

Polymer angiogenic factor carrier. Part I. Chitosan-alginate membrane as carrier PDGF-AB and TGF- β

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Summary

Microcrystalline chitosan (MCCh) characterise by many valuable features, including biocompatibility, antibacterial, fungicidal and high adhesive properties, high water retention value (WRV) factor, potent sorption, high bioactivity, biodegradability, ability to create membranes directly out of water suspensions. This biopolymer due to its biodegradability and safety in application no cytotoxic or immune interaction was assessed as a very effective haemostatic factor.

MCCh with such properties form useful initial material for biological bandages; it can be used in surgery as growth factor carrier. Biodegradable membranes are applied in surgery for guided tissue regeneration, what contributed to make the manufacturing technology of modified membranes out of microcrystalline chitosan.

Performed research was to show how certain angiogenic factors are released - platelet derived angiogenic factor (PDAF-AB) and transforming factor β (TGF- β) from doubled-layered alginate-chitosane membrane which was already used in dental surgery. Assessment of angiogenic factors release (PDGF-AB and TGF- β) from polymer base may become helpful in choosing the right carrier for growth factors.

Key words: growth factors (PDGF-AB, TGF- β), polymers (microcrystalline chitosan, calcium alginate), alginate-chitosan membrane, release rate

INTRODUCTION

Angiogenic growth factors may significantly aid and modify tissue regeneration [1, 2]. Main role in this process is played by factors released from platelet rich plasma (PRP). Research over platelet rich plasma enabled to identify platelet derived growth factor (PDGF), insulin growth factor (IGF), epidermal growth factor (EGF) and transforming growth factor (TNF). Platelet derived growth factor stimulates mitogenesis of bone marrow stem cells and osteoblasts leading to increase in its number significantly. PDGF enhances endothelial cell mitosis, initiates angiogenesis and blood vessel proliferation to damaged tissue begins [3]. TGF- β stimulates fibroblast growth, enhances collagen matrix synthesis and suspends blood vessel proliferation.

Initiation of bone cell growth is contributed by means of certain growth factors - PDGF, TGF- β and IGF. Its source is mainly platelet rich plasma (PRP), which recently is applied as ingredient of platelet rich gel (PRG). For most efficient usage of PRG it was joined with osteoconductive material; as a result demineralized bone matrix was obtained widely used for regeneration of bone lesions [3].

Recent research indicates that blood platelets have the ability to secret huge amounts of platelet derived growth factor (PDGF-AB) and transforming growth factor (TGF- β) [4, 5]. Activity of newly created three-layered alginate-chitosan-alginate membrane was also assessed by fluctuations in PDGF-AB and TGF- β concentration in platelet rich plasma. New three-layered membrane used in guided bone regeneration stabilize PRPs' osteoconductive activity by protective influence in relation to PDGF-AB and TGF- β release from platelets.

Current methods of treatment use knowledge of tissue engineering (biomimetics) achievements, which is based on embryogenetic processes application in tissue healing [6]. For proper conduction of regeneration three fully dependent on themselves factors are required: carriers, cells and extracellular cell matrix (ECM), which includes growth factors, morphogenes, adhesines, hormones and vitamins.

Significant carrier of healing substances and growth factors is chitosan - natural polymer used for artificial organ manufacturing [7-9]. Carried research enabled to apply chitosan for tissue engineering purposes as "biological scaffolding"- base for cell culture breeding [10]. Experimental research indicate also that it is possible to use chitosan as "biological glue", which could be applied in bone tissue healing and massive haemorrhage [11].

One of many materials used in surgery is also fibrin gel (fibrin glue) [12]. Connected with endothelial growth factor (ECGF), fibrin glue, is good haemostatic, bandage-like material. In vitro scientific studies shown, that fibrin gel in combination with hyaluronic acid and chondroitin sulphate (GHC6S) is perfect factor promoting matrix cell secretion reaction and inhibiting degradation processes [13,14]. Both ingredients of studied complex and fibrin network structure have potent influence for level of release growth factors.

Research over new materials for membranes used in guided tissue regeneration is still in progress. In presented case was shown how to obtain two-layered membrane out of natural polymers mixture: microcrystalline chitosan and calcium alginate; as well as assessment of properties of obtained membrane for binding and release particular growth factors.

Aim of study was level assessment of particular growth factors release (PDGF-AB and TGF- β) from polymer base (two-layered chitosan-alginate membrane).

MATERIAL AND METHODS

Microcrystalline chitosan was used (MCCh M-66) in form of a hydrogel contains 2.83% of polymer, deacetylated in 80% and of average viscosimetric particle mass approx. 200 kDa and calcium alginate (ALG LF 10/60) in form of 0.5% water suspension (Institute of Biopolymer and Chemical Fibres, Łódź).

Physio-chemical modification improved quality of chitosan and led to microcrystalline chitosan form. Production process is protected by means of international patents, which are owned by Institute of Biopolymer and Chemical Fibres in Łódź. In this process some physio-chemical factors are used; such as neutralisation, coagulation or glucosamine particles aggregation. MCCh characterize with many usable features: biocompatibility, antiviral and antifungal properties, potent adhesive force, high WRV factor value, high sorption and high bioactivity as well.

Preparation of polymer films

Double-layered alginate-chitosan polymer films (MCCh-ALG) were prepared by pouring mixture of MCCh hydrogel (approx. 4.0 g) and suspension of calcium alginate (approx. 2.0 g) with one drop of glycerol (0.03 g) and two drops of propylene glycerol (0.05 g) onto clean Teflon® plates (5 cm \times 6 cm. Plates with deposited layer of polymer mixture were dried for 24 hours at room temperature. On such prepared films another layer of polymers

(approx. 6.0 g) and plastificators (approx. 0.08 g) mixture was poured. During mixing all the ingredients appropriate growth factor was introduced of known concentration (100 μ l solution obtained by dissolving 10 μ g PDGF-AB in 1.11 ml 0.1 mol/l PBS phosphate buffer, pH 7.4 and 5 μ g TGF- β dissolved in 3.921 ml PBS phosphate buffer. After water was vaporized from hydrogel, chitosan-alginate xerogel was obtained.

Chitosane constituted 92% and alginate 8% of mass of the mixture. Polymer film samples (1 cm x 1cm) were eluted to the buffer PBS 0.1 mol/l pH 7.4 and growth factor marking was performed after 30 minutes, 1-th, 2-nd, 3-rd and 5-th hour in eluate. Concentration of released growth factors was marked by means of immunoenzymatic method Elisa.

Marking of platelet derived growth factor (PDGF-AB) and transforming growth factor beta (TGF- β) binded with carrier was made by means of immunoenzymatic method using Elisa tests R&D System Reagent, which contains polyclonal antibodies against PDGF-AB conjugated to horseradish peroxidase (PDGF-AB Conjugate) and recombinant human PDGF-AB in buffered protein (PDGF-AB Standard). A monoclonal antibody specific for PDGF-AB and TGF- β has been precoated onto a microplate. Standards and sample are pipetted into the wells and any PDGF-AB and TGF- β present is bound by the immobilised antibody.

Recombinant TGF- β , 25.0 kDa (Serotec, Immunological Excellence), Recombinant Human platelet derived growth factor PDGF-AB, 25.5 kDa (Chemicon® Internatoinal a Serological Company), Elisa Test by R&D System. Absorption reading was done at 540 nm with a use of Elx 800 Reader, Bio-Tek Instruments, Inc. Values of average and standard deviation were computed using Excel calculating sheet, Microsoft Office 2007. Released growth factor concentration is presented in charts and on drawing.

RESULTS AND DISCUSSION

High molecular compounds of large particle size are more widely used in pharmacy as substances which are helpful both in manufacturing new type of medications as well as target therapeutic systems with controlled release of healing substances [15]. Structure “healing substance-polymer” provides more convenient usage and decrease of doses, restricts side effects and simplifies administration of medications.

As polymer carriers for medications different natural polymers are used such as: fibrin glue, polysacharides - dextrane, hyaluric acid, cellulose, chitin, protein polymers - albumin,

gelatin and synthetic polymers - vinyl polyalcohol and their modification products [16]. Natural polymers and its derivatives constitutes valuable material for many forms of carriers.

Chitosan is biocompatible and biodegradable natural biopolymer. It is capable of absorbing liquids and it has some water permeability properties. There are many ways chitosan can be applied both in medicine or pharmacy as medications and growth factors carrier [17, 18]. In literature different concentration of chitosan-modified in composition with glutaraldehyde and collagen membranes were described [19 - 20].

Other way of modification was to construct multi-layered membranes with usage of different neutral polymer of higher viscosity-methylcellulose and alginate with addition of plasticators (glycerol or polypropylene glycol). Usage of mixture of two polymers and small amounts of plasticators has significantly improved flexibility of obtained membranes [21].

In our research alginate-chitosan membrane was used. In the figure 1 view is presented with scanning electron microscope (Quanta 200 SEM). Membrane characterise with developed surface, roughness and porosity (Figure 1).

Profiles of growth factors release (PDGF-AB and TGF- β) to the PBS buffer (0.01 mol/l, pH 7.4) from newly created membrane was assessed (Figure 2). The amount of released PDGF-AB platelet factor was significantly higher in comparison with TGF- β . Greatest amount of released factor was after 3-rd hour of incubation (255.41 ± 8.82 ng/ml, binding factor 3.53). After 5 hours level of release was slightly lower, about 217.24 ± 13.90 ng/ml, binding factor 4.14 (Table 1).

Concentration of released factor TGF- β was highest during 1-th hour of incubation (114.20 ± 9.61 ng/ml, binding factor 1.12, as it takes place *in vivo* conditions [22]. In 3-rd and 5-th hour of incubation TGF- β release to eluent was respectively 11.53 ± 2.70 ng/ml, binding factor 11.06 and 19.40 ± 2.00 ng/ml, binding factor 6.58 (Table 2).

Results from carried out research suggest, that platelet derived growth factor is released faster from studied membrane than TGF- β (Figure 2). It seems that chitosan-alginate membrane is more valuable for platelet derived growth factor (PDGF-AB). These results suggested that PDGF-AB loaded with chitosan may be beneficial to enhance for example periodontal bone regeneration [23].

On basis of results (charts and drawings) it was concluded, that obtained membrane indicates greater effectiveness in binding TGF- β factor and simultaneously has lower effectiveness in its release. Opposite situation has been observed in case of PDGF-AB factor. Binding force of this factor is weaker, therefore release is more effective – almost 30% after 3 hours, than it decreases up to 23% after 5 hours of this process (Figure 2). It was stated, that

specific surface of the membrane has an influence on the amount of binded growth factor and its affinity to specific growth factor.

CONCLUSIONS

1. Chitosan-alginate membrane might be used in dental surgery as carrier for angiogenic growth factor: platelet derived growth factor (PDGF-AB) and transforming growth factor (TGF- β).
2. Release level of platelet derived growth factor (PDGF-AB) is significantly higher in comparison with TGF- β .
3. Lower degree of release of TGF- β arise from higher affinity of TGF- β to polymer base.
4. Choice of appropriate carrier, growth factor and degree of release in particular time period may be used in surgery depending on needs.

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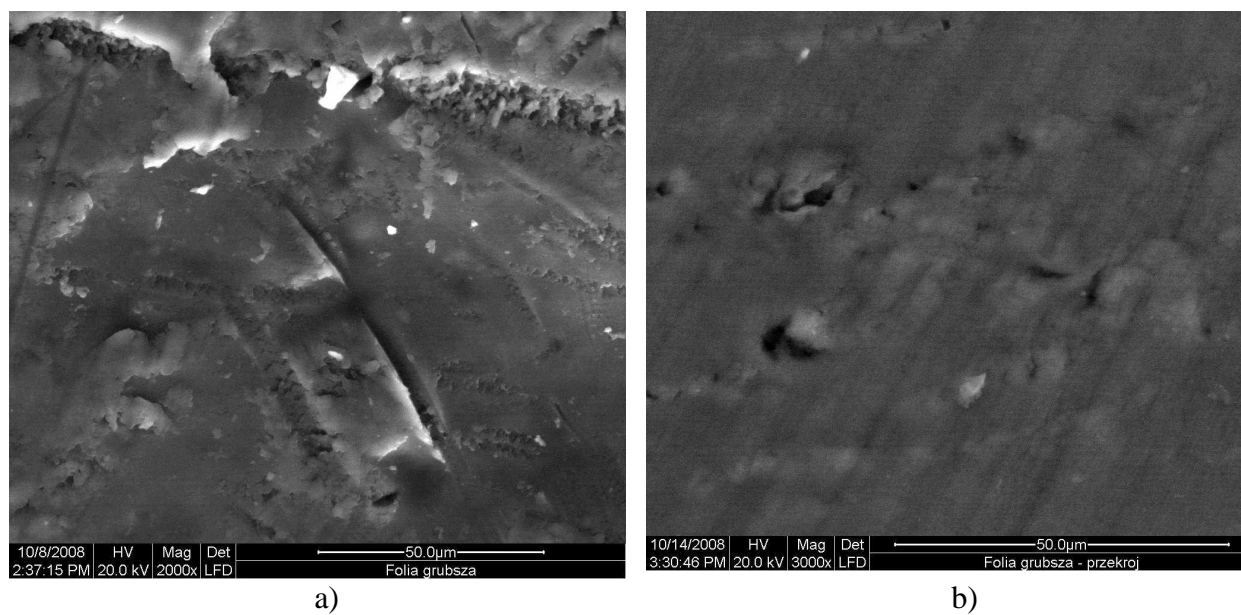
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RYC. 1. Mikroskopowy obraz membrany chitozanowo-alginianowej; a) widok powierzchni, b) przekrój poprzeczny

FIG. 1. Microscopic view chitosan-alginate membrane; a) microscopic view; b) cross-section

TABELA 1. Stężenie uwolnionego PDGF-AB [ng/ml] do buforu w czasie procesu elucji do fosforanowego buforu PBS 0,1 [mol/l] pH 7,4 i współczynnik wiązania (WW) PDGF-AB z nośnikiem chitozanowo-alginiowym.

TABLE 1. Concentration of release PDGF-AB [ng/ml] into PBS phosphate buffer 0.01 [mol/l] pH 7.4 and PDGF-AB binding factor (WW) with chitosan–alginate carrier

Czas Time [h]	0,5	1	3	5
\bar{X} [ng/ml]	112.75	100.22	255.41	217.24
\pm SD n = 8	4.77	5.35	8.88	13.90
WW	7.99	8.90	3.53	4.14

WW = całkowite stężenie/stężenie uwolnionego PDGF-AB [ng/ml]

WW = total concentration /concentration of release PDGF-AB [ng/ml]

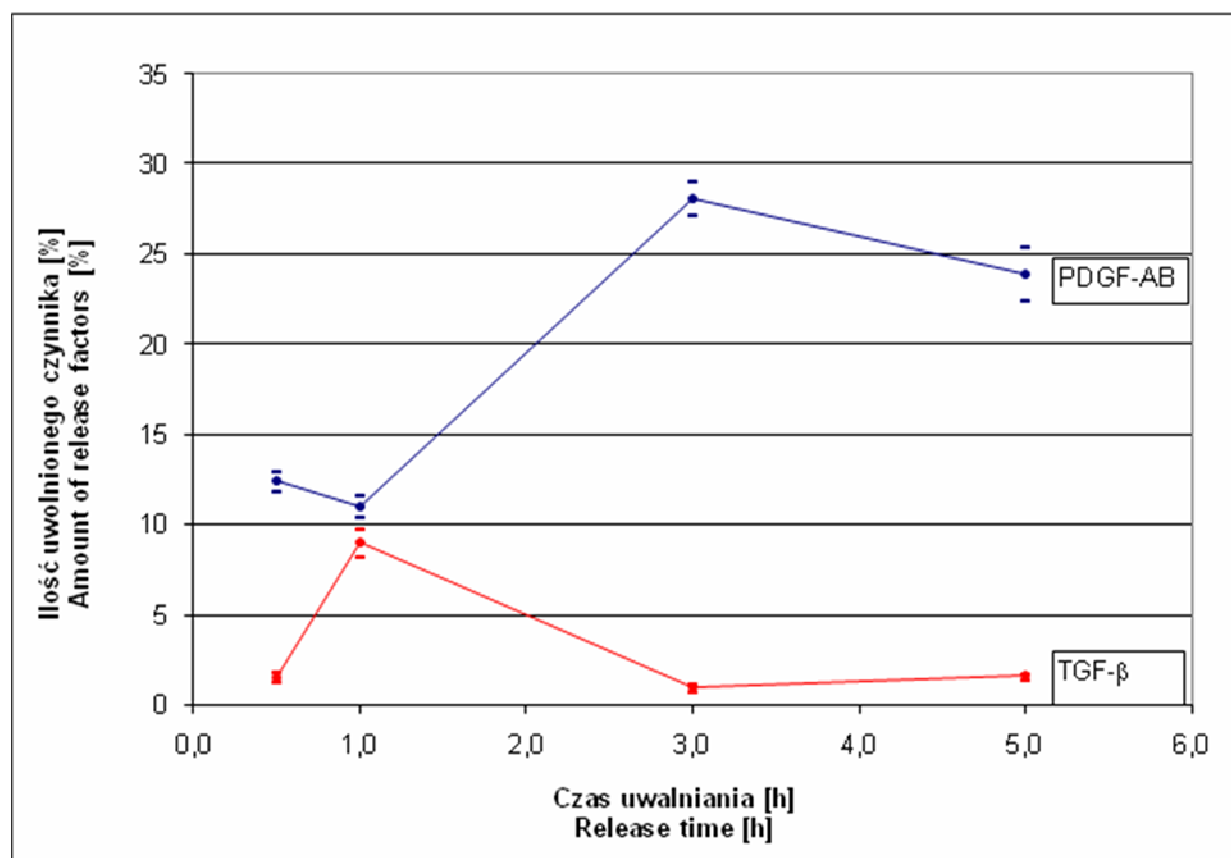
TABELA 2. Stężenie uwolnionego TGF- β [ng/ml] do buforu w czasie procesu elucji do fosforanowego buforu PBS 0,1 [mol/l] pH 7,4 i współczynnik wiązania (WW) TGF- β z nośnikiem chitozanowo-alginiowym.

TABLE 2. Concentration of release TGF- β [ng/ml] into PBS phosphate buffer 0.01 [mol/l] pH 7.4 and TGF- β binding factor (WW) with chitosan–alginate carrier

Czas Time [h]	0,5	1	3	5
\bar{X} [ng/ml]	18.82	114.20	11.53	19.40
\pm SD n= 8	3.29	9.62	2.70	2.01
WW	6.78	1.12	11.06	6.58

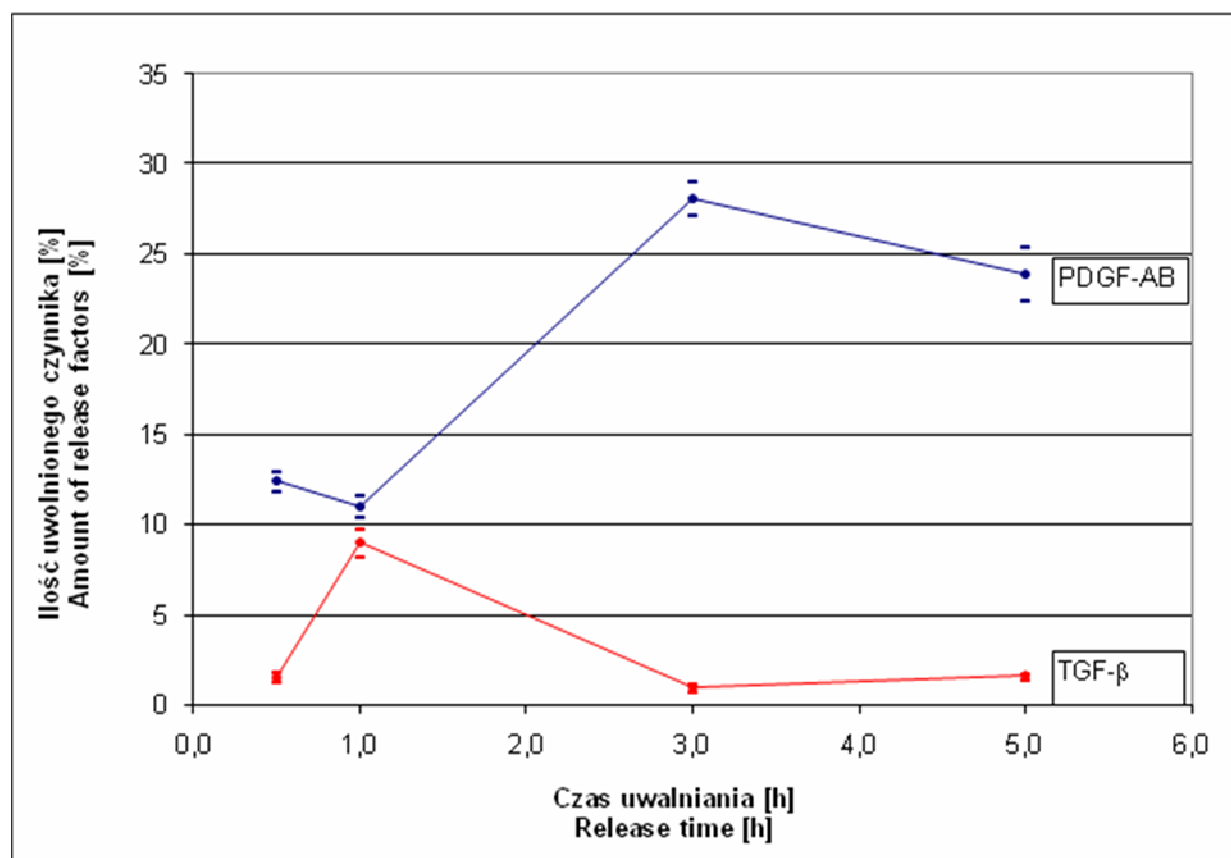
WW = stężenie całkowite/stężenie uwolnionego TGF- β [ng/ml]

WW = total concentration /concentration of release TGF- β [ng/ml]



RYC.2 . Profile uwalniania czynników wzrostu z membrany chitozanowo-alginianowej

FIG. 2. Release profile growth factors from chitosan-alginate membrane



RYC.2 . Profile uwalniania czynników wzrostu z membrany chitozanowo-alginianowej

FIG. 2. Release profile growth factors from chitosan-alginate membrane