionic complexation with bFGF.[15]

The aim of this study is to design a novel wound dressing containing bFGF delivery system (microspheres to provide sustained release): a well-formed porous and biodegradable inner layer matrix that would serve as the host for the proliferating cells and would degrade spontaneously without creating any adverse effects, while the materials of the outer layer would enhance the intensity and permeability of the wound dressing; and the tissue regenerates were planned to act as the underlying dermal layer. bFGF was added into gelatin microspheres that would act as sustained release vehicles. Thus, bFGF was expected to activate cell proliferation while the porous matrix would form the medium for these cells to adhere. In this study, gelatin was chosen as the porous soft layer material, since it plays a potential role in carrying the growth factor and has been used in wound dressings.[6,24–29] Gelatin is known to exhibit activation of macrophage and a high hemostatic effect.[25] As a denatured form of collagen, gelatin is known to have practically no antigenicity, while collagen expresses some in physiological conditions.[6] Furthermore, gelatin is far more economical and convenient than collagen.[29] The soft and porous gelatin sponges would have direct contact with the tissue and are expected not to cause any damage to the wound area. Because of their high absorptive capacity, they would prevent fluid accumulation. Therefore, excess water (exudate composed of wound fluids) and cell debris would be absorbed and retained inside the sponges. Tissue ingrowth would take place in the matrix and the regenerating wound tissue and implant would not be separated. The sponge is biodegradable, therefore, it would degrade and be replaced by the newly regenerated tissue. Polyurethane membranes were used as the external layer because of their biocompatibility, highly elastomeric (extensible) strength, and permeability to gaseous substances. They create an inert environment for the blood, control water and heat transfer through the wound area, and prevent bacterial invasion. They are mechanically strong and protect the wound from the external effects.[30,31,32] The study concentrated on the synthesis of the wound dressing and on *in vivo* testing for its effects on wounds experimentally created on the backs of york pigs.

EXPERIMENTAL

Materials

An aqueous solution of human recombinant bFGF was purchased from Sigma (USA); gelatin (P.I. = 5.0)was obtained from Sigma (USA); polyurethane (Tendra®) was obtained from Mölnlycke Health Care (Sweden). All other reagents or chemicals were purchased from Sigma (USA) and were of analytical grade. All were used without any further treatment or purification.

Preparation of bFGF-impregnated gelatin microspheres

Gelatin microspheres were prepared by a modified coacervation technique reported by Nastruzzi's.[33] Briefly, an aqueous gelatin solution was added dropwise into paraffin oil while the mixture was mechanically stirred at 1000 rpm to form a water–oil emulsion. Then the solution was rapidly cooled by immersing in ice–water medium. The formed-gelatin microspheres were filtered, washed with acetone, and dried at room temperature. Then the noncrosslinked microspheres prepared were placed in glutaraldehyde aqueous solution, and gelatin crosslinking was allowed to proceed for 12 h. After crosslinking, the microspheres were placed in glycine aqueous solution for 30 min to block unreacted residual glutaraldehyde.

The resulting microspheres were washed twice with 0.1 wt % Tween 80-solution, centrifuged, and freeze-dried at room temperature.

To prepare the bFGF containing microspheres, bFGF (5 μg in 1 mL phosphate buffer, pH = 7.4) was added into the aqueous gelatin solution at the beginning of microsphere preparation stage. The resultant microspheres were designated as GM-bFGF.

Gelatin sponge preparation

Preparation of gelatin sponges

Aqueous gelatin solutions were stirred at about 2000 rpm for 30 min at room temperature, and glutaraldehyde solutions were added to form crosslinkings. Then the solutions were poured into molds, frozen in liquid nitrogen, and freeze-dried for 24 h. The thickness of the resultant sponges was 1 cm. The gelatin sponges were exposed to ^{60}Co to achieve sterilization prior to *in vivo* applications and were labeled as GS.

Preparation of gelatin sponges with bFGF

bFGF (5 μg bFGF in 1 mL phosphate buffer, pH = 7.4) was added into the gelatin solutions (prepared as mentioned earlier) and poured into molds. The general procedure of sponge formation was followed. These sponges were labeled as GS-bFGF.

Preparation of gelatin sponges with bFGF-loaded microspheres

Microspheres containing bFGF were added into the gelatin solutions and the general procedure of sponge formation was followed. These sponges were labeled as GS-GM-bFGF. Here, the GS means gelatin sponges without bFGF loaded.

Preparation of bilayer wound dressing

The bilayer wound dressing was constructed at the wound site, by initially applying the sponges and then covering with adhesive polyurethane (Tendra).

Characterization of microspheres and bilayer wound dressing

Morphological analysis

The morphology of microspheres (bFGF-not loaded or bFGF-loaded microspheres) and the prepared bilayer wound dressing were examined using a light microscope (Olympus BX-41) and a scanning electron microscope (SEM, HITACHI *S*-2700) after coating the samples with a thin layer of gold under vacuum.

Microstructure analysis

The average sizes and size distribution of microspheres were measured by a particle size analyzer (Leica), using acetone as the solvent. The sponge microstructures were investigated by geometrical measurement on SEM. The porosity and average diameter of pores were measured using an image analyzer (Leica).

Release studies and bFGF analysis by ELISA

Microspheres containing bFGF (GM-bFGF) and gelatin sponges with bFGF (GS-bFGF) were placed in phosphate buffer (pH = 7.4) and were incubated on a rotating incubator at 37°C, respectively. The buffer was replaced daily and the amount of bFGF in releasing media was determined by an ELISA. ELISA was performed according to the procedure of the kit (Quantikine® human fgf basic immunoassay; R and D systems, Minneapolis, MN).

Bioassay for bFGF

The biological activity of the released bFGF was determined by testing its ability to stimulate the proliferation of cultured neonatal rat skin fibroblasts. The cells were isolated, purified, and cultured on two-dimensional culture dishes. The fibroblasts were plated at a density of 5000 cells/cm^2 on tissue culture dishes and incubated overnight in phosphate buffer (pH = 7.4) medium at 37°C in 5% CO_2. After cell adhesion, the culture supernatant was replaced by a fresh medium and 100 μL of the releasing medium was added into each well. After incubation for 96 h, the cells were detached with EDTA and the cell number was counted using a hemocytometer. The results of cell number are reported as means ± standard deviations. Statistical analysis of data was performed by analysis of variance single factor.

In vivo studies

Animal model and surgical procedure

Ten, 1-month-old male york pigs, weighing about 10–15 kg, were used for *in vivo* experiments. The animals were fed with a standard diet and housed individually in the animal care facilities of the University.

The pigs were anesthetized by intraperitoneal injection of a mixture (1:1) of nembutal and atropine sulfate (3.5 mg/0.07 mL/york pig). After shaving and depilation, four full-thickness skin defects (diameter = 3.2 cm, area = 8.0 cm^2) were made, preserving the panniculus carnosus, on the back of each york pig. On each animal, vaseline gauze was applied to the first wound area and it was kept as control. The second wound was covered with gelatin sponge (GS). The

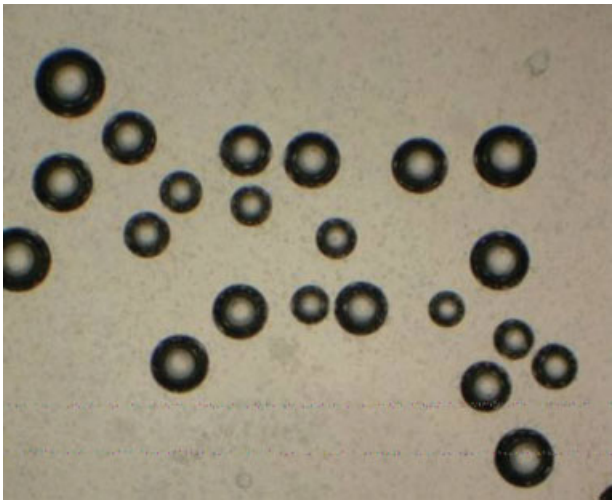


Figure 1 Photomicrographs of microsphere samples (×40). The microspheres did not aggregate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

third and fourth wounds were covered with free-bFGF containing sponges (GS-bFGF) and bFGF-loaded microsphere containing sponges (GS-GM-bFGF), respectively. Then the wounds were completely covered with a commercially available adhesive polyurethane (Tendra). Finally, the total wound area was covered with gauze containing fradiomycin sulfate, and the edges of the gauze were sutured to the skin with 4–0 nylon monofilament.

Everyday, the pigs were checked and the length and width of the lesions were measured. To evaluate the biocompatibility and efficiency of the systems, specimens encompassing the whole area were removed under general anesthesia on 7th, 14th and 21st days after the operation. Specimens were fixed in formaldehyde to be processed for histology.

Wound area measurements

Length and width of the wound regions were measured using a microruler that was sterilized prior to every measurement. Assuming that the lesion is in the shape of an ellipse (due to skin anisotropy), the following equation for the area of an ellipse was used as suggested by Baker and Haig[34]:

$$\text{Wound area (cm}^2\text{)} = [\text{long axis (cm)} \times \text{short axis (cm)} \times \pi]/4$$

Statistical analysis

Data are expressed as means ± SD. Analysis of variance (ANOVA) followed by the t test was used to determine the significant differences among the groups (p-values less than 0.05 were considered significant).

Histological examination

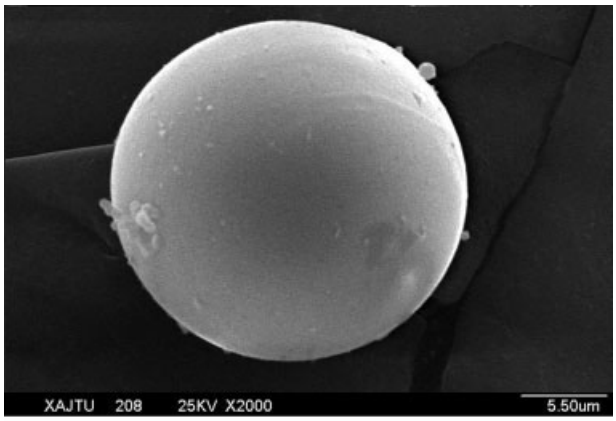
Skin samples containing the whole wound area were removed in the first, second, and third weeks of post-operation, and all specimens were immersed in 10% buffer formalin. They were dehydrated in a graded series of ethanol and were embedded in paraffin. Five to seven micrometer thin sections were prepared and stained with hematoxylin and eosin, Masson's Trichrome, and Gomori's. Photomicrographs were measured using an Olympus BH-7 light microscope.

RESULTS AND DISCUSSION

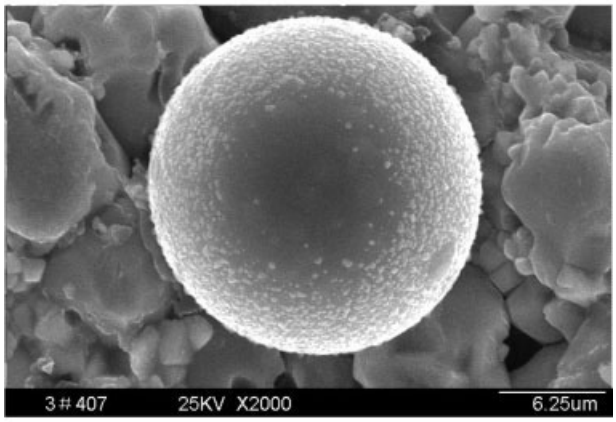
Characterization of microspheres

Morphological analysis results

Photomicrographs and SEM micrographs of microsphere samples are given in Figures 1 and 2. The

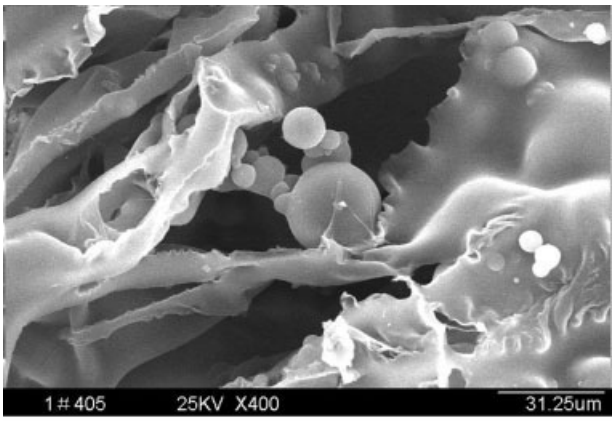


(a)

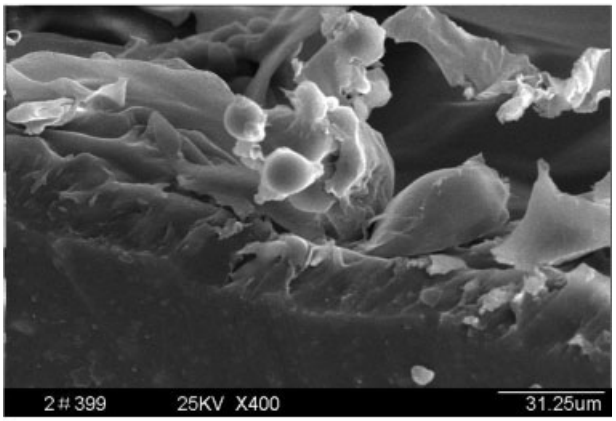


(b)

Figure 2 SEM micrographs of microsphere samples (×2000). (a)The samples not loaded with bFGF were quite spherical with no crack on the smooth surfaces. (b) bFGF-loaded microspheres were spherical and rough on the surfaces.



(a)



(b)

Figure 3 The SEM photographs of the gelatin sponges (×400). (a)The gelatin sponges show the porous and interconnected network structures. The microspheres inside pores can be clearly seen. (arrow:GM). (b) The full structure of a bilayer-wound dressing shows that the polyurethane film (PU) did not affect the porous structure of the gelatin sponges and the two layers adhered firmly to each other (arrow:PU).

micrographs indicate that the microspheres did not aggregate (Fig. 1). From the SEM micrographs, it was evident that the microspheres were quite spherical. It was noticed that the samples not loaded with bFGF had no crack on the smooth surfaces [Fig. 2(a)], but loading of bFGF into microspheres caused roughness on the surfaces [Fig. 2(b)].

Particle size analysis of microspheres

It is possible to obtain different-sized microspheres by changing the experimental conditions such as stirring speed, concentration of gelatin, or addition of surface active materials to the reaction medium during preparation.[35] In this study, the prepared microspheres had mean diameters in the range 11.80–37.60 μm, and the mean diameters of microsphere samples were 14.36 ± 3.56 μm. Prior to *in vivo* applications, the microspheres were put through a sieve with an aperture of 16 μm to achieve uniformity. Since the addition of surfactants might cause some adverse effects *in vivo* and decrease biocompatibility, no surfactants were used in this study.

Characterization of bilayer wound dressing

The SEM photographs of the bilayer wound dressing are shown in Figure 3. The gelatin sponges show the porous and interconnected network structures, and they are characterized by the porosity ranging between 40 and 70% with an average pore size of 80–160 μm. The microspheres inside pores could be clearly seen in the sponge structure and did not interfere with the pore structure of the sponge [Fig. 3(a)]. The full structure of a bilayer wound dressing is composed of a thin polyurethane film (PU) over which the gelatin sponge containing bFGF-loaded microspheres were attached. The polyurethane film did not affect the porous structure of the gelatin sponges and the two layers adhered firmly to each other [Fig. 3(b)].

Kinetics of bFGF release and bioactivity

The release rate of bFGF from the microspheres(GM-bFGF) was fairly constant, and the cumulative release increased linearly with time, as judged by ELISA (Fig. 4.).By day 7 of the release study, 92.9% of the impregnated bFGF was released to the external medium. The release profile obtained from bFGF-containing microspheres incorporated into the gelatin sponges was similar to that. In contrast, incorporating bFGF directly into the gelatin sponges (GS-bFGF) resulted in a rapid release after an initial burst of approximately 70% from the sponges, and 91.3% of the impregnated bFGF was released by day 2 of the study.

The released bFGF was further assessed for its ability to stimulate fibroblasts proliferation *in vitro*. The growth factor was collected at three time points during the 7-day release study. The results presented in Figure 5 reveal that the releasing media collected from bFGF containing matrices during the 7-day study

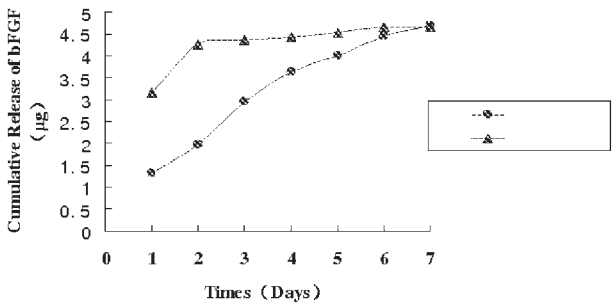


Figure 4 Kinetics of bFGF release judged by ELISA. The cumulative release from microspheres increased linearly with time.

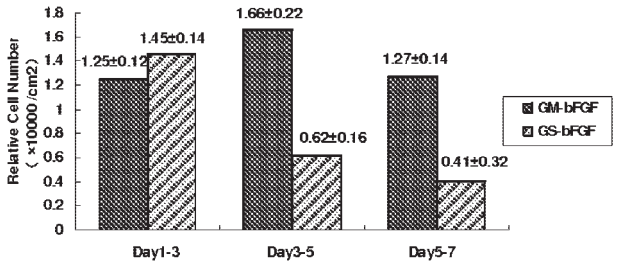


Figure 5 Bioactivity of the released bFGF. The bioactivity was determined using fibroblast proliferation assay. *Y*-axis is the relative cell proliferation resulting from the addition of bFGF released from the microspheres and the sponges at days 1–3, 3–5, and 5–7, as compared with controls. Values represent mean and standard deviation ($n = 3$). The difference between the two groups for each time is significant (analysis of variance, $p < 0.05$).

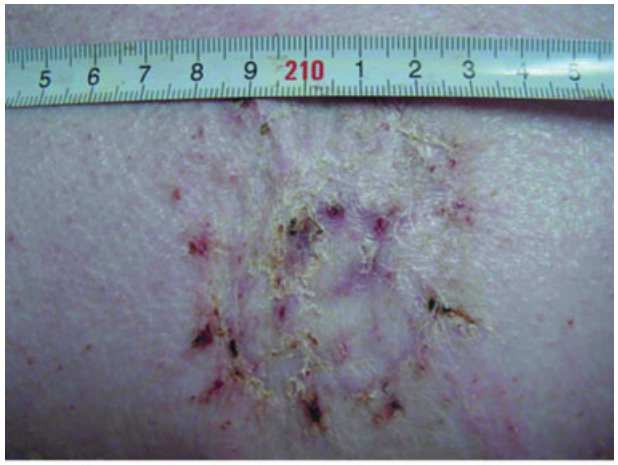


Figure 6 Macroscopic observations of lesions after 21 days post operation. (a) For the control wounds (lesion 1), hemorrhagic crusts hadn't disappeared completely. (b) In the GS-GM-bFGF applied wounds, there was an increase of vascularization in the surrounding area. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

stimulated the proliferation of fibroblasts *in vitro*. The highest proliferation response was obtained when the cells were exposed to the cumulative releasing media from days 3–5 of the experiment. This result is in accordance with the ELISA results, which revealed the highest amount of released bFGF at middle days. But the highest proliferation response of media collected from GS-bFGF was obtained at initial days (days 1–3). Comparison of the effective concentration of bFGF from the proliferation assay with the amount released by ELISA shows that the released bFGF from the microspheres(GM-bFGF) is more than 90% active at all times.

In vivo studies

The prepared sponges were applied on the full-thickness skin defects created on the dorsal regions of york pigs and controlled daily. The wound areas were measured and are presented below.

Macroscopic observations

One week after the operation, thick, depressed hemorrhagic crusts existed on almost all these wounds with white- and gray-colored centers. The sizes of skin defects were recorded as the originals. In lesion 1, the wound areas were more depressed and covered with hemorrhagic crust with a dark center. Lesion 2 was similar to lesion 1, although the skin defects were not as deep as the lesion 1. Two weeks after the operation, the skin defects and crusts were partially observed for the lesions 1, 2 in all animals. For other lesions, decreases in the wound areas were observed. There was a thin hemorrhagic crust with a thicker and dark-colored center in the lesions 3. In lesion 4, where higher bFGF-loaded microsphere containing sponges were applied, skin defect and crust were hardly observed, and the periphery of the wounds were well-

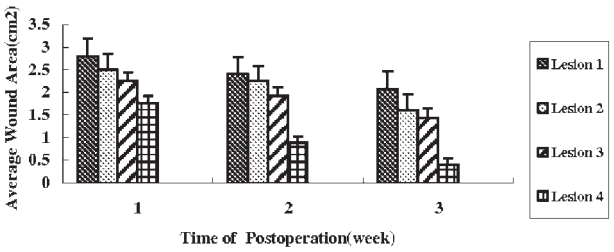


Figure 7 Change in wound area of lesions treated with various dressings 1, 2, and 3 weeks after operations. Lesion 1(vaseline gauze), lesion 2 (GS), lesion 3 (GS-bFGF), and lesion 4 (GS-GM-bFGF). There was a progressive decrease in the wound areas with time. The healing competency could be valued in the order of GS-GM-bFGF, GS-bFGF, GS, and vaseline gauze. Values represent mean and standard deviation ($n = 3$). The difference between lesion 3 and lesion 4 is significant (analysis of variance, $p < 0.05$).

TABLE I
The Decrease Percentages in the Wound Areas of Each Groups (%)

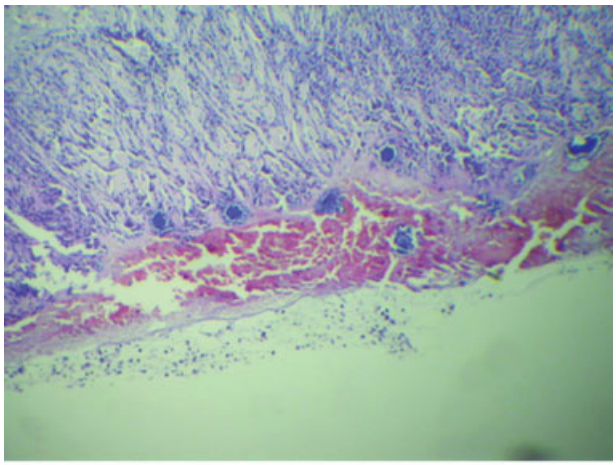
Groups	Postoperation		
	Week 1	Week 2	Week 3
Control group	65 ± 0.43	70 ± 0.38	74 ± 0.38
GS group	69 ± 0.42	72 ± 0.35	80 ± 0.32
GS-bFGF group	72 ± 0.19*	76 ± 0.30*	82 ± 0.21*
GS-GM-bFGF group	78 ± 0.21*	89 ± 0.19*	95 ± 0.17*

Values are expressed as mean ± SD for ten animals.
* Compared with control groups, the decrease of areas of wounds on which GS-bFGF and GS-GM-bFGF applied was higher, and the difference between them was considered significant, $P < 0.05$.

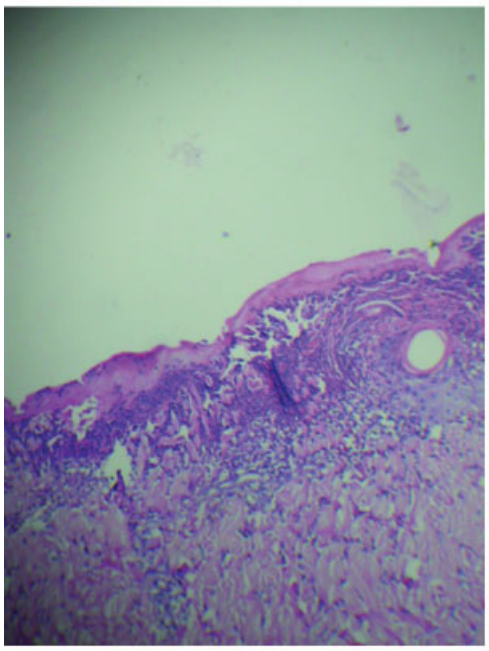
vascularized. Three weeks after the operation, a majority of skin defects seemed almost re-epithelialized. Macroscopically, however, each case of groups are somewhat different in healing stage. For the control wounds (lesion 1), hemorrhagic crusts hadn't disappeared completely from all the lesions [Fig. 6(a)]. Compared with the control group, the decrease of wound area of the lesion 2 and the lesion 3 were remarkable. In lesion 4, the wound regions were covered with epidermis, the defect had disappeared and the wound area had closed. And there was an increase of vascularization in the surrounding area [Fig. 6(b)].

It is known that, one of the most frequent complications of the wounds and wound dressing is the infection. It is very important that, in this study, no infection was observed for any of the animals throughout the experiments.

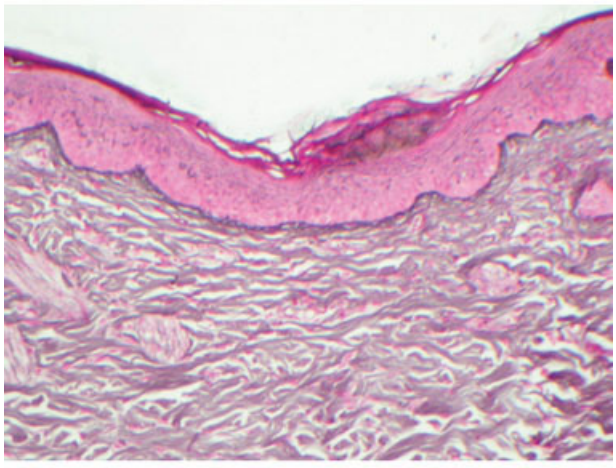
The area of the wounds originally produced was 8.0 cm^2. At the 7th, 14th, 21st postoperative day, the wound areas were calculated from the measured values and the average values are given in Figure 7. And the decrease percentages in the wound areas of each group are shown in Table I. In summary, there was a progressive decrease in the wound areas with time. It was also observed that the decrease of areas of wounds on which GS-bFGF and GS-GM-bFGF were applied was higher than those of the control groups. In this regard, the overall score of healing competency could be valued in the order of GS-GM-bFGF, GS-bFGF, GS, and vaseline gauze. The effective difference between free bFGF and microsphere-loaded bFGF was very remarkable, that is, the difference between GS-bFGF and GS-GM-bFGF was significant. But



(a)



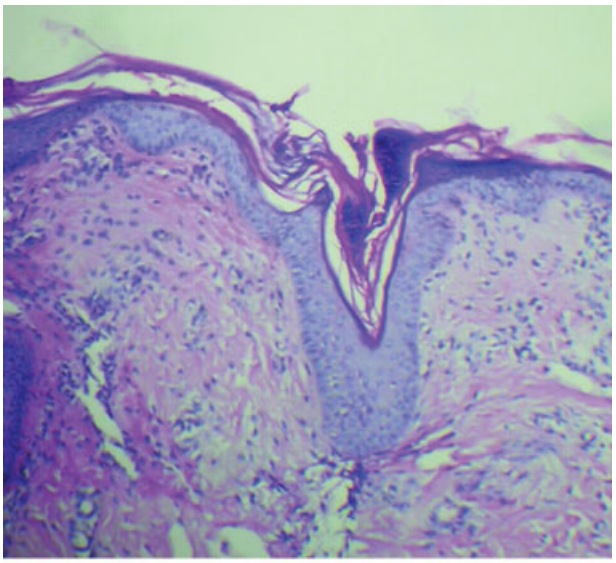
(b)



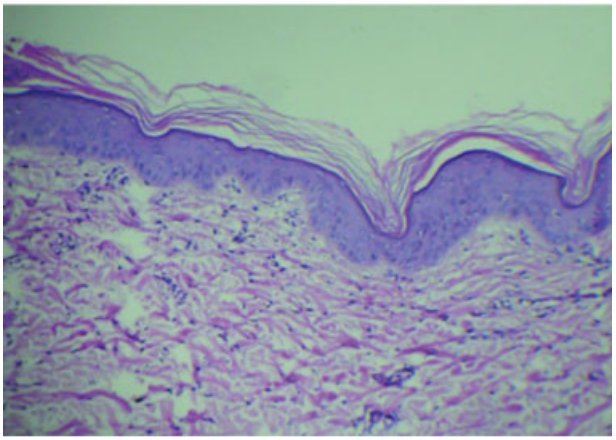
(c)

Figure 8 Histological findings after 7 days post operation. (a) Wound region of the control groups after 1 week. The epithelium on the wound region was missing (HE, ×4). (b) Wound region of the GS-bFGF group after 1 week. The wound regions were covered with thick crusts and epidermal parts were missing under the crust (HE, ×4). (c) Wound region of the GS-GM-bFGF group after 1 week. The wound region is filled with an organized granulation tissue (Gomori's, ×10). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

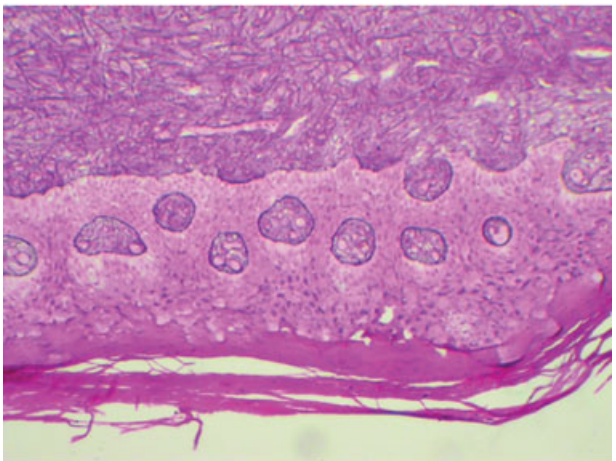
the difference was not distinct between GS and vaseline gauze. Furthermore, the final difference of curative effect between GS and GS-bFGF was not considered significant.



(a)



(b)



(c)

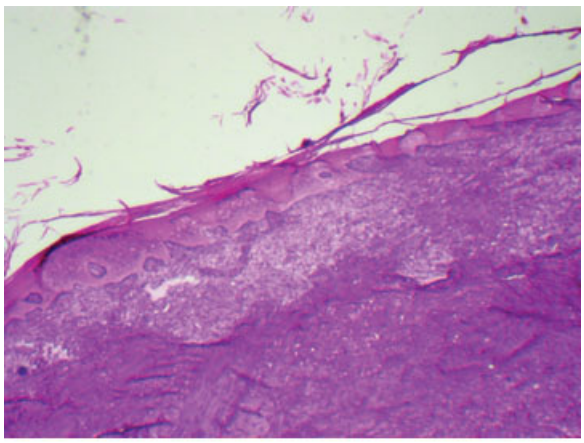
Histological findings

Seven days after the operation, the epithelium on the wound region was missing in the control group. Most of the crust covering the wound cavity was removed during sectioning. In other examined groups, the wound regions were covered by thick crusts and epidermal parts were missing under the crust. In some of the sections, the scab on the wound was detached from its place during sectioning. The structure of the dermis was not affected by the operation, and it was observed to be the same as the normal dermis in all groups examined. As shown in the pictures, no hair follicles were present in the wound regions and no mononuclear cells were observed [Figs. 8(a)–8(c)].

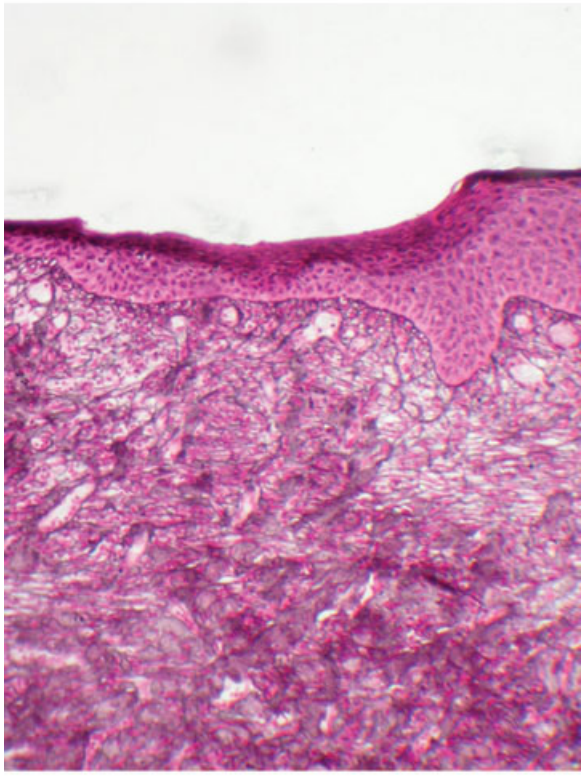
Fourteen days after the operation, the crust had disappeared and some of the wound area was covered with a continuous epidermis. In the GS and GS-bFGF applied wounds, the stratified epithelium that formed the epidermis was much thicker than that of the normal skin, and the wound regions were filled with the connective tissue of the dermis [Fig. 9(b)]. The dermis consisted of thin collagen fibers which were not as a feature of the normal pig skin. The junctions of the normal dermis and the dermis in the wound regions were very prominent in all groups examined. In the GS-GM-bFGF applied wounds, epithelization was mostly complete and appeared to be thick at the periphery of the wound region. Thick, coarse collagen fibers of the normal dermis continued horizontally with the newly-formed thin collagen fibers. The collagen fibers in the dermis layer were in a more organized form [Fig. 9(c)]. In the control group, the skin surface was not repaired and did not gain the normal appearance [Fig. 9(a)].

Twenty one days after the operation, the structure of the epidermis in the experimental groups became almost the same as that of the normal in appearance and thickness. No mononuclear cell infiltration and foreign body reactions were observed for any of the examined groups. In the control group, the junctions of the normal and newly formed dermis were still prominent though not so distinct as the 14th day groups [Fig. 10(a)]. In GS and GS-bFGF applied group, the junction was less well demarcated with some horizontal fibers extending between the two regions. In

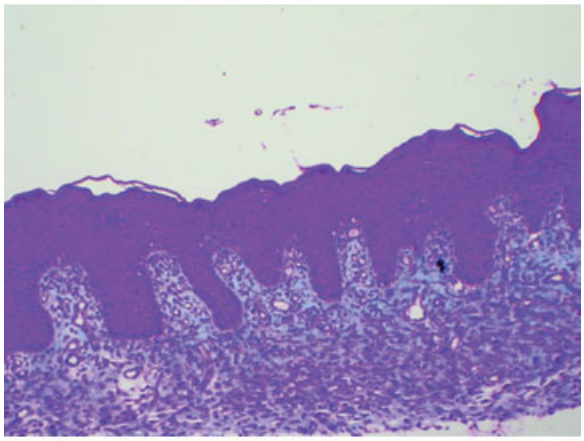
Figure 9 Histological findings after 14 days post operation. (a) Wound region of the control groups after 2 weeks. The crust had disappeared (HE, ×4). (b) Wound region of the GS-bFGF group after 2 weeks (HE, ×4). The stratified epithelium that formed the epidermis was much thicker than that of the normal skin. (c) Wound region of the GS-GM-bFGF group after 2 weeks. Thick, coarse collagen fibers of the normal dermis continued horizontally with the newly formed thin collagen fibers (Gomori's, ×10). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



(a)



(b)



(c)

these groups, the horizontal organization of the fine collagen fibers in the superficial dermis was still observed [Fig. 10(b)]. In the GS-GM-bFGF group, there was a marked difference. The wound had healed completely and was covered with a newly formed dermis. The structure of the dermis was rather loosened and the junction was much less prominent. The collagen fibers of the two regions continued and intermingled gently with each other. [Fig. 10(c)]

In summary, GS-GM-bFGF group was found to show the best wound healing properties as a wound dressing, from histological findings. The wound dressings containing bFGF-impregnated microspheres have a substantial effect on decreasing the wound area and removal of histological signs of tissue damage in experimentally created pig skin lesions.

CONCLUSIONS

The aim of this work was to prepare a bilayer wound dressing containing basic fibroblast growth factor delivery system as a novel covering in healing skin defects. For this purpose, gelatin microspheres were prepared to provide sustained release, and the compatibilities of bilayer wound dressings were designed and tested with a series of preliminary *in vitro* protein release and bioactivity experiments and *in vivo* experiments by applying them on full-thickness skin defects created on york pigs.

As a result, the prepared microsphere and sponges were found to be biocompatible. bFGF-impregnated microspheres can serve as delivery vehicles for controlled release of bFGF, and the released growth factor can enhance fibroblasts proliferation *in vitro*. The amount released by ELISA and the effective concentration of bFGF from the proliferation assay show that the released bFGF from the microspheres(GM-bFGF) is more than 90% active in 7 days.

The well-formed porous structure allowing growth of fibroblasts and capillaries into the matrix, with good affinity, adhesiveness, and nonirritability for tissues, make it usable as the inner layer material of an ideal wound dressing. Mononuclear cell infiltration or foreign body reactions were not observed for any of

Figure 10 Histological findings after 21 days post operation. (a) Wound region of the control groups after 3 weeks (Gomori's, ×10). The surface of the wound is covered with a thin epidermis. Dermis consists of thin collagen fibers. (b) Wound region of the GS-bFGF group after 3 weeks. (Gomori's, ×10). The epidermis and dermis appear to be almost the same as the normal skin except the collagen fibers are parallel to the surface and less organized. (c) Wound region of the GS-GM-bFGF group after 3 weeks. Masson's trichrome (×10) epithelization is completed. Dermis is infiltrated with collagen fibers in a mesh structure. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

wounds. Furthermore, the materials of the outer layer enhanced the tensile strength of the wound dressing. Besides the repair time of epidermis was the shortest in all groups, the wound cavity was filled with a dermis that appeared to have a structure resembling the normal dermis, without any scar tissue formation in the GS-GM-bFGF applied lesions. The high quality of the newly formed dermis in the wound cavity was attributed to the application of sustained-release bFGF.

In our experimental model, histological investigations showed that these bFGF-loaded, biodegradable materials made the skin defect almost re-epitheliaze at the 21st postoperative day with a minor infection and good integrity of dermis. And they are biocompatible. In our study, GS-GM-bFGF group was found to show the best wound healing properties as a wound dressing from all the macroscopic observations and histological findings. The other properties of these materials are as follows: the preparation process is relatively simple; they have a high body fluid absorption capacity; the release rate of macromolecules and growth factors from them are controllable; they are very soft and therefore cause no disturbances during the application. *In vivo* experiments showed that the rate of wound-area decrease was much faster compared to that of the control groups and the quality of the newly formed dermis as judged by the histological examinations was almost as good as the normal skin. Results on the application of these novel coverings in pig's skin defect models indicated that the bilayer wound dressing containing bFGF-loaded microspheres provided an optimum healing milieu for the proliferating cells and regenerating tissues.

In case of very deep wounds or ulcers, the applied dose of bFGF could be increased or frequent application of fresh bFGF-loaded dressings could be made. It would also be more effective, if a mixture of growth factors is used instead of only bFGF. We believe that after the optimization of the composition and testing *in vivo* for other signs of wound healing (such as DNA, collagen and glucoseaminoglycans contents and wound tensile strength), these constructs would meet the demand for wound dressings in the medical field.

References

1. Smith, D. J.; Thomson, P. D.; Bolton, L. L.; Hutchinson, J. J. Plast Reconstr Surg 1993, 91, 1094.
2. Shibata, H.; Shioya, N.; Kuroyanagi, Y. J Biomater Sci Polym Ed 1997, 8, 601.
3. Yannas, I. V.; Burke, J. F. J Biomed Mater Res 1980, 14, 65.
4. Matsuda, K.; Suzuki, S.; Isshiki, N.; Yoshioka, K.; Wada, R.; Hyon, S. H.; Ikada, Y. Biomaterials 1981, 3, 119.
5. Jorgensen, S. J.; Hendriksen, C. Ugeskr Laeger 2005, 167, 263.
6. Purna, S. K.; Babu, M. Burns 2000, 26, 54.
7. Bruin, P.; Jonkman, M. F.; Meijer, H. J.; Pennings, A. J. J Biomed Mater Res 1990, 24, 217.
8. Draye, J. P.; Delaey, B.; Van de Voorde, A.; Van Den Bulcke, A.; De Reu, B.; Schacht, E. Biomaterials 1998, 19, 1677.
9. Kaya, A. Z.; Turani, N.; Akyuz, M. J Wound Care 2005, 14(1), 42.
10. Tsou, T. L.; Tang, S. T.; Huang, Y. C.; Wu, J. R.; Young, J. J.; Wang, H. J. J Mater Sci Mater Med 2005, 16, 95.
11. Lynch, S. E.; Colvin, R. B.; Antoniades, H. N. J Clin Invest 1989, 84, 640.
12. Shirakata, Y.; Tokumaru, S.; Yamasaki, K.; Sayama, K.; Hashimoto, K. J Dermatol Sci 2003, 32, 209.
13. Fujita, N.; Sakaguchi, I.; Kobayashi, H.; Ikeda, N.; Kato, Y.; Minamino, M.; Ishii, M. Biol Pharm Bull 2003, 26, 329.
14. DeBlois, C.; Cote, M. F.; Doillon, C. J. Biomaterials 1994, 15, 665.
15. Watts, S. A. Adv Nurse Pract 2001, 9, 60.
16. Andreatte-van, L. S.; Smith, D. J.; Bulgrin, J. P.; Schafer, I. A.; Eckert, R. L. J Biomed Mater Res 1993, 27, 1201.
17. DaCosta, M. R.; Aniceto, C.; Jesus, F. M.; Mendes, M. Lancet 1994, 344, 481.
18. Hui, E. P.; Yeo, W.; Wickham, N. W. R. Aust New Zealand J Med 1995, 26, 420.
19. Brown, G. L.; Curtsinge, L. J.; Brightwell, J. R.; Ackerman, D. M.; Tobin, G. R.; Polk, H. C.; George-Nascimento, C.; Vanezuela, P.; Scholtz, G. S. New Engl J Med 1989, 321, 76.
20. Puolakkainen, P. A.; Twardzik, D. R.; Ranchalis, J. E.; Pankey, S. C.; Reed, M. J.; Gombotz, W. R. J Surg Res 1995, 58, 321.
21. Sheardown, H.; Clark, H.; Wedge, C.; Apel, R.; Rootman, D.; Cheng, Y. L. Curr Eye Res 1997, 16, 183.
22. Wang, H. J.; Wan, H. L.; Yang, T. S.; Wang, D. S.; Chen, T. M.; Chang, D. M. Burns 1996, 22, 10.
23. Kawai, K.; Suzuki, S.; Tabata, Y.; Ikada, Y.; Nishimura, Y. Biomaterials 2000, 21, 489.
24. Cortesi, R.; Esposito, E.; Osti, M.; Squarzoni, G.; Menegatti, E.; Davis, S. S.; Nastruzzi, C. Eur J Pharm Biopharm 1999, 47, 153.
25. Choi, Y. S.; Hong, S. R.; Lee, Y. M.; Song, K. W.; Park, M. H.; Nam, Y. S. Biomaterials 1999, 20, 409.
26. Lee, C. H.; Singla, A.; Lee, Y. Int J Pharm 2001, 221, 1.
27. Matsuda, K.; Suzuki, S.; Isshiki, N.; Yoshioka, K.; Wada, R.; Okada, T.; Ikada, Y. Biomaterials 1990, 11, 351.
28. Lu, W. W.; Ip, W. Y. Br J Plast Surg 2000, 53, 225.
29. Noorjahan, S. E.; Sastry, T. P. J Biomed Mater Res B 2004, 71, 305.
30. Ulubayram, K.; Hasirci, N. J Colloid Surf B: Biointeractions 1993, 1, 261.
31. Nomi, M.; Atala, A.; Coppi, P. D.; Soker, S. Mol Aspects Med 2002, 23, 463.
32. Ulubayram, K.; Hasirci, N. Polymer 1992, 33 2084.
33. Nastruzzi, C.; Pastesini, C.; Cortesi, R.; Esposito, E.; Gambari, R.; Menegatti, E. J Microencapsulation 1994, 11, 249.
34. Baker, P. G.; Haig, G. Practitioner 1981, 225, 569.
35. Esposito, E.; Cortesi, R.; Nastruzzi, C. Biomaterials 1996, 17, 2009.