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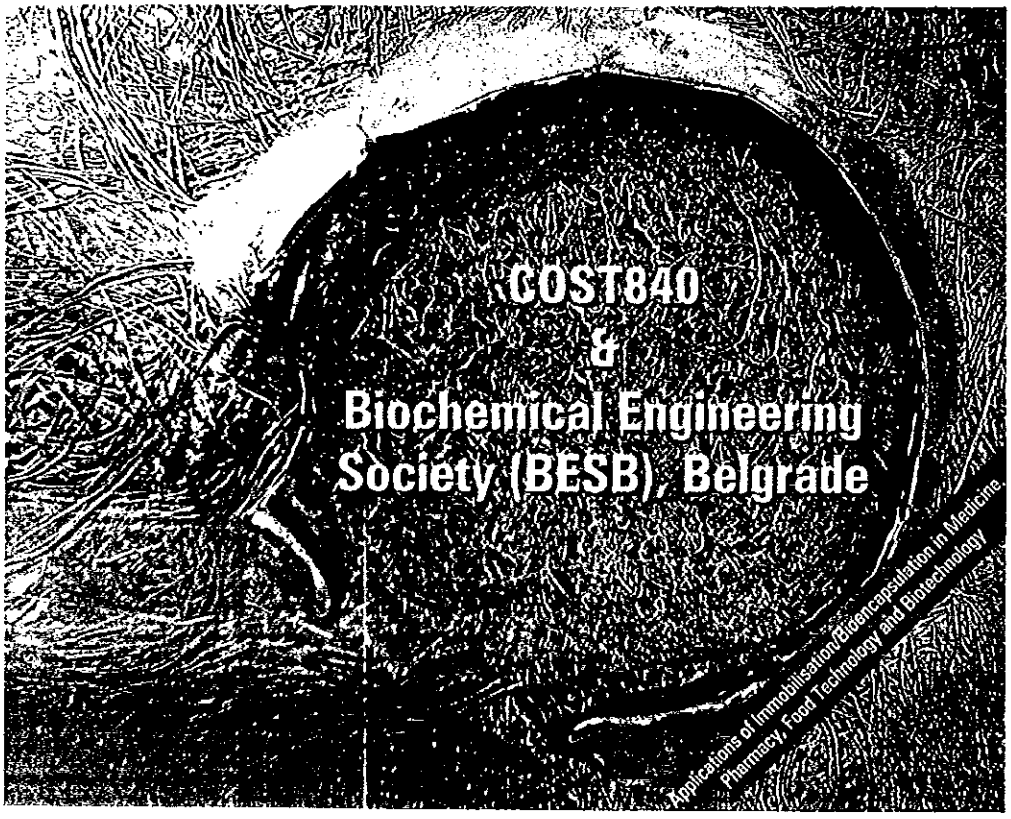

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TABLE OF CONTENTS

12 APPLICATION OF BIOENCAPSULATED PROTEINASES AND PEPTIDES FOR WOUND HEALING

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1. Introduction

Tissue repair remains a central problem in cell biology and represents one of the most complicated challenges in medicine [1]. Wound healing is a complex dynamic process involving an integrated action of a number of cell types, cytokines and the extracellular matrix. It includes a series of successive but overlapping phases needed for complete healing. The phases are as follows: 1) an initial inflammatory response accompanied by accumulation of neurophils, monocytes and lymphocytes in the wound area; 2) a proliferative (new-tissue formation) phase in which fibroblasts, endothelial and other cell types migrate into the wound, where they begin to proliferate, to secrete matrix material, and to form functional microvessels while epithelial cells migrate as a layer over the open wound surface; and 3) a maturation phase (tissue remodeling) when collagen and other matrix materials are secreted and remodeled to restore tissue strength [2].

D. W. Thomas (ed.), Advanced Biomaterials for Medical Applications, 165–175.

Thrombin is a serine proteinase which belongs to trypsin family and plays a central role in blood coagulation mechanism. Additionaly, it has a numerous biological functions that are related to inflammation, tissue remodeling and healing. Thrombin is concentrated at the sites of vascular injury in the fibrin clots and is protected from inhibitors. With time it is gradually released into the wound and regulates a number of the phases of tissue repair [3-5]. Thrombin increases the adherence of neutrophils, monocytes and lymphocytes to capillary endothelial cells. It can also enhance vascular permeability and transmigration of inflammatory cells to tissue [6]. Moreover thrombin may stimulate the release of a number of growth/chemotactic factors e.g. platelet-derived growth factor (PDGF; released from platelets and endothelial cells following thrombin treatment) [7,8]. Thrombin has been shown to directly affect migration, proliferation and cellular ability to secrete other growth factors and inflammatory mediators [3, 9]. The fibrin network formation provides a matrix to support cell migration, adhesion and proliferation within the wound [10].

To date there are at least 2 formulations based on bovine thrombin on the market for wound healing. These are fibrin glue or sealant (Immuno, Austria) [11] and Tachocomb (Nycomed). Fibrin glue can be prepared directly on the wound surface by mixing freshly prepared fibrinogen and thrombin solutions in the presence of some additives (calcium ions, some adhesive proteins and inhibitors of fibrinolysis). This procedure may represent thrombin bioencapsulation into the fibrin network; serving as a scaffold for growing fibroblasts and other cells. It should be noted that these compounds are expensive due to their isolation and prufication from fibrinolytics and virus contaminations. Another approach would be to employ thrombin via its bioencapsulation (entrapment) into hydrogel films [12]. There are two specific advantages of such a strategy. Firstly the ability to stabilize thrombin activity and, secondly, providing thrombin controlled release from the polymer dressings. The interaction of thrombin with cells is mediated by thrombin binding to a seven-transmembrane domain, G-protein-coupled cell surface receptor which belongs to the PAR family (protease activated receptors). Three receptors of four representatives of this family, namely PAR-1, PAR-3 and PAR-4, can be activated by thrombin. The fourth one needs thrypsin or tryptase secreted by mast cells to be activated.

Briefly, the activation mechanism is as follows. Thrombin posesses the ability to cleave the peptide bond (e.g. Arg 41-Ser 42 bond in humans) in the extracellular domain of the receptor and expose a new N-terminus. This newly generated N-terminus acts as a "tethered ligand" which activates the receptor by binding to its second extracellular loop; inducing intracellular signal transduction [13-14]. Synthetic peptides which are homologous in their structure with tethered ligands are called PAR agonists. These peptides are known to mimic some of thrombin activities in terms of wound healing. In our research our attention has been focused on 2 peptides, namely TRAP-6

(thrombin receptor agonist peptide) which is PAR-1 agonist (ag-PAR-1), and PAR –2 agonist (ag-PAR-2). Both TRAP-6 and ag-PAR-2 contain 6 amino acids in their structures which are SFLLRN and SLIGKV, respectively. Synthetic peptides which can replace these functions of thrombin in clinical wound healing could potentially be cheaper, more stable, and may be more promising than thrombin for clinical use.

Peptides bioencapsulated in polymer matrices (films, microparticles) can therefore, be envisaged as an effective approach to the development of new bioactive dressings for wound therapy. Via the replacement of thrombin with synthetic peptides one can avoid all disadvantages of thrombin, whilst gainng benefits related to the unique peptide ability to stimulate wound healing.

2. Preparation of polymer dressings with bioencapsulated thrombin and peptides

In our research we proposed entrapment of thrombin or peptides in composite hydrogel dressings (films). These polymer matrices are based on biocompatible synthetic polymers, such as poly(N-vinyl caprolactam), (PVCL), poly-L-lysine, and natural polymers (alginate, chitosan).

Temperature-sensitive poly(N-vinyl caprolactam) has been selected due to an unique combination of its properties (water solubility and biocompatibility) and, moreover, its ability to produce hydrogels at physiological conditions (i.e. pH, and temperature) with simultaneous mild entrapment of proteins or cells. PVCL phase separation together with simultaneous protein or cell bioencapsulation into forming hydrogels can easily be achieved by adding dropwise polymer solution containing protein/cells to previously heated (37-39° C) water medium [13]. Earlier PVCL-based hydrogels were successfully applied by us for animal cell immobilization [15] as well as for entrapment of various enzymes, such as trypsin, chymotrypsin, carboxypeptidase B [16]. Two natural polysaccharides, namely alginate and chitosan which have been widely employed in various biomedical fields, have been proposed as other components of composite polymer dressings. The use of these materials could allow to improve the properties of polymer dressings in terms of their biocompatibility, mechanical stability, to prolong peptide release from the dressing, finally, they could result in increasing positive effect of bioencapsulated thrombin and peptides on wound healing.

3. Study of the effects of thrombin or peptides bioencapsulated in polymer dressings on wound healing

To study the effect of polymer dressings with entrapped thrombin or peptides a series of experiments in mouse and a rat models have been carried out. The dressings were applied to a back wound of experimental animals (1x1 cm^2 in the case of mice and 2x3 in a rat model). Control animals were treated with dressings without thrombin/peptides (blank films). The healing process was estimated using several criteria, such as : 1) study of histologic samples of granulation tissue (on day 3 and day 7 after wounding) and 2) determination of wound size (also on day 3 and day 7). As mentioned above, the tissue repair process includes 3 phases, namely inflammation, new-tissue formation (proliferative) and tissue remodelling. The inflammation phase proceeds about 3 days and is characterized by a large number of polymorphonuclear leucocytes and some other cells. All the changes that occur to cells during the proliferative phase have been thoroughly described recently [17]. In the proliferative (new-tissue formation) phase one can observe an increasing number of fibroblasts. Fibroblasts are the principal cell type in the dermis, although others such as histocytes, lymphocytes and mast cells can be present. These fibroblasts which are responsible for the synthesis of all the dermal extracellular matrix components and for a number of the enzymes which degrade and remodel the extracellular matrix macromolecules. Fibroblast can be observed in the wound area already on day 3 after injuring, but its number reaches maximum value on day 7. The dermal vasculature is composed of endothelial cells. These tightly linked cells form a branching, three-dimensional network of blood vessels that penetrate the dermis and supports both fibroblast and keratinocyte activities.

To study these phenomena in this model system we measured wound size directly and studied granulation tissue samples to estimate proliferation activities of various cells (macrophages, fibroblasts, endothelial and epithelial cells) twice during wound healing process (day 3 and day 7 post injury). Data were means from the number of various proliferating cells in granulation tissue patterns from wounds and were assessed by radioautography [18]. As can be seen from Table 1, the best results were obtained for granulation tissue samples taken from wounds coated with polymer dressings containing TRAP-6.

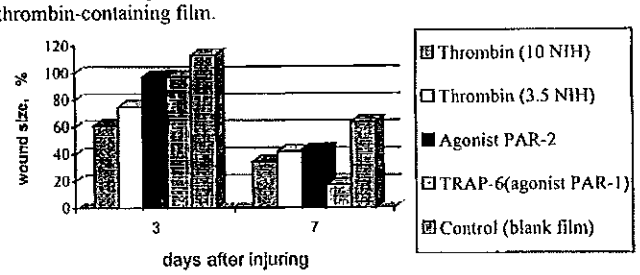
TABLE I. The effect of composite PVCL-Ca alginate (PVCL-AlgCa) hydrogel dressings with entrapped thrombin/peptides on cell number in granulation tissue patterns.

Dressings with bioencapsulated thrombin or peptides	Fibroblast: Macrophage	Cell number/field		
		fibroblasts	Epithelial cells	Endothelial cells
Thrombin (10 NIH)	3.7	3.0	19.0	1.3
Thrombin (3.5 NIH)	0.7	1.8	12.7	0.9
PAR-1 agonist (TRAP-6)	5.8	2.8	20.0	1.8
PAR-2 agonist	0.7	2.3	10.1	1.4
Control film	1.0	2.5	13.4	1.0

The data are means from 3 to 5 independent experiments

The changes in wound size in the case of various polymer dressings with thrombin or peptide entrapped in the mouse model are demonstrated in Fig.1. On day 7 after injuring the smallest wound size was observed in the case of PVCL-CaAlg dressing with TRAP-6 entrapped (17 % compared to initial wound size of 100 %). At the same time for the control animal group (dressing - thrombin/peptides) the wound size was larger (63 %).

On day 7 the wound under TRAP-containing dressing was approximately twice those under thrombin-containing coating and approx. 3.7 times smaller than the wounds in the control group (dressings without thrombin or peptides). Thus, the TRAP-6-containing dressing accelerated wound healing even over that seen in a thrombin-containing film.



FFig.1. Wound sizes under PVCL-CaAlg dressings with entrapped thrombin/peptides in a mouse model.. Initial wound size just after injury was taken as 100%. The data are means from 3 to 5 independent experiments.

For the next series of experiments we utilised TRAP-6, and have modified the dressing surface with an upper layer to prolong the peptide release from the dressings. Two positively charged polymers, namely chitosan and poly-L-lysine have been selected; both being widely used in biomedical fields.

Chitosan is reported to increase fibroblast proliferation *in vitro* [19]. Both polymers easily interact with negatively charged alginate giving polyelectrolyte membrane (the upper layer) on the surface of PVCL-CaAlg dressing. As can be seen from Table 2, on day 7 the relative wound size in case of PVCL-CaAlg-chitosan dressing with TRAP-6 was about 16 %, what was 1.6 times smaller than that for the control PVCL-CaAlg-chitosan film without TRAP-6 (26 %) and for poly-L-lysine-containing dressing with TRAP (25%). Histological examinations of granulation tissue samples from the wounds coated with PVCL-CaAlg-chitosan and with PVCL-CaAlg–poly-L-lysine dressings demonstrated

TABLE 2. The effect of PVCL-CaAlg dressings with chitosan or poly-L-lysine upper layer on wound size.

Parameters of wound healing	PVCL-CaAlg dressings with poly-L-lysine upper layer		PVCL-CaAlg dressings with Chitosan upper layer	
	TRAP-6	*Control**	TRAP-6	*Control**
3 days after injuring				
Relative wound size, % **	93.6	77.0	**64.6**	79.3
Fibroblasts/macrophages ratio	0.75	0.31	**0.78**	0.35
7 days after injuring				
Relative wound size, % **	24.9	31.2	**16.2**	26.2
Fibroblasts/macrophages ratio	7.85	1.75	**5.9**	1.63

*The dressings without peptide were used as a controls.

** Initial wound size was taken as 100% . The data were means from 3 to 5 independent experiments.

that all parameters were significantly enhanced. Light microscopy of control granulation tissue samples revealed differences in granulation tissue formation between e.g. in PVCL-CaAlg-chitosan -TRAP-6 and control PVCL-CaAlg-chitosan (without TRAP-6) dressings. Thus, for the control sample (dressing without TRAP-6) on day 7 we observed that the granulation tissue exhibited many macrophages and few neutrophils. In contrast to this picture the sample covered with TRAP-6-containing dressing had developed a keratinised

epithelial covering and well-developed tissue with evidence of organisation of the extracellular matrix [20].

4. PREPARATION OF PLGA MICROPARTICLES WITH BIOENCAPSULATED TRAP-6

Currently polymer matrices based on biocompatible biodegradable lactic acid (PLA) or poly(lactic-co-glycolic) acid microparticles (PLGA) are widely employed for bioencapsulation of a wide spectrum of therapeutic agents, including proteins and peptides [21-24].

Bioencapsulation of TRAP-6 in biodegradable poly(D,L-lactide-co-glycolide) (PLGA) microparticles opens the possibility to use these peptide delivery devices for wound healing. Preparation technique of PLGA microparticles with bioencapsulated TRAP-6 and the evaluation of their some physico-chemical properties (microparticle size distribution, structure etc) have been reported recently [25].

5. Study of the effect of PLGA microparticles with bioencapsulated TRAP-6 on wound healing

The animal studies of healing on day 3 and day 7 after injury (Fig.2,3) revealed marked reductions in macrophage number in the test versus the control animals. In contrast with this the TRAP-containing microparticle treatment increased the fibroblast numbers in the wound bed. Since the fibroblast/macrophages ratio may indicate the proliferation/ inflammation process it would appear that they may havce the potential to stimulate wound healing.

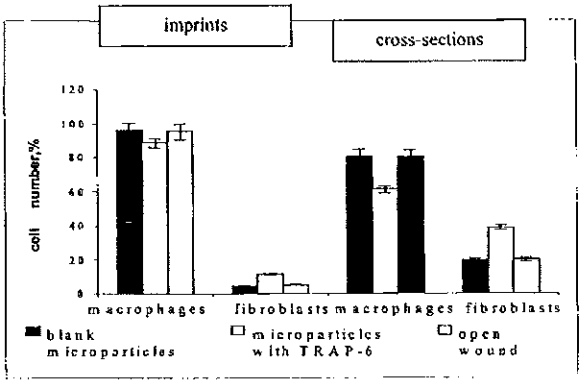


Fig.2. Macrophages and fibroblasts in the wound on day 3. The data were means from 3 to 5 independent experiments.

Moreover, in granulation tissue cross-sections we observed 7-8 layers of keratinized epithelium in case of the wounds covered with TRAP-6-contaning dressings and 4/5 layers for the control and untreated wounds.

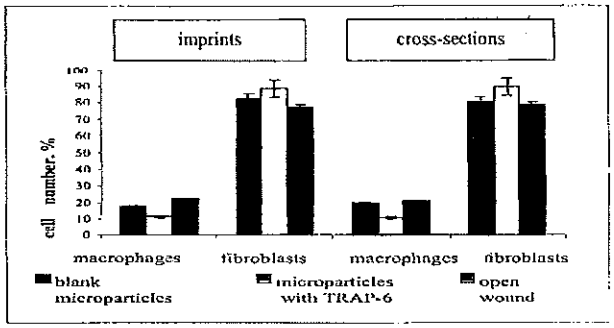


Fig.3. Macrophages and fibroblasts in the wound on day 7.

Does calculation of these cells really reveal the dynamics of wound healing process? In our research we established that changes in cell composition in imprints and cross-sections were similar. We also employed imprints to assess the wound healing potential of these agents in addition to histological sections.

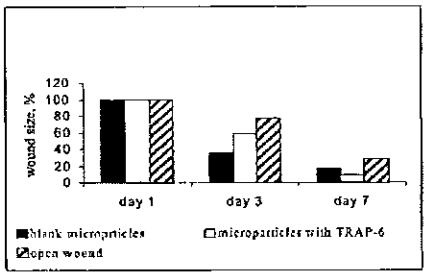


Fig.4. The effect of TRAP-6 encapsulated in PLGA microparticles on wound size. Initial wound size was taken as 100%. The data were means from 3 to 5 independent experiments.

As can be seen from Fig.4, on day 7 the wound size under microparticles with bioencapsulated TRAP-6 was half that observed in control- (8.5 and 15.6 % respectively) or untreated animals (28 %).

Summary

These data in an animal model demonstrate that thrombin or synthetic peptides bioencapsulated in PVCL-CaAlg polymer dressings or PLGA microparticles may be of therapeutic use for clinical situations. These data demonstrate the role of these agents in the inflammatory and proliferative phases of tissue repair in vivo. These data show that these agents may accelerate a number of wound healing processes in vivo. It is anticipated that the development of new formulations based on the proposed bioencapsulated peptides will contribute to a new generation of effective dressings with drug controlled release for wound therapy.

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