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Evaluation of the Use of Fibrin and Microcrystalline Chitosan Membranes as Carriers for Transforming Growth Factor Beta-1

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ABSTRACT: The aim of this study was to describe the mechanical features of homogeneous and layered chitosan (Ch) and fibrin–chitosan (Fb–Ch) membranes as well the kinetics of transforming growth factor beta-1 (TGF-β1) release from five types of polymer carriers. Composites in the form of a film containing physiologically clotted fibrin (Fb) and microcrystalline chitosan (MCCh) were prepared and then crosslinked with calcium chloride. The films were characterized by Infrared (IR) spectroscopy, mechanical tests (film thickness, maximal tensile force, breaking strength, and elongation at break), and SEM images. The results reveal that Ch film demonstrates higher efficiency in binding TGF-β1 and, at the same time, is less effective in its release—1.25% of the total amount between 6 h and 14 days. However, the Fb membrane binds TGF-β1 not as strongly, which leads to more effective release of the compound—25% after 6 h and 28.98% of the total amount after 14 days. The factor TGF-β1 is released *in vitro* from Fb–Ch membranes with different kinetics. The most efficient release of TGF-β1 was observed in the case of the layered Fb–Ch (M4 L) membrane (after 14 days it reached a maximal value of 14.08% of the total amount). The release was lower with increasing Ch concentrations in the film, suggesting a high affinity of TGF-β1 with the Fb–Ch component. The Fb–Ch membrane with incorporated TGF-β1 may prove to be a very useful scaffold in the tissue regeneration process. This study demonstrates that Fb and MCCh gels could be used as carrier matrices for the controlled release of bioactive TGF-β1. It was found that the degree of TGF-β1 release from the membrane is influenced by the physiochemical and mechanical characteristics of the films and by its affinity to growth factor TGF-β1. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: fibrin–chitosan membranes; mechanical tests; transforming growth factor beta-1; kinetics of TGF-β1 release

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INTRODUCTION

The tissue regeneration process can be accelerated by membranes soaked with different protein factors; their effectiveness is strictly dependent on four elements: the structure of the factors, the kinetics of their release and degree of their penetration into the cell, as well as the framework of the carrier used.[1–3] To regenerate damaged bones and tissue, one may use biodegradable carriers with growth factors inserted,[4,5] which stimulate tissue healing and regeneration.

The loss or damage of organs and tissue due to aging or pathological conditions is a major human health problem.[6] Tissue engineering, a new field in biomedical science, combines cellular and molecular biology on the one hand, and material and mechanical engineering on the other, to provide an alternative to organ and tissue transplants from a limited supply of donors. Tissue formation is a very complex process, which involves the action of a variety of different factors such as hormones, cytokines, and growth factors—compounds that transmit signals, cause cells to migrate toward the damage site, and amplify the regeneration process. Among them, transforming growth factor beta-1 (TGF-β1) seems to be very important. Recombinant human TGF-β1 (rhTGF-β1) stimulation has been found to increase the amount and thickness of bone trabeculae.[7]

Our interest in the use of fibrin (Fb) and microcrystalline chitosan (MCCh) as a carrier of TGF-β1 rhTGF-β1 was first aroused by the wide range of practical applications of membranes in tissue engineering (organ damage) described in the work of Langer and Vacanti,[4] which also inspired further research. The

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use of ceramic composites and scaffolds in dentistry, orthopedics, and plastic surgery has been presented by Habraken et al.,[3] who showed that these materials are effective carriers for drugs whose release is strictly dependent on their chemical structure, type of drug, and drug loading. Their studies also used biodegradable polymers such as polylactic acid (PLA), gelatin, or Ch as matrices for ceramic particles or as adjuvants in calcium phosphate cements; the application of these polymers can improve biodegradation and drug release to the ceramic material. In our study, we measured the rate of release of TGF-β1 rhTGF from membranes made with Ch, Fb, or mixtures of the two with various polymer contents and preparation methods.

The type and concentration of growth factor, as well as proper carrier choice, have been the subject of numerous studies and scientific discussions.[3] However, there are also a number of other questions that remain to be answered regarding selective membrane choice (natural, synthetic, homogeneous, and layered), physiochemical properties (viscosity, resistance to deformation, porosity, and hygroscopicity), and the kinetics of growth factor release. There have also been attempts at using membranes (scaffolds) in studies on an animal (dog, rabbit, and rat) model.[7–9]

In the processes of embryogenesis and healing, tissue development is regulated by cytokines and requires the presence of proper carriers (scaffolds), which are crucial for the regeneration processes. Their practical application is limited by a few conditions: the scaffold must be biodegradable, allow reasonable cell adhesion, should provide sufficient mechanical support to withstand *in vivo* forces,[10–13] should be biocompatible, and it must not exhibit either cytotoxic or immunogenic properties.[14,15] A special requirement that a carrier has to meet is pore density, which must be high enough to guarantee infiltration of cells and their growth into tissue.

Among polymers used for scaffold production, the most suitable are collagen, Fb, Ch, heparin, alginates, silk, and hyaluronic acid,[14,16,17] which are both biocompatible and biodegradable. However, the most commonly used polymers are synthetic polymers such as polyglycolic acid, PLA, copolymers of glycolic and lactic acids (PLGA), polyhydroxybutyrate, and polyurethanes as well as natural polymers such as Ch, glycosaminoglycans, Fb, and collagen.

Chitosan was chosen as a biomaterial because of its biocompatibility, biodegradability, and nontoxicity.[18,19] It can be used as an individual compound in a carrier or in combination with other polymers and inorganic substances. MCCh is a special multifunctional polymeric material prepared by the aggregation of glucosamine macromolecules from an aqueous solution of organic acid.[20] It is the only form of polyaminosaccharide characterized by the presence of free unbound amine groups, which can be present in a liquid dispersion form with direct film-forming behavior. In the literature available, there are a number of reports on the application of MCCh in medicine, pharmacology, and other areas.[21–24] When a mixture of MCCh and another neutral polymer—alginate, methylcellulose, or Fb—is used in a membrane or hydrogel, a carrier of platelet derived growth factor (PDGF-AB), TGF-β, and basic fibroblast growth factor (bFGF) growth factors with a diverse release rate is obtained.[25,26] Previous studies did not include the impact of membrane structure on the mechanical properties and degree of release of growth factors. Therefore, it was advisable to produce membranes that differ in their preparation and percentage share of Fb and Ch in the membrane.

Fibrinogen[27,28] is a soluble plasma glycoprotein synthesized by the liver, which, in the presence of thrombin and calcium ions, is converted into Fb monomers. Fb has been used extensively as a biopolymer scaffold in tissue engineering.[17,29] It is a biopolymer that shows great potential in tissue regeneration and wound healing and can be used in surgery for patients with hemophilia. Fb is an effective hemostatic and wound dressing material that can be made in the form of a sponge, film, powder, or sheet. The stability of Fb hydrogel can be prolonged using a number of strategies: optimizing the pH, fibrinogen, and calcium ion concentration as well as creating a highly cross-linked, dense, and denaturated three-dimensional Fb matrix. Fb, either alone or in combination with other materials, has been used as a biological scaffold for stem or primary cells to regenerate adipose tissue, bone, cardiac tissue, cartilage, liver, nervous tissue, ocular tissue, skin, tendons, and ligaments.

This study demonstrates that Fb and MCCh gels could be used as carrier matrices for controlled release of bioactive TGF-β1 by adjusting the concentrations of Fb–Ch in the gels. In this article, an assessment is made of the impact of the physicochemical and mechanical properties of Ch, Fb, and fibrin–chitosan (Fb–Ch) membranes on the release of rhTGF-β1 from the polymer carrier.

EXPERIMENTAL

Materials

Fibrinogen, fraction I, Type I from human plasma was supplied by Sigma-Aldrich Chemical (St. Louis, MO). MCCh/LA Fg-90 (weight-average molecular weight $M_w = 2.8 \times 10^5$ Da) in the form of hydrogel of definite polymer content of 3.0 wt %, degree of deacetylation (DD) of 83.2%, and water retention value of 587% was prepared with the previously published unconventional method[20] of the Institute of Biopolymers and Chemical Fibres, Łódź, Poland. The DD, necessary to estimate the content of $-NH_2$ groups in the samples, was determined by the method of first derivative UV spectrophotometry, according to Khor and coworkers.[30]

rhTGF-β1 and human TGF-β1 Quantikine Immunoassay ELISA Kit were supplied by R&D System (Minneapolis, MN). Thrombin EC 3.4.4.13 was supplied by Biomed (Lublin, Poland). Factor XIIIa (Fb-stabilizing factor, FSF), fragment 72–97, and aprotinin from bovine lung 5 tissue inhibitor (TI) U mg^{-1} protein were supplied by Sigma-Aldrich Chemical. Aprotinin is a nonspecific protease inhibitor that inhibits plasmin function and, as a result, prevents plasmin-mediated activation of TGF-β1.[31] It is used as medication administered by injection to reduce bleeding during complex surgery such as heart and liver surgery.

Preparation of Polymer Carriers

Layered and mixed membranes with growth factor TGF-β1 were prepared from biodegradable MCCh and Fb polymers in aseptic conditions. For comparison, one set of polymer membranes was

Table I. Component of Chitosan, Fibrin–Chitosan, and Fibrin Membrane Systems

	Systems							
Components	M1 H	M1 L*	M2 L	M3 L	M3 M	M4 L	M4 M	M5 H
Chitosan (mg)	30	60	30	30	30	30	30	–
Fibrinogen (mg)	–	–	10	20	20	30	30	30
TGF-β1 (0.1 μg)	+	–	+	–	+	+	–	+
Aprotinin (20 μg)	+	–	+	–	+	+	–	+
FSF (1.0 μg)	–	–	+	+	+	+	+	+
Thrombin (16 NIH)	–	–	+	+	+	+	+	+

In all systems: $CaCl_2$ (1.11 mg), plasticizers: glycerol and propylene glycol in equal amounts (25 mg).

H, homogeneous; M, mixed; L, bilayer;
*L, triple layer.

prepared with Ch in the absence of Fb, whereas another was prepared with Fb in the absence of Ch. Two types of these membranes were produced: layered and homogeneous.

Chitosan. Homogenous Ch films (M1 H) (Table I) were prepared by pouring MCCh hydrogel (1 g) with 20 μL of $CaCl_2$ (0.5 mol L^{-1}) and plasticizers, glycerol and propylene glycol (GP), in equal amounts (25 mg) into a Teflon® well. While the constituents were being stirred, 100 μL of TGF-β1 (1 μg mL^{-1}) was quickly added in aseptic conditions. The films were then desiccated for 24 h in an incubator at 28°C once the solvent had evaporated.

Triple-layer polymeric Ch films (M1* L) (Table I) were prepared by pouring MCCh hydrogel (1 g), 20 μL of $CaCl_2$ (0.5 mol L^{-1}), and plasticizers, glycerol and GP, in equal amounts (25 mg) into a Teflon® well. The films were desiccated for 24 h in an incubator at 28°C once the solvent had evaporated. Finally, another two layers of MCCh hydrogel (0.5 g) with 20 μL of $CaCl_2$ (0.5 mol L^{-1}) and plasticizers, glycerol and GP, in equal amounts (25 mg) were poured on top of these films. The films were desiccated for 24 h in an incubator at 28°C once the solvent had evaporated.

Fibrin–Chitosan. To prepare a complex carrier (Fb–Ch) containing Fb and MCCh, a mixture of these polymers in the form of a fibrinogen solution (10.0 mg mL^{-1}) and MCCh hydrogel (3.0 wt %) was used (Table I).

Bilayer polymeric Fb–Ch membranes (M2 L, M3 L, and M4 L) were prepared by pouring a mixture of MCCh hydrogel (1 g) with 20 μL of $CaCl_2$ (0.5 mol L^{-1}) and plasticizers, glycerol and GP, in equal amounts (25 mg) into a Teflon® well. The films were desiccated for 24 h in an incubator at 28°C once the solvent had evaporated. A Fb layer containing fibrinogen solution in the PBS buffer (0.01 mol L^{-1}, pH 7.4), 20 μL of aprotinin (1 mg mL^{-1}), 10 μL of FSF (0.1 mg mL^{-1}), 100 μL of TGF-β1 (1 μg mL^{-1}), 20 μL of $CaCl_2$ (0.5 mol L^{-1}), and 0.4 mL of thrombin (40 National Institutes of Health (NIH) U mL^{-1}) was added and applied on the dried Ch film in aseptic conditions. Once the solvent had evaporated, a bilayer Fb–Ch xerogel was obtained (Table I).

Mixed Fb–Ch membranes (M3 M and M4 M) were prepared by the addition of fibrinogen solution in PBS buffer (0.01 mol L^{-1}, pH 7.4) to a suspension of MCCh hydrogel (1 g) with 20 μL of $CaCl_2$ (0.5 mol L^{-1}), plasticizers, glycerol and GP, in equal amounts (25 mg), 20 μL of aprotinin (1 mg mL^{-1}), 10 μL of FSF (0.1 mg mL^{-1}), 100 μL of TGF-β1 (1 μg mL^{-1}), and 0.4 mL of thrombin (40 NIH U mL^{-1}) in aseptic conditions. Once the solvent had evaporated after storing the mixture at 28°C for 24 h, a single-layered, mixed Fb–Ch xerogel was obtained (Table I).

Fibrin. A Fb film (M5 H) (Table I) was produced by pouring 3 mL of fibrinogen solution (10 mg mL^{-1}) in PBS buffer (0.01 mol L^{-1}, pH 7.4) with an addition of 10 μL of FSF (0.1 mg mL^{-1}), 20 μL of aprotinin (1 mg mL^{-1}), and plasticizers, glycerol and GP, in equal amounts (25 mg) into a Teflon well. While the constituents were being stirred, 100 μL of TGF-β1 (1 μg mL^{-1}), 20 μL of $CaCl_2$ (0.5 mol L^{-1}), and 0.4 mL of thrombin (40 NIH U mL^{-1}) were quickly added in aseptic conditions. Once the solvent had evaporated, a homogeneous Fb membrane (M5 H) was obtained (Table I).

Methods

Investigation of Mechanical Parameters of the Membranes. The mechanical properties were evaluated at the Accredited Metrological Laboratory, Institute of Biopolymers and Chemical Fibres, Łódź, Poland (IBWCh), which holds an accreditation certificate—number AB 338. The basic mechanical parameters of the polymer materials, which were in the form of membranes, were determined in accordance with the following standards: thickness in mm—PN-EN ISO 4593 : 1999, tensile strength in MPa, and elongation at break in %—PN-EN ISO 527-3 : 1998 using a tensile testing machine Instron (Norwood, MA) and Uster Type C, Lanametr. Parameters of condition were as follows: temperature 20°C ± 2°C and relative humidity (RH) 65% ± 4%.

Scanning Electron Microscopy. Scanning electron micrographs were taken for Ch and Fb–Ch films using an ESEM type Quanta 200 scanning electron microscope (SEM) from FEI (Hillsboro, OR).

Fourier Transform Infrared Spectrophotometric Measurements. Polymer films (Ch, Fb, and Fb : Ch—2 : 1) were prepared for use in IR studies. The water dispersion of MCCh and a solution of coagulated fibrinogen or a coagulation mixture of these polymers were put on a Teflon plate and left to

Table II. Thickness and Mechanical Parameters of Membrane Systems Derived

No.	Parameters	Membrane						
		M1 H	M1 L*	M2 L	M3 L	M3 M	M4 L	M4 M
1	Thickness (mm)	0.056	0.097	0.103	0.112	0.090	0.080	0.083
	Variation coefficient of thickness (%)	5.05	2.99	0.00	0.00	8.69	0.00	8.43
2	Max tensile force (N)	6.88	27.0	6.69	2.75	2.15	7.57	12.5
	Variation coefficient of tensile force (%)	46.8	24.2	31.9	6.40	22.2	42.8	35.6
3	Breaking strength (MPa)	8.10	18.5	4.33	1.63	1.63	6.31	9.88
4	Elongation at break (%)	1.75	6.85	28.0	85.3	41.5	2.38	6.17
	Variation coefficient of elongation at break (%)	37.8	40.0	13.8	9.61	10.9	5.75	10.9

H, homogeneous; M, mixed; L, bilayer; *L, triple layer. $T = 20°C \pm 2°C$, RH = 65% ± 4%.

dry at room temperature. Then, the polymer film was removed and used in Fourier transform infrared (FTIR) measurements on an ATI Mattson Infinity Series FTIR spectrophotometer.

Determination of TGF-β1 *In Vitro* Release. The TGF-β1 release was performed in five selected systems (Table I) at room temperature. For measurement of kinetics, the membranes (30 mg) were placed in tightly closed test tubes containing 1 mL of PBS buffer (0.01 mol L^{-1}, pH 7.4) and were agitated. Consecutive samples of 110 μL were extracted after 0.5, 1, 2, 6, 24, 48, and 96 h and after 14 days. Eluent membranes were directly frozen and stored at −20°C until subsequent analysis. The volume extracted was always replaced with 0.01 mol L^{-1} PBS, pH 7.4, buffer. The amount of TGF-β1 was measured immunoenzymatically using Elisa (R&D System). The absorbance was measured at 450 nm using an Elx800 Elisa Reader, BIO-TEK Instruments (Winooski, VT). The study was done in triplicate, and results obtained from the release studies were statistically validated.

Statistical Analysis. Statistical analysis was performed using the Microsoft Excel Analysis Tool Pak in Microsoft Office Excel 2007 and Statistica 9.

RESULTS AND DISCUSSION

Investigation of Mechanical Parameters of the Membranes

The mechanical properties of Ch and Fb–Ch membranes (M1 H—homogeneous; M1 L*—three-layered; M2 L, M3 L, and M4 L—bilayered; and M3 M and M4 M—mixed) were investigated and compared (Table II). The Fb membrane (M5 H) was not evaluated because of its high brittleness (fragility and disintegration). The results are presented as the combined value of 10 independent samples.

Thickness. The membranes obtained differ in thickness (Table II) as a result of the formation process (Table I). The thickest (>0.8 mm) are Fb–Ch membranes (M4 L, M3 L, and M2 L—bilayered), M3 M and M4 M—mixed, and the M1 L* one (a three-layered Ch membrane). Among them, the thickest (>0.11 mm) appeared to be the two-layered M3 L membrane, whereas the one-layer M1 H Ch membrane was the thinnest (0.056 mm).

Maximal Tensile Force. In strength studies, one determines the maximal force (F) that has to be applied to tear a sample, a membrane strap of known width. However, when membranes that differ in breadth are studied, values of the force (F) cannot be compared. In this case, the values of breaking strength are compared (Bs), which is a quotient of the maximal tensile force and the area of the foil's section

$$\mathrm{Bs} - \frac{F}{d \times h}\left[\frac{\mathrm{N}}{\mathrm{mm} \times \mathrm{mm}}\right]$$

where Bs is the breaking strength (MPa), F is the maximal tensile force (N), d is the thickness (mm), and h is the width of stretched membrane (mm).

Breaking Strength. The following membranes appeared to have fairly high breaking strength (>6 MPa): the one-layer Ch M1 H, the twofold M4 L (bilayers), the M4 M (mixed), and the three-layer Ch M1 L* membrane. The most durable was the three-layer Ch M1 L* membrane, with a breaking strength > 18 MPa. The rest of the membranes, M3 M, M3 L, and M2 L, are characterized by considerably lower breaking strength values (<6 MPa). Very low breaking strength (1.63 MPa) was recorded for the M3 M and M3 L membranes.

Elongation at Break. The one-layer M1 H and three-layer M1 L* Ch membranes as well as the bilayer M4 L and mixed M4 M Fb–Ch membranes were characterized by a significantly low elongation at break value (<10%), which makes them very brittle. However, for the bilayer M2 L membrane, the value of this parameter was about 30%. Significantly higher (more than 30%) values were recorded for the mixed M3 M and the bilayer M3 L membranes, especially in the case of membrane M3 L, where the elongation at break reached more than 80%.

All membranes whose elongation at break > 30% are extensible, but their breaking strength is low.

Scanning Electron Microscopy

Pictures of the mixed and layered membranes obtained from the SEM (Quanta 200 SEM) are shown in Figure 1. The layered membranes (M1 L*, M3 L, and M4 L) with a well-developed surface are porous and coarse. The porous surface of a membrane is crucial for the diffusion of growth factors and free oxygen penetration; these processes are essential in enhancing tissue growth and regeneration. Aggregates or precipitated material

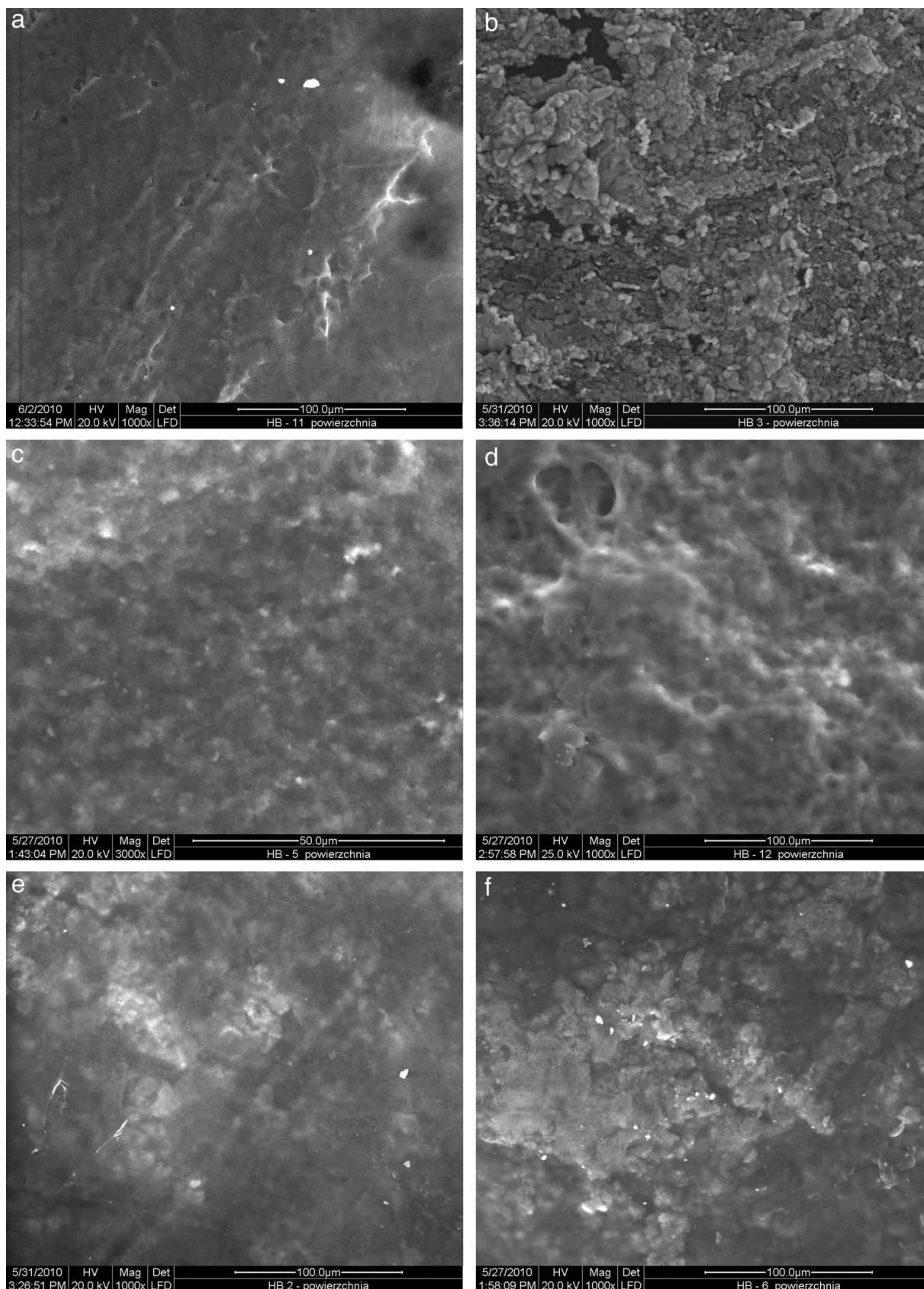

Figure 1. SEM images of a Ch and Fb–Ch composite membrane: (a) M1 H—×1000, (b) M3 L—×1000, (c) M4 L—×3000, (d) M1 L*—×1000, (e) M3 M—×1000, and (f) M4 M—×1000.

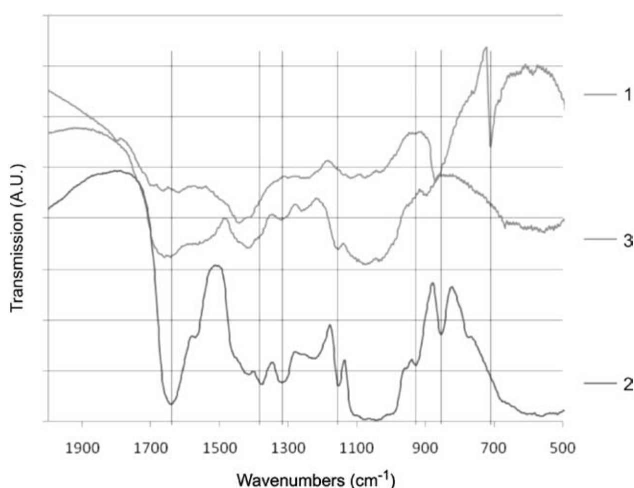

Figure 2. FTIR spectra of Fb (1), MCCh film (2), and Fb–Ch system (2 : 1) (3).

may be seen on the surfaces of Ch homogeneity (M1 H) and Fb–Ch mixture (M3 M and M4 M) membranes.

FTIR Spectroscopy Analysis

The spectrum of MCCh film (Figure 2, spectra 2) shows the characteristic peaks of amide I (C=O) absorption at 1650 cm^{-1}, —NH amide II at 1560 cm^{-1}, and amide III at 1320 cm^{-1}. In addition, the spectrum shows absorption bands at ca. 1380 cm^{-1}, referring to the crystalline area of the polymer, characteristic absorption bands of high intensity (800–1200 cm^{-1}) due to the repeating Ch pyranose rings, as well as peaks at ca. 1150 and 1050 cm^{-1}, corresponding to the interring ethereal bond C—O—C.[32,33]

Analysis of IR spectroscopy for the Fb–Ch composite (Figure 2, spectra 3) shows that while the —NH amide II band at ca. 1560 cm^{-1} fades away, there is also a band at ca. 1650 cm^{-1}; however, it is less distinct and of lower intensity when compared with the characteristic peak of amide I (C=O). The bands at ca. 1150, 1200, and 1320 cm^{-1}, characteristic of Ch, are still visible but of lower intensity. Although the band at 1380 cm^{-1}, related to the crystal structure of the polymer, dies away, a new broad band at 1400 cm^{-1} appears. Similarly, the band at ca. 850 cm^{-1}, characteristic of Ch and Fb, fades away and a new, less developed one appears at ca. 900 cm^{-1}. Also, the band at ca. 700 cm^{-1}, characteristic of Fb (Figure 2, spectra 1), disappears and a poorly developed band at ca. 665 cm^{-1} is observed. This clear shift of bands toward lower wave numbers as well as the disappearance of the amide II peak at ca. 1560 cm^{-1} show the weakening of chemical bonds and may also suggest that linkages between the Ch —NH_2 and Fb —COOH groups are formed.

In Vitro Release Kinetics of TGF-β1

The release profile of TGF-β1 was determined for five kinds of carriers (M1 H, M2 L, M3 M, M4 L, and M5 H)—homogeneous, layered, and mixed, which contain Fb and Ch in various proportions (Table I). The kinetics of TGF-β1 release from these polymer carriers are illustrated in Figure 3.

The results show that TGF-β1 is released most easily and efficiently (25% after 6 h and 28.98% of the total amount after 14 days) from the homogeneous Fb M5 H membrane. After 14 days, 7.77 and 14.8% of the total amount of the growth factor are released from the layered M2 L and M4 L membranes, respectively. In the case of the mixed M3 M membrane, 12.3% of TGF-β1 is released, whereas from the homogenous M1 H membrane, very little tissue factor is released (1.25% of the total amount after 6 h and 14 days).

Correlation between the Structure, Composition, Mechanical Properties, and Release Kinetics of the Membranes

The results obtained indicate that the amount of growth factor bound by a membrane is highly influenced by the specific area

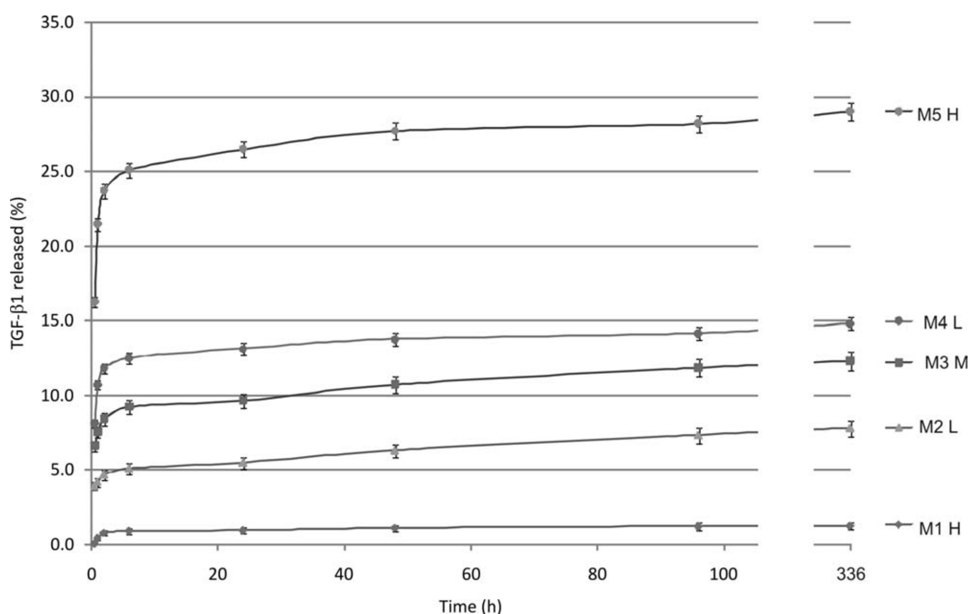

Figure 3. Release profile of TGF-β1 from Ch—M1 H, Fb–Ch—M2 L, M3 M, and M4 L, and Fb—M5 H membrane systems.

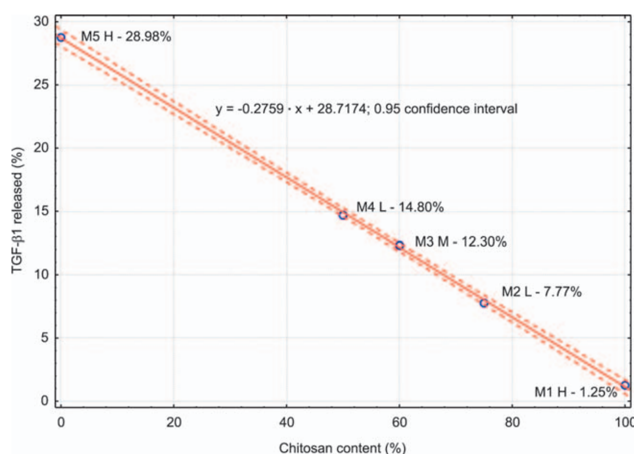

Figure 4. Influence of Ch content on TGF-β1 release profile from Ch—M1 H, Fb–Ch—M2 L, M3 M, and M4 L, and Fb—M5 H membrane systems. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of the membrane and its affinity to growth factor (Figure 4). The less efficient release profile of TGF-β1 from the Ch membrane shows that the factor is more firmly bound to the carrier. The Ch membrane reveals higher efficacy in the binding of the TGF-β1 and, at the same time, lower efficacy in its release (1.25% after 14 days). The opposite situation was observed in the case of the Fb membrane—the TGF-β1 binding is weaker, and therefore its release is more efficient—28.98% after 14 days. The correlation between the amount of TGF-β1 released and the Ch content in the membrane is shown in Figure 4, which can be described with the following equation:

$$y = -0.2759x + 28.7174,$$

where y is the TGF-β1 released (%) and x is the Ch content in the membrane (%).

The release was lower with increasing Ch concentrations in the membrane, suggesting a binding affinity of TGF-β1 with the Fb–Ch component.

Tissue engineering combines cell and molecular biology with materials and mechanical engineering to replace damaged or diseased organs and tissue. Commercially available fibrinogen and thrombin can be combined to form a Fb hydrogel; the functionality of Fb as a scaffold can be improved by incorporation of bioactive peptides and growth factors. A number of allogenic Fb sealants, such as Tisseel®, Evicel™, and Crosseal™, have been approved by the Food and Drug Administration for clinical use as hemostatic agents.[11]

It has been shown that macromolecules prepared from biological origins can be used for various clinical purposes. For application, a biomaterial should be nontoxic, nonantigenic, and adequately available. Both Ch and Fb are in accordance with these requirements and seem to meet all the necessary demands to serve as biomaterials.

Because growth factors are such powerful regulators of biological function, their presence in tissue is highly regulated according to time and space. TGF-β1 has a potential role in wound healing. Scaffolds that release TGF-β1 for reconstructing bones use poly(DL-lactic-*co*-glycolic) acid (PLGA) and demineralized bone matrix as carriers.[34] This study was focused on the influence on the bone regeneration process of TGF-β1 incorporated into PLGA scaffold.[2] Studies on biomedical composites revolve around the development of technologies for membrane material production, such as the proper selection of components, which takes into consideration their physicochemical features, guarantees a good mechanism of release, and allows to evaluate different scaffolds as possible bases for growth factor incorporation.[35]

In our previous research, a bilayer membrane (MCCh–ALG) produced from MCCh, calcium alginate (ALG),[25] and hydrogel of Fb, methylcellulose, MCCh was used.[26] The most rapid release of bFGF in the presence of ketoprofen was observed from the Ch matrix. The varied release of growth factors (PDGF-AB, TGF-β, and b-FGF) from polymer carriers observed in our earlier studies was the reason for our continued interest in the kinetics of the release of selected transforming growth factors (rhTGF-β1) from natural biodegradable polymers (MCCh and Fb) in Fb–Ch membranes.

The aim of this study was to evaluate the structure of single constituent (Fb or Ch) or bilayer Fb–Ch membranes as well as to determine the release profile of the growth factor from the membranes. The membranes investigated were homogenous, mixed, bilayer, and triple layer. Measurements of the thickness, maximal tensile force, breaking strength, and elongation at break were carried out. The texture of the membrane surface was observed with a SEM (Quanta 200 SEM) and evaluated by FTIR spectral analysis. The FTIR spectrum of the Fb–Ch composite (Figure 2, spectra 3) contains characteristic absorption peaks of Fb and Ch. The shift in these absorption bands may be attributed to intermolecular crosslinking between —CHO groups on the backbones of Fb and free NH_2 group of the Ch molecule.

A significant part of the research was the formation of bilayer Fb–Ch membranes (Table I). The mechanical strength test showed that the combination of these two carriers enhanced the mechanical properties of the membrane and efficacy of growth factor release. Studies on the activity of a combination of carriers have been performed by other authors.[17,36] In this study, a combination of carriers on one membrane was found to enhance the characteristics of the biomaterial.

Our Fb–Ch membrane with incorporated TGF-β1 may prove to be a very useful scaffold in the tissue regeneration process. The TGF-β1 release profile from our membranes (of a composition shown in Table I, Figure 3) after 14 days was 7.77% for M2 L and 12.3% of the total amount for M3 M. The most efficient release of TGF-β1 was observed in the case of the Fb–Ch M4 L membrane; the TGF-β1 factor release was at a controlled rate, and after 14 days it reached a maximal value of 14.08% of the total amount. Moreover, this membrane shows higher mechanical strength, which may be the reason for delayed TGF-β1 release when compared with the Fb membrane.

CONCLUSIONS

A rise in Ch content in layered membranes increases their elasticity, expressed by the elongation at break value, and does not influence their breaking strength to any appreciable degree. Although layered membranes will lengthen, mixed membranes have a low resistance and will break >30%. Increased Fb concentration within the membrane leads to elevated fragility and disintegration, which in turn enhances growth factor release. However, in comparison to Fb, a higher amount of Ch leads to higher viscosity and moisture content as well as to a delay in the release of the growth factor.

The results indicate that factor TGF-β1 is released *in vitro* from Fb–Ch membranes with different kinetics. Assessment of the angiogenic TGF-β1 release profile from a polymer base may be valuable in choosing a proper carrier for a growth factor. Membranes of mechanical strength > 10 MPa may be applied in practice. The membranes tested could be used as carriers for growth factors in clinical treatment, especially in bone regeneration.

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