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Research paper

The use of fucosphere in the treatment of dermal burns in rabbits ☆

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Abstract

The aim of this study was to prepare a new microsphere (fucosphere) system based on polyion complexation of fucoidan with chitosan, and to evaluate its treatment efficiency on dermal burns.

The physicochemical properties such as mean particle size and distribution, zeta potential and bioadhesive properties of the microspheres were investigated. The formulation which had the high surface charge, narrow size distribution and the highest bioadhesive property was selected and applied on seven male New Zealand white rabbits with dermal burns. Biopsy samples were taken on day 7, 14 and 21. Each burn site was evaluated macroscopically and histopathologically and the findings were compared with controls of fucoidan solution and chitosan microspheres.

The microspheres between the size ranges of 367 and 1017 nm were obtained. The work of bioadhesion of microspheres, with the surface charges +6.1 to +26.3 mV, changed between 0.081 and 0.191 mJ cm^{-2}. Macroscopically and histopathological observations indicated that the fastest healing of the burns was obtained in group treated with fucosphere after 21 days of treatment ($P < 0.05$). Rete peg formation values and nuclear organize regions (NORs) were higher with treated fucospheres than the other groups on day 14.

In conclusion, *in vitro* and *in vivo* evaluation of fucospheres indicated that the new microsphere system shortened the treatment period of burns and provided fast and effective healing by improving regeneration and re-epithelization. Hence fucosphere may find application in the treatment of dermal burns.
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Keywords: Fucoidan; Chitosan; Biopolymer; Fucosphere; Microsphere; Burn healing; Animal experiment

1. Introduction

Third-degree burn is the most serious injury because it destroys all the layers of the skin and the healing begins with the formation of granulation tissue, often associated with hypertrophic scars [1,2]. Recently, dermal substitution and burn healing have become one of the most exciting research areas in biomaterial sciences. Although there have been many recent advances in this field, commercially available products and the biological materials currently described in experimental studies are still incapable of fully substituting for natural living skin [2,3]. On the other hand, healing of dermal wounds with macromolecular agents such as natural polysaccharides is preferred as a skin substitute as they possess useful properties such as high biocompatibility and non-toxicity [4].

There are two stages of burn healing. The first stage involves the inflammatory phase and the second stage is the new tissue formation phase [5]. During the inflammatory phase, infiltrating neutrophils aid in the removal of foreign agents in the burn area. It was found that

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polysaccharides such as chitin and chitosan could accelerate the infiltration of inflammatory cells, consequently accelerating wound cleaning [6]. When the new tissue formation phase occurs, fibroplasia begins by the formation of granulation tissue within the wound space.

Chitosan is a mucopolysaccharide with structural characteristics similar to glycosamines. This biopolymer, produced by deacetylation of chitin, is derived from the exoskeleton of crustaceans. Chitosan has been used as a wound dressing in burn healing for proliferation and activation of inflammatory cells in granulation tissue [7]. Fucoidan is a sulphated polysaccharide extracted from brown seaweeds (e.g., *Fucus vesiculosus*, *Ascophyllum nodosum*). Fucoidan is made up of α-L-fucose units linked by (1 → 4) and (1 → 3) glycosidic bonds and sulphated at positions 2 and/or 3 and/or 4 depending on the algal species. Fucoidan is endowed with significant gel contraction-promoting ability and integrin expression-enhancing heparinic activity [8]. A great number of studies on pharmacological properties of fucoidan have been carried out but there is limited research on the use of fucoidan for the treatment of burn healing [9–11].

The aim of this study was to prepare a new microsphere (fucosphere) system based on polyion complexation of negatively charged fucoidan with positively charged chitosan, and to evaluate its treatment efficiency on dermal burns.

2. Materials and methods

2.1. Materials

Chitosan (MW 250 kDa, deacetylation degree ⩾90%, Pronova A/S, Norway; MW 400 kDa, deacetylation degree ⩾60%, Fluka, Germany; MW 750 kDa, deacetylation degree ⩾75%, Sigma, USA), fucoidan (MW 80 kDa, from *Fucus vesiculosus*), sodium sulphate (Merck, Germany) and lactic acid (85% m/v) were purchased from Sigma, USA. All other reagents used were of analytical grade.

2.2. Preparation of the microspheres

Fucospheres were prepared by mixing positively charged chitosan and negatively charged fucoidan using a polyion complexation method [12,13]. Briefly, 10 ml aqueous solution of fucoidan was added dropwise into 10 ml chitosan solution in 1% m/v lactic acid using high shear homogenisation (Ika, Euroturrax T20, Germany) at 20,000 rpm. Formed microspheres were separated by centrifugation at 15,000 rpm and then freeze-dried (Lyovac GT 2E, Steris, Germany). For comparison, chitosan microspheres were prepared by precipitation method as previously described [14]. To prepare chitosan particles, 10 mL of sodium sulphate solution (20% m/v) was dropped into 10 mL of acidic solution of chitosan (2% m/v) and stirred (Ika-Werke GmbH & Co, Germany) for 1 h at 500 rpm. Chitosan microspheres were washed, separated by centrifugation at 12,000 rpm and then freeze-dried. A number of variables were investigated for the purpose of optimization of the microsphere formulations (Table 1).

2.3. Scanning electron microscopy (SEM)

Particles were mounted on the metal grids using double-sided adhesive tape and coated with gold about 500×10^{-8} cm in thickness using SC7640 Sputter Coater (Quorum technologies, Newhaven, UK) under high vacuum, 0.1 Torr, 1.2 kV and 50 mA at 25 ± 1 °C. The surface morphology of microspheres was investigated with scanning electron microscopy (SEM) (Joel, JSM-5200, Japan) at 20 kV.

2.4. Determination of particle size

Measurements were performed at 25 °C, using a Climet submicron particle size analyzer (Climet CI-1000 liquid counter system, USA) with a 180 Series Laser Diode illuminated light scatter submicron sensor (Measurement range: 0.2–50 μm). The particle suspensions were sonicated in an ultrasonic bath for 5 min prior to analysis and bidistilled water was used as a dilution medium. Analyses were performed in three different batches and the results were expressed as a mean of three measurements.

2.5. Zeta potential

The zeta potential values of microspheres were determined in a 0.2 M KCl solution after measurement of the electrophoretic mobility for 20 s at 25 °C using a Zetasizer

Table 1
Mean particle diameter, zeta potential and bioadhesion values of the microspheres

Formulations	Fucoidan concentration (%)	Chitosan concentration (%)	Chitosan origin	Mean particle size (nm ± SD)	Polydispersity index ± SD	Zeta potential (mV ± SD)	Work of bioadhesion (mJ cm^{-2} ± SD)
A1	1.50	0.50	Sigma	367 ± 34	0.268 ± 0.032	23.1 ± 0.9	0.104 ± 0.003
A2	2.00	0.50	Sigma	575 ± 43	0.146 ± 0.021	13.3 ± 0.4	0.164 ± 0.006
A3	2.50	0.50	Sigma	901 ± 61	0.234 ± 0.041	8.2 ± 0.5	0.187 ± 0.005
A4	–	0.50	Sigma	616 ± 23	0.318 ± 0.074	26.3 ± 0.4	0.082 ± 0.004
B1	2.00	0.25	Sigma	416 ± 31	0.213 ± 0.045	9.7 ± 0.5	0.081 ± 0.005
B2	2.00	0.75	Sigma	768 ± 22	0.098 ± 0.021	16.7 ± 0.4	0.191 ± 0.005
C1	2.00	0.50	Protan 243	801 ± 40	0.314 ± 0.054	6.1 ± 0.3	0.134 ± 0.005
C2	2.00	0.50	Fluka (M.W)	1017 ± 73	0.358 ± 0.081	10.5 ± 0.6	0.141 ± 0.006

3000 HSA (Malvern Ltd., Malvern, UK). Zeta potential measurements were carried out in triplicates.

2.6. In vitro bioadhesion studies

The bioadhesion of microspheres was measured by using modification of a previously described method of Kockisch et al. [15]. The measurement was conducted using a texture analyzer (TA.XT *Plus*, Stable Micro Systems, Haslemere, Surrey, UK) equipped with a 5 kg load cell and bioadhesion test rig. Freshly excised chicken back skin was used as a model tissue after removing all fats and debris. The dermal tissue was fitted on the bioadhesion test rig and then 100 μl of distilled water was applied on the surface of the tissue before starting the bioadhesion test. Microspheres (5 mg) were attached to the lower surface of the probe (P10 perspex, θ: 10 mm, contact area: 0.785 cm^2) of the instrument with double-sided adhesive tape. The tests were conducted at 37 °C. The probe lowered onto the surface of the tissue at a constant speed of 1.0 mm s^{-1} and contact force of 1.0 N applied. After 2.0 min of contact time, the probe was then moved vertically upwards at a constant speed of 1.0 mm s^{-1}. The area under the curve (AUC) was calculated from force–distance plot as the work of adhesion (Fig. 1) using *Texture Exponent 4.0.4.0* software package of the instrument. The formulation given below was used to calculate work of adhesion per cm^2 (mJ cm^{-2}) (Eq. (1)). Each measurement was carried out in triplicates.

$$\text{Work of adhesion}(\text{mJ cm}^{-2}) = \frac{\text{AUC}}{\pi r^2} \quad (1)$$

πr^2 = the skin surface being in contact with particles.

2.7. Skin burn wounds

Experimental design and treatment of animals were approved by the Animal Care Committee of Selçuk University. Seven male New Zealand white rabbits (4.2 ± 0.3 kg) were used for the evaluation of dermal burn wounds. The back of the rabbits were depilated, and ketamine (25 mg/kg) and xylazine (1 mg/kg) were injected intramuscularly into the rabbits to induce sedation before a heated aluminium stamp was applied. The electrically heated stamp was maintained at a temperature of 80 °C and applied for 14 s to form a dermal burn wound (burn area: 3.8 cm^2) described by Knabl et al. [16]. Burn wound depth was assessed by histopathological determination of the depth of damage to the following skin elements: hair follicle (epithelial cells), connective tissue collagen (a change in collagen staining), nerves and smooth muscles (mesenchymal cells) and blood vessels (endothelial cells). The depth was measured starting from the epithelium basal layer. It was confirmed that all animals had deep dermal burns. Each rabbit had four burn wounds; the first was treated with fucospheres (B2, 20.6 mg/3 days), the second was treated with fucoidan solution of 0.75% m/v (2 mL/3 days) and the third was treated with chitosan microspheres (20.6 mg/3 days) for 21 days. The fourth wound was used as a control and hence did not receive any treatment. Formulations were applied onto the wet fascia of the wound area after escharectomy. The biopsy samples were taken on 7th, 14th and 21st days from six rabbits and the degree of healing was evaluated macroscopically and histopathologically. One rabbit was separated to take photographs of the wound areas at days 7, 14 and 21.

2.8. Histopathological examination

The biopsies of skin samples (0.5×1.5 cm^2) were fixed in a 10% buffered formaldehyde solution, then embedded in paraffin block and sectioned to 4 μm increments. The sections were made perpendicular to the anterior–posterior axis and perpendicular to the surface of the wounds. The sections were positioned on a slide and stained with haematoxylin–eosin and Masson's trichrome reagents. The measurement of "wound epithelial elongation" was carried out on the line between two irregular zones for as many as needed depending on the microscopic length on the epithelial line in the wound epithelial elongation region and they were added to each other to get the final elongation. The measurement for "wound epithelial thickness" was done at five different locations excluding rete pegs on the line in the wound epithelial elongation section. The number of rete pegs suggesting migrating of cells from epidermal appendages was counted in the wound epithelial elongation section. Results were given as a mean of six rabbits.

Acute inflammatory cells (polymorphonuclear leukocytes), chronic inflammatory cells (mononuclear leukocytes), fibroblast and collagen proliferation were evaluated separately. Each item was graded by two pathologists according to a semi-quantitative approach as absent (0), mild (1), moderate (2) and severe (3) without the knowledge of the specimen groups.

2.9. AgNOR staining and quantification study

The nucleolar organiser regions (NORs) stained by silver and the argyrophilic NOR-associated proteins are called AgNORs, respectively. The samples were prepared according to an earlier study [17]. Four micrometers section of samples were cut from the paraffin block, dewaxed with xylene and hydrated. The silver staining solution (0.3 mL) containing one part by volume of 2% m/v gelatine in 1% m/v formic acid and two parts of 25% m/v aqueous silver nitrate solution was immediately poured on each slide. After staining (20 min), the solution was poured off and the slides were washed with bidistilled water, placed for 10 min in a 5% m/v sodium thiosulphate solution, rinsed with bidistilled water and dried. The AgNOR proteins appeared as well-defined black dots that counted in 50 cells of each sample. The analysis was performed in triplicates for each batch of microspheres.

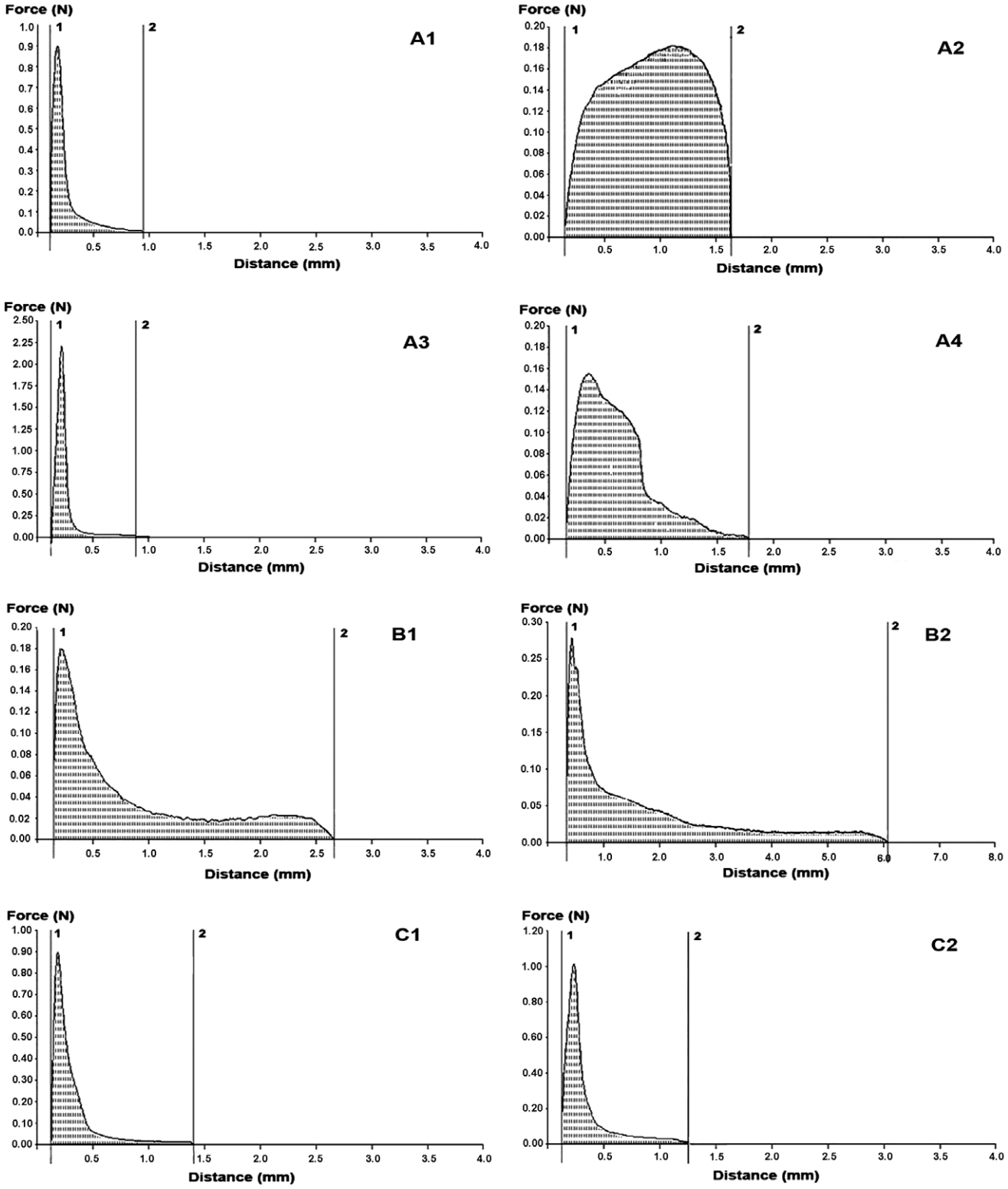

Fig. 1. Work of bioadhesion graphs of the formulations.

2.10. Statistical analysis

In vitro data obtained from each experiment were subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparisons test. Differences between the groups were tested for significance by the chi-squared test (χ^2) for the *in vivo* studies. $P < 0.05$ was considered to be indicative of significance.

3. Results and discussion

3.1. The characterization of fucospheres

Fucospheres prepared by polyion complexation method utilizing the opposite electrostatic charges of positively charged chitosan and negatively charged fucoidan had a spherical and non-porous structure (Fig. 2).

The particle size of the fucospheres obtained was between 367 and 1017 nm. Formulation variables were found to affect particle size. By increasing the concentration of chitosan and fucoidan in the formulation, particle sizes of fucospheres were also increased (Table 1). Particles in formulation A1 containing 1.5% fucoidan were 367 nm in diameter, while particle sizes of the formulations containing 2.0% (A2) and 2.5% (A3) fucoidan were 575 and 901 nm ($P < 0.05$), respectively. Similarly, formulations containing 0.25% (B1), 0.50% (A2) and 0.75% (B2) chitosan were 416, 575 and 768 nm in size, respectively, and such increase in particle sizes was considered to be statistically significant ($P < 0.05$). The particle size was increased with increasing of the polymer concentration. The results were in accordance with the literature [18–20]. The origin of the chitosan used in the formulations was also affecting the particle size. While microspheres prepared with Sigma's chitosan (A2) had the smallest particle size (575 nm), particle sizes of the formulations prepared with Protan 243 chitosan (C1) and Fluka chitosan (C2) were 801 and 1017 nm, respectively (Table 1) ($P < 0.05$). The effect of increasing molecular weight of the chitosan on increasing particle size of microspheres can also be a contributing factor. The homogenous particle size distribution administered to wound surface plays an important role in improving the efficacy of treatment. In order to prevent the secondary infection of bacteria, an ideal wound dressing not only absorbs wound fluid but also disperses on the wound surface homogeneously to cover whole wound area [21]. Small particle size and narrow size distribution will increase the surface area of the particles which contact burn area. This will improve the spreadability of the particles on the wound surface, increase the retention time of the particles at the application site and patient compliance by reducing the frequency of administration. The particle size and distribution of the microspheres were investigated in our study. Narrow particle size distribution was obtained by increasing the concentrations of fucoidan and chitosan in the formulation. An ideal particle size distribution was observed in formulations A2 (P.I.: 0.146 ± 0.023) and B2 (P.I.: 0.098 ± 0.021) (Table 1).

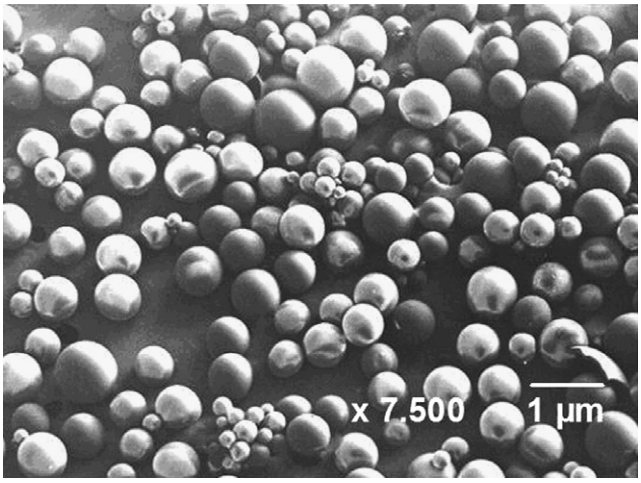

Fig. 2. Scanning electron micrograph of fucospheres (B2 formulation).

The zeta potential values of the microspheres were between +6.1 and +26.3 mV (Table 1). The surface charge was decreased by increasing the concentration of fucoidan in the formulation (A1–A3, +23.1 to +8.2 mV), and an increase was observed by increasing the chitosan concentration (B1–B2, +9.7 to +16.7 mV). Positive zeta potentials were recorded for the microsphere formulations although the concentration of negatively charged fucoidan was higher than that of positively charged chitosan in the formulations [22,23]. This indicates that the outer surface of the fucospheres consists of chitosan [24,25]. It was suggested that, as a result of interaction between protonated amine groups of chitosan and non-protonated sulphate groups of fucoidan, excess of chitosan chain forms the outer layer of the fucospheres, and chitosan and fucoidan complex constitutes a hydrophobic core. The amount of protonated amine groups of chitosan on the outer surface of fucospheres significantly changed the surface charge of the microspheres, as seen with chitosan–glycyrrhetic acid nanoparticles [26] and chitosan–heparin complexes [27,28]. The charges of coating materials applied to surface in wound healing are important determinants of mucoadhesion ability, biocompatibility and to ensure interaction on the wound surface [1,2,29]. Polymers which are negatively charged and have hydrogen-bonding capabilities are known to have mucoadhesion ability. However, positively charged polymeric materials could possibly develop additional molecular attraction forces by electrostatic interactions with negatively charged wound mucosal surfaces [29]. Therefore, increased surface of fucospheres has been a favorable condition with regards to administration, and the microspheres with the highest chitosan concentration had the highest surface charge (B2, 16.7 mV) (Table 1).

Bioadhesion values of the microspheres varied between 0.081 and 0.191 mJ cm^{-2} (Table 1). Bioadhesion of fucospheres was affected by all formulation parameters other than chitosan origin ($P < 0.05$). Bioadhesion of the particles was found to increase when the concentrations of chitosan and fucoidan in the formulation were increased (Fig. 1). Increasing the chitosan concentration from 0.25 to 0.75% increased the bioadhesion values of the particles approximately by 2.5-fold (B1: 0.081 mJ cm^{-2}; B2: 0.191 mJ cm^{-2}). In addition to this, by increasing the fucoidan concentration from 1.5% to 2.5%, higher bioadhesion value was obtained (A1: 0.104 mJ cm^{-2}; A3: 0.187 mJ cm^{-2}) (Table 1) ($P < 0.05$). Chitosan demon-

strates high bioadhesive properties due to the interaction of positively charged amino groups of chitosan with negatively charged tissue proteins in the mucus. Therefore, the use of chitosan in higher concentrations in the formulations has increased the bioadhesion of the microspheres. The results were similar to the findings of Kockisch et al. and Chowdary and Rao [15,30]. In order to achieve effective treatment of wound, coating material should have high bioadhesion with the ability of not losing the integrity after contact with the tissue [1,2]. The findings showed that the formulation having the highest bioadhesion was that of B2 microspheres containing 2.0% fucoidan and 0.75% chitosan (0.191 ± 0.005 mJ cm^{-2}).

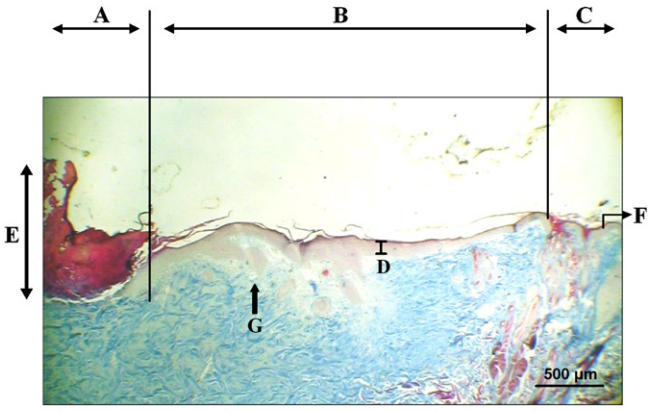

Fig. 4. The scheme of the microscopic evaluation of the wound area. (A) The eschar area of the wound, (B) the wound epithelial elongation, (C) non-burned epithelial area, (D) wound epithelial thickness (the healing area of the wound), (E) the eschar thickness of the wound, (F) non-burned epithelial thickness, (G) the papillary structures called rete pegs or finger like projections.

3.2. In vivo wound healing study

The optimized formulation B2 with appropriate particle size and distribution, high surface charge and bioadhesive properties in *in vitro* studies was chosen for use in *in vivo* experiments and treatment efficacy on rabbits with dermal burns of partially was investigated. Edema was observed only in the control group within the first seven days, and no significant difference was observed between the groups

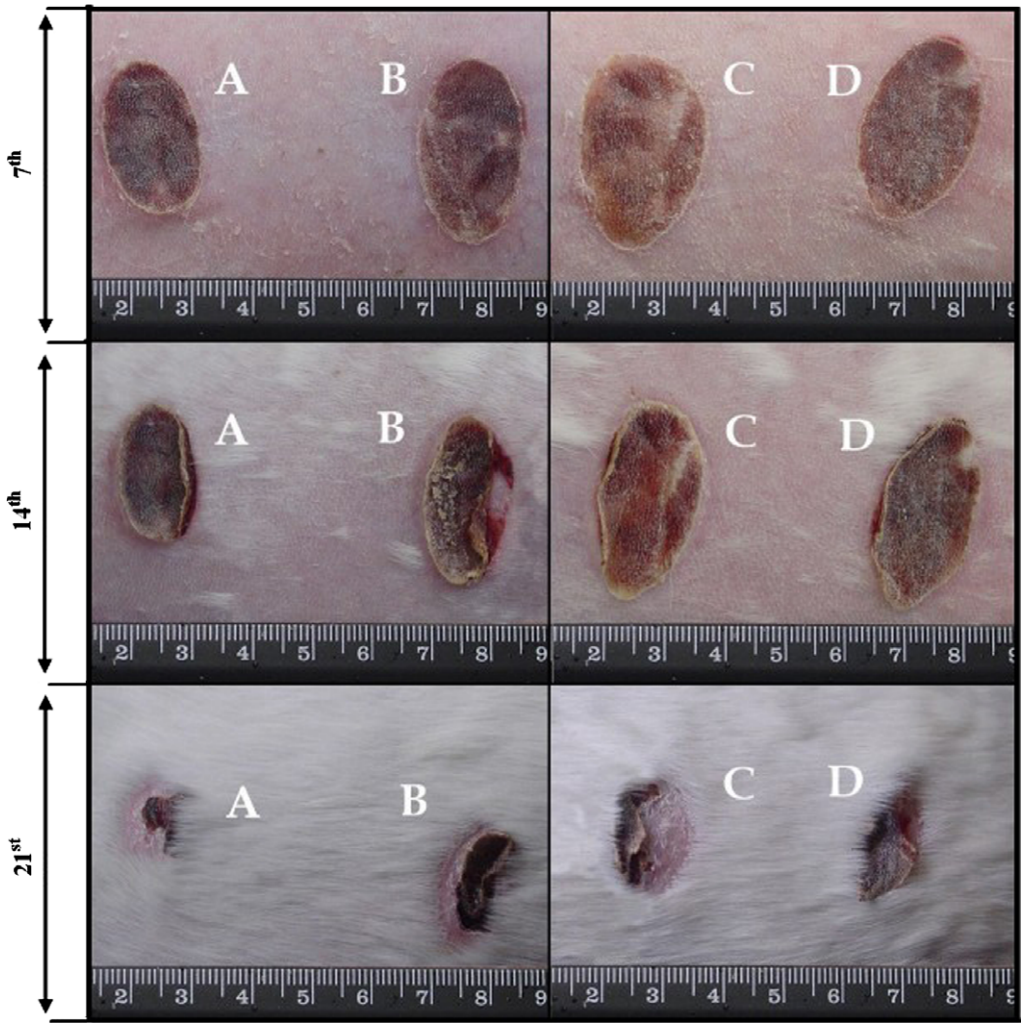

Fig. 3. The photographs of the wound areas treated with fucosphere (A), fucoidan solution (B), chitosan microsphere (C) and control wounds (D) at days 7, 14 and 21.

by means of contraction area and amount of hair growth up to day seven (Fig. 3). The findings at day 14 did not reveal any edema in any of the groups including the control. The degree of contraction area and level of healing is given by the following sequence in descending order: fucospheres > chitosan microspheres ⩾ fucoidan solution > control. No edema and inflammation were observed in the early phase of the dermal burn healing in the groups excluding controls due to antibacterial and anti-inflammatory effect of chitosan and fucoidan as reported in the previous studies [31–33]. On the other hand, when 21-day macroscopic findings were examined, the highest level of healing was noted in groups treated with fucosphere (Fig. 3). Histopathological evaluation parameters of the wounds are given in Fig. 4. While epithelial lengths in all groups increased by time, mean epithelial lengths of fucosphere treated groups reached the highest value (2733 μm) with a length difference of 1367 μm between days 7 and 14, and epithelial length increase slowed between the days 14 and 21 (Fig. 5) (Table 2). Epithelium thickness values of the fucosphere treated groups demonstrated a similar profile as that of epithelial length up to day 14, and epithelium thickness values of fucosphere were shown to be higher than that of other groups by day seven (193 μm) (Table 2) ($P < 0.05$). These changes in epithelial length and thickness observed on days 7 and 14 were considered to be related to increases in fibroblast, collagen and epithelial cells (Table 3). It was observed that fibroblast and collagen amounts in fucosphere treated groups had increased at day 14 compared to that at day seven, but decreased at day 21. The results of increased fibroblast migration and collagen synthesis with accelerated epithelial regeneration obtained from this study appeared to be similar to those reported earlier (Table 3) [34,35]. Again, the effect of fucoidan on the relationship between fibroblast migration and tissue repair on wound site was investigated and fucoidan was reported to be suitable for use in wound healing due to its binding to fibroblasts and to its relation

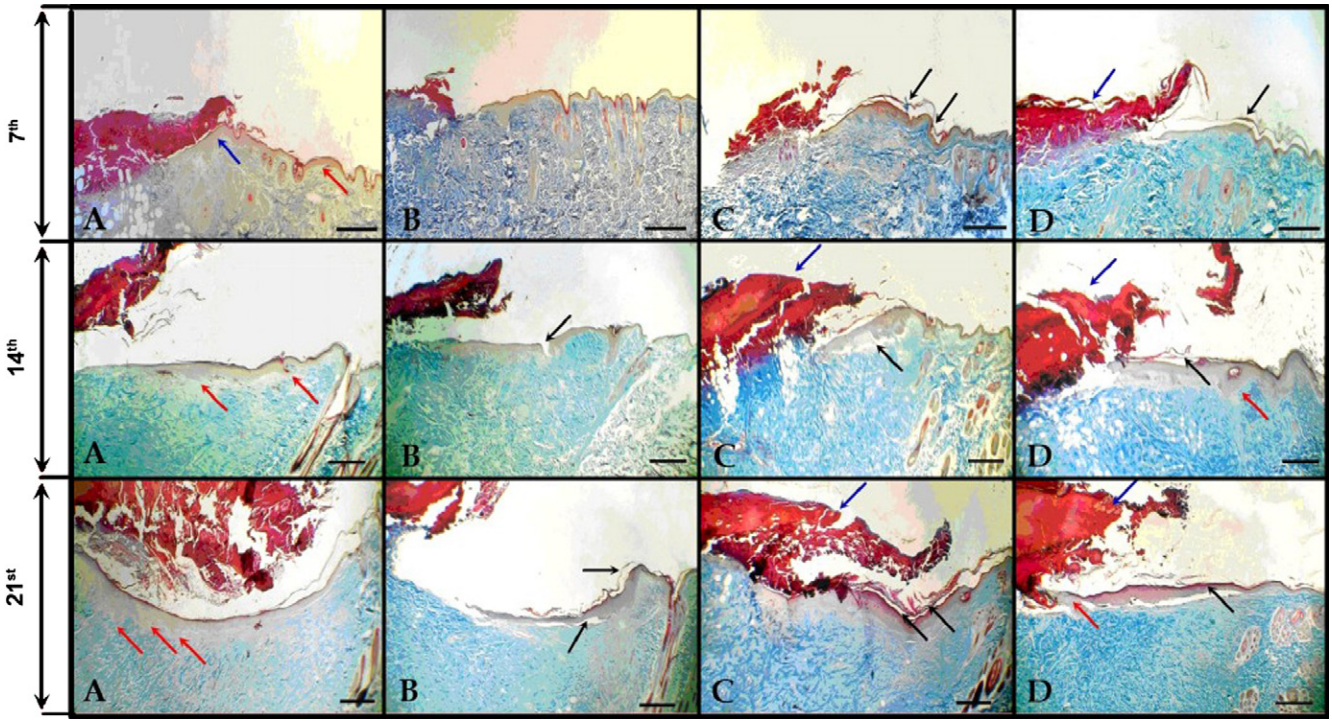

Fig. 5. The histopathological photographs of the burn epithelial tissues stained with haematoxylin & eosin at days 7, 14, and 21 (A, fucosphere; B, fucoidan solution; C, chitosan microsphere treated; and D, control wounds; the arrows were shown: rete pegs (red), elevation of epithelium from dermis (black), scars (blue), the bars are 500 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Wound epithelial elongation and thickness values at days 7, 14 and 21 after treatment

Days	Wound epithelial elongation values (μm ± SE)				Wound epithelial thickness values (μm ± SE)			
	Fucosphere group	Fucoidan solution group	Chitosan microsphere group	Control group	Fucosphere group	Fucoidan solution group	Chitosan microsphere group	Control group
7	1366 ± 44	1455 ± 64	1358 ± 57	1302 ± 90	193 ± 15	121 ± 8	118 ± 6	111 ± 10
14	2733 ± 213	2086 ± 134	2316 ± 244	1950 ± 82	220 ± 27	187 ± 9	119 ± 14	154 ± 5
21	3275 ± 730	3586 ± 149	3250 ± 319	3533 ± 196	157 ± 13	146 ± 10	123 ± 15	134 ± 8

*All results were means of the wounds that belong to six rabbits.

Table 3
The histological changes graded from absent to severe in the four groups at days 7, 14 and 21

Histological alteration	7th day number (percentage)				14th day number (percentage)				21st day Number (percentage)			
	Fucosphere group	Fucoidan solution group	Chitosan microsphere group	Control group	Fucosphere group	Fucoidan solution group	Chitosan microsphere group	Control group	Fucosphere group	Fucoidan solution group	Chitosan microsphere group	Control group
Bleeding												
Grade												
0	1 (16.7)	1 (16.7)	0 (0.0)	0 (0.0)	4 (66.7)	4 (66.7)	2 (33.3)	0 (0.0)	5 (83.3)	6 (100.0)	4 (66.7)	3 (50.0)
1	4 (66.7)	4 (66.7)	6 (100.0)	3 (50.0)	2 (33.3)	2 (33.3)	4 (66.7)	5 (83.3)	1 (16.7)	0 (0.0)	2 (33.3)	2 (33.3)
2	1 (16.7)	1 (16.7)	0 (0.0)	3 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)
3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Fibroblast proliferation												
Grade												
0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (50.0)	6 (100.0)	0 (0.0)	0 (0.0)
1	6 (100.0)	0 (0.0)	6 (100.0)	6 (100.0)	0 (0.0)	1 (16.7)	4 (66.7)	4 (66.7)	3 (50.0)	0 (0.0)	1 (16.7)	4 (66.7)
2	0 (0.0)	6 (100.0)	0 (0.0)	0 (0.0)	5 (83.3)	5 (83.3)	2 (33.3)	2 (33.3)	0 (0.0)	0 (0.0)	4 (66.7)	1 (16.7)
3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (16.7)
Collagen formation												
Grade												
0	3 (50.0)	1 (16.7)	1 (16.7)	5 (83.3)	0 (0.0)	0 (0.0)	0 (0.0)	4 (66.7)	3 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)
1	3 (50.0)	5 (83.3)	5 (83.3)	1 (16.7)	2 (33.3)	3 (50.0)	6 (100.0)	2 (33.3)	3 (50.0)	4 (66.7)	3 (50.0)	3 (50.0)
2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (66.7)	3 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	3 (50.0)	3 (50.0)
3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Mononuclear leukocyte												
Grade												
0	4 (66.7)	5 (83.3)	6 (100.0)	5 (83.3)	0 (0.0)	2 (33.3)	4 (66.7)	4 (66.7)	5 (83.3)	4 (66.7)	3 (50.0)	0 (0.0)
1	2 (33.3)	1 (16.7)	0 (0.0)	1 (16.7)	3 (50.0)	4 (66.7)	2 (33.3)	2 (33.3)	1 (16.7)	2 (33.3)	3 (50.0)	6 (100.0)
2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Polymorphonuclear leukocyte												
Grade												
0	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	6 (100.0)	4 (66.7)	4 (66.7)	0 (0.0)	6 (100.0)	4 (66.7)	6 (100.0)	5 (83.3)
1	1 (16.7)	4 (66.7)	2 (33.3)	2 (33.3)	0 (0.0)	2 (33.3)	2 (33.3)	3 (50.0)	0 (0.0)	2 (33.3)	0 (0.0)	1 (16.7)
2	5 (83.3)	0 (0.0)	4 (66.7)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
3	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

0, absent; 1, mild; 2, moderate; 3, severe.

Table 4
The number of rete pegs and NORs at days 7, 14 and 21 after treatment

Days	Number of rete pegs ± SE				Number of NORs ± SE			
	Fucosphere group	Fucoidan solution group	Chitosan microsphere group	Control group	Fucosphere group	Fucoidan solution group	Chitosan microsphere group	Control group
7	4.8 ± 0.4	3.1 ± 0.4	3.0 ± 0.3	3.4 ± 0.5	3.3 ± 0.1	2.9 ± 0.2	2.9 ± 0.1	2.7 ± 0.2
14	6.8 ± 0.5	6.1 ± 0.2	5.0 ± 0.5	3.9 ± 0.2	5.5 ± 0.1	3.6 ± 0.5	4.2 ± 0.1	2.6 ± 0.2
21	5.3 ± 1.0	4.6 ± 0.7	2.3 ± 0.3	3.8 ± 0.4	2.6 ± 0.1	3.5 ± 0.8	4.3 ± 0.1	2.6 ± 0.3

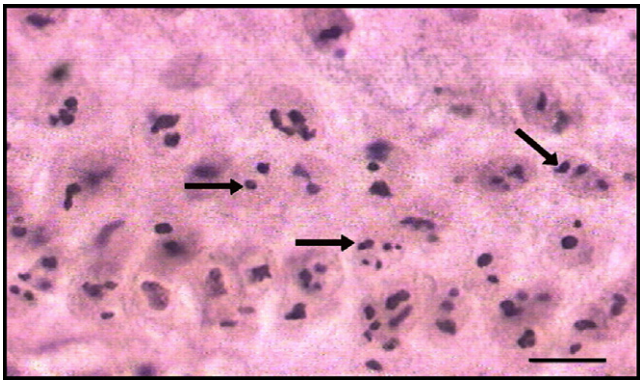

Fig. 6. The NOR dots of the epithelial cells of the wound areas treated with fucosphere at day 14 (the arrows show NOR dots, the bar is 20 μm).

with various growth hormones and cytokines [10,36]. Chitosan was also demonstrated to accelerate re-epithelization by accelerating infiltration on the wound site and providing fibroplasia [5–7]. Therefore, the higher treatment efficacy observed with fucospheres prepared using fucoidan and chitosan compared to other groups was suggested to be the result of the synergic effect provided by these two polymers.

At the dermal–epidermal junction, the contour of the bottom of the epidermis is irregular with numerous projections known as rete pegs or finger like structures (Fig. 4). These projections help to anchor the epidermis to the dermis [37,38]. Our results demonstrated that the number of the rete pegs in fucosphere treated groups was higher than the other groups through the course of therapy ($P < 0.05$) and reached highest level (6.8 ± 0.5) by day 14 (Table 4 and Fig. 5).

The highest NOR value (5.5 ± 0.1) was also observed on day 14, on the fucosphere administered groups when all groups were compared ($P < 0.05$) (Table 4 and Fig. 6). As a result, data obtained proved that fucospheres could increase the healing and re-epithelization of the wound between days 7 and 14.

4. Conclusions

The *in vitro* and *in vivo* studies investigating the efficacy of fucospheres in the treatment of experimental dermal burns demonstrated that the use of chitosan in the treatment of burns was due to its ability to re-epithelize and encourage fibroblast migration. Although fucoidan solution alone was not as effective as chitosan, the burn healing effect of fucoidan–chitosan microspheres was observed to be better than chitosan microspheres. In conclusion, fucospheres may be suitable as a wound coating material and can be used in wound healing.

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References

[1] A.F.P.M. Vloemans, A.M. Soesman, R.W. Kreis, E. Middelkoop, A newly developed hydrofibre dressing in the treatment of partial-thickness burns, Burns 27 (2001) 167–173.
[2] R.L. Sheridan, R.G. Tompkins, Skin substitutes in burns, Burns 25 (1999) 97–103.
[3] M. Balasubramani, T.R. Kumar, M. Babu, Skin substitutes: a review, Burns 27 (2001) 534–544.
[4] L.G. Griffith, Polymeric biomaterials, Acta Mater. 48 (2000) 263–277.
[5] Y. Usami, S. Minami, Y. Okamoto, A. Matsuhashi, Y. Shigemasa, Influence of chain length of *N*-acetyl-D-glucosamine and D-glucosamine residues on direct and complement-mediated chemotactic activities for canine polymorphonuclear cells, Carbohydr. Polym. 32 (1997) 115–122.
[6] H. Ueno, H. Yamada, I. Tanaka, N. Kaba, M. Matsuura, M. Okumura, T. Kadosawa, T. Fujinaga, Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs, Biomaterials 20 (1999) 1407–1414.
[7] G.I. Howling, P.W. Dettmar, P.A. Goddard, F.C. Hampson, M. Dornish, E.J. Wood, The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes *in vitro*, Biomaterials 22 (2001) 2959–2966.
[8] J. Giraux, J. Tapon-Bretaudiere, S. Matou, A.M. Fischer, Fucoidan, as heparin, induces tissue factor pathway inhibitor release from cultured human endothelial cells, Thromb. Haemost. 80 (1998) 692–695.
[9] S. Hideyuki, N. Masato, K. Toshiko, S. Haruji, H. Hidesuke, Y. Terou, Complex of fibroblast growth factor, Japan Patent (JP) application no: JP/1996/10017498.
[10] T. Fujimura, Y. Shibuya, S. Moriwaki, K. Tsukahara, T. Kitahara, T. Sano, Y. Nishizawa, Y. Takema, Fucoidan is the active component of *Fucus vesiculosus* that promotes contraction of fibroblast-populated collagen gels, Biol. Pharm. Bull. 23 (2000) 1180–1184.
[11] R. O'Leary, M. Rerek, E.J. Wood, Fucoidan modulates the effect of transforming growth factor (TGF)-β_1 on fibroblast proliferation and wound repopulation in *in vitro* models of dermal wound repair, Biol. Pharm. Bull. 27 (2004) 266–270.
[12] A.D. Sezer, Fucoidan multiparticulate drug carrier systems, Patent Cooperation Treaty (PCT) and World Intellectual Property Organization (WIPO) application no: PCT/TR05/00015, WO/2006/091180.
[13] A.D. Sezer, J. Akbuğa, Fucosphere – New microsphere carriers for peptide and protein delivery: preparation and *in vitro* characterization, J. Microencapsul. 23 (2006) 513–522.

[14] A. Berthold, K. Cremer, J. Kreuter, Preparation and characterization of chitosan microspheres as drug carriers for prednisolone sodium phosphate as model for anti-inflammatory drugs, J. Control. Release 39 (1996) 17–25.
[15] S. Kockisch, G.D. Rees, S.A. Young, J. Tsibouklis, J.D. Smart, Polymeric microspheres for drug delivery to the oral cavity: an *in vitro* evaluation of mucoadhesive potential, J. Pharm. Sci. 92 (2003) 1614–1623.
[16] J.S. Knabl, W. Bauer, H. Andel, I. Schwendenwein, P.F. Dado, M. Mittlböck, W. Römer, M.S.S. Choi, R. Horvat, G. Meissl, M. Frey, Progression of burn wound depth by systemical application of a vasoconstrictor: an experimental study with a new rabbit model, Burns 25 (1999) 715–721.
[17] D. Trere, AgNOR staining and quantification, Micron 31 (2000) 127–131.
[18] A. Polk, B. Amsden, K. De Yao, T. Peng, M.F. Goosen, Controlled release of albumin from chitosan–alginate microcapsules, J. Pharm. Sci. 83 (1994) 178–185.
[19] V.R. Sinha, A.K. Singla, S. Wadhawan, R. Kaushik, R. Kumria, K. Bansal, S. Dhawan, Chitosan microspheres as a potential carrier for drugs, Int. J. Pharm. 274 (2004) 1–33.
[20] A.D. Sezer, F. Hatipoğlu, Z. Oğurtan, A.L. Baş, E. Cevher, J. Akbuğa, New nanosphere system for treatment of full-thickness burn on rabbit, 31th FEBS Congress, June 24–29, 2006, p. 185, Istanbul, Turkey.
[21] S.Y. Lin, K.S. Chen, L.R. Chu, Design and evaluation of drug-loaded wound dressing having thermoresponsive, adhesive, absorptive and easy peeling properties, Biomaterials 22 (2001) 2999–3004.
[22] L. Chevolot, A. Foucaulta, S. Colliec-Jouaulta, J. Ratiskola, C. Sinquina, Improvement purification of sulfated oligofucan by ion-exchange displacement centrifugal partition chromatography, J. Chromatogr. A 869 (2000) 353–361.
[23] A. Grenha, B. Seijo, C. Remunan-Lopez, Microencapsulated chitosan nanoparticles for lung protein delivery, Eur. J. Pharm. Sci. 25 (2005) 427–437.
[24] Y. Hu, X. Jiang, Y. Ding, H. Ge, Y. Yuan, C. Yang, Synthesis and characterization of chitosan-poly(acrylic acid) nanoparticles, Biomaterials 23 (2002) 3193–3201.
[25] C. Larez, F. Canelon, E. Milan, I. Katime, Interpolymeric complexes of poly (itaconic acid) and chitosan, Polymer Bull. 48 (2002) 361–366.
[26] Y. Zheng, Y. Wu, W. Yang, C. Wang, S. Fu, X. Shen, Preparation, characterization, and drug release in vitro of chitosan–glycyrrhetic acid nanoparticles, J. Pharm. Sci. 95 (2006) 181–191.
[27] A. Zhu, M. Zhang, J. Wu, J. Shen, Covalent immobilization of chitosan/heparin complex with a photosensitive hetero-bifunctional crosslinking reagent on PLA surface, Biomaterials 23 (2002) 4657–4665.
[28] X.H. Wang, D.P. Li, W.J. Wang, Q.L. Feng, F.Z. Cui, Y.X. Xu, X.H. Song, Covalent immobilization of chitosan and heparin on PLGA surface, Int. J. Biol. Macromol. 33 (2003) 95–100.
[29] C.M. Lehr, J.A. Bouwstra, E.H. Schacht, H.E. Junginger, *In vitro* evaluation of mucoadhesive properties of chitosan and some other natural polymers, Int. J. Pharm. 78 (1992) 43–48.
[30] K.P. Chowdary, Y.S. Rao, Mucoadhesive microspheres for controlled drug delivery, Biol. Pharm. Bull. 27 (2004) 1717–1724.
[31] M.I. Bilan, A.A. Grachev, N.E. Ustuzhanina, A.S. Shashkov, N.E. Nifantiev, A.I. Usov, Structure of a fucoidan from the brown seaweed *Fucus evanescens* C.Ag, Carbohydr. Res. 337 (2002) 719–730.
[32] K. Miyatake, Y. Okamoto, Y. Shigemasa, S. Tokura, S. Minami, Anti-inflammatory effect of chemically modified chitin, Carbohydr. Polym. 53 (2003) 417–423.
[33] H. Sashiwa, S. Aiba, Chemically modified chitin and chitosan as biomaterials, Prog. Polym. Sci. 29 (2004) 887–908.
[34] G. Erdağ, R.L. Sheridan, Fibroblasts improve performance of cultured composite skin substitutes on athymic mice, Burns 30 (2004) 322–328.
[35] M.J. Eichler, M.A. Carlson, Modeling dermal granulation tissue with the linear fibroblast-populated collagen matrix: a comparison with the round matrix model, J. Dermatol. Sci. 41 (2006) 97–108.
[36] J.L. Giraux, J. Tapon-Bretaudiere, S. Matou, A.M. Fischer, Fucoidan, as heparin, induces tissue factor pathway inhibitor release from cultured human endothelial cells, Thromb. Haemost. 80 (1998) 692–695.
[37] Z. Oğurtan, F. Hatipoğlu, C. Ceylan, The effect of *Alkanna tinctoria* tausch on burn wound healing in rabbits, Dtsch. Tierarztl. Wschr. 109 (2002) 481–485.
[38] H. Matsumura, N. Yoshizawa, T. Kimura, K. Watanabe, N.S. Gibran, L.H. Engrav, A burn wound healing model in the hairless descendant of the Mexican hairless dog, J. Burn Care Rehab. 18 (1997) 306–312.