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Chitosan-thioglycolic acid conjugate: a new scaffold material for tissue engineering?

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Abstract

It was the aim of this study to evaluate chitosan-thioglycolic acid (chitosan-TGA) conjugate as scaffold material in tissue engineering. Chitosan was modified by the introduction of thiol groups. Briefly, TGA was introduced to chitosan via amide bond formation mediated by a carbodiimide. The properties of the resulting polymer were thereby altered in regard to water solubility, mucoadhesion, biodegradability and in situ gelling compared to the original polymer. Due to the immobilised thiol groups (240 ± 30 μmol thiol groups per gram polymer), the viscosity of a 1.5% chitosan-TGA solution was improved 4.3-fold. This can be explained by the formation of disulphide bonds within this polymeric network. The conjugate was tested as scaffold material in form of a gel and sheets. Furthermore, the influence of the thiol groups on the viability of L-929 mouse fibroblasts was evaluated. It was shown that the L-929 mouse fibroblasts grew on both scaffolds despite the thiol groups, although the different surface conditions seemed to have an influence on the growing rate. Chitosan-TGA sheets seemed to be the more preferred layer. The improved in situ gelling may be important for ongoing developments. Direct injectable matrices at the site of tissue damage mimicking the tissue being restored may be a future trend on this topic. Hence, chitosan-TGA is a promising candidate as scaffold material in tissue engineering.

Keywords: Chitosan; Biopolymer; Tissue engineering; Thiolated chitosan; Scaffold material; In situ gelling

1. Introduction

The expanding field of tissue engineering applications has accelerated the need of materials which are tissue compatible, biodegradable and with mechanical properties similar to the target tissues. Biodegradable and biocompatible polymers, respectively, have been attractive candidates for scaffolding materials because they degrade as the new tissues are formed, eventually without inflammatory reactions or toxic degradation (Ma and Choi, 2001). The scaffold material has an essential function concerning cell anchorage, proliferation and tissue formation in three dimensions (Langer and Vacanti, 1993). Performance of these properties demands usually a porous scaffold structure, with the porosity characteristics being application specific. A number of synthetic and biological

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materials, such as poly-lactid acids, poly-glycolic acids, collagen and chitin, for example, are currently being used as tissue scaffolds (Hutmacher, 2000). The microstructures of these systems range from hydrogels, to open-pore structures, to fibrous matrices (Madihally and Matthew, 1999). Parallel to this there is a continuous search for materials which fulfil all these broadly defined demands on controllable design of shapes and in situ gelling.

The biopolymer chitosan may provide an answer to this search. It is a copolymer of glucosamine and *N*-acetylglucosamine. Chitosan is a partially deacetylated derivative of chitin, mostly derived from the exoskeleton of crustaceans (Singla and Chawala, 2001). Chitosan is described to possess biological and material properties suitable for clinical applications, since it has been reported to be non-toxic and biocompatible when used in human and animal models (Illum, 1998; Lahiji et al., 2000; Muzzarelli et al., 1988).

To improve the material properties of chitosan concerning the scaffold structure, a previously developed thiolated chitosan (chitosan-thioglycolic acid (chitosan-TGA) conjugate) was tested as scaffold material (Kast and Bernkop-Schnürch, 2001). By the introduction of thiol groups the basic polymer could be altered resulting in a polymer with properties which might be useful for tissue engineering, like water solubility, controllable biodegradation and in situ gelling. In this study, we focused on the growth behaviour of L-929 mouse fibroblasts seeded on either chitosan-TGA gels or on lyophilised polymer conjugate (i.e. sheets). Furthermore, the influence of the thiol groups on the viability and proliferation of these fibroblasts was investigated in vitro.

2. Materials and methods

2.1. Synthesis of the chitosan-TGA

The synthesis of the chitosan-TGA was carried out as described previously (Kast and Bernkop-Schnürch, 2001). Briefly, TGA was attached covalently to chitosan. First, 500 mg of chitosan (chitosan from crab shell; degree of deacetylation >85%; Fluka, Switzerland) was hydrated in 4 ml of 1 M HCl and dissolved by the addition of demineralised water to obtain a 1% solution of chitosan hydrochloride. Thereafter, 500 mg of TGA (Aldrich) was added. After TGA was completely dissolved in the chitosan hydrochloride solution, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC; Sigma, St. Louis, MO) was added in a final concentration of 125 mM in order to activate the carboxylic acid moieties of TGA. The reaction mixture was incubated at pH 5 for 3 h at room temperature under stirring. Samples prepared in exactly the same way but omitting EDAC during the coupling reaction served as controls for the analytical studies.

In order to eliminate unbound TGA and to isolate the polymer conjugates, the reaction mixtures were dialysed five times in tubings (molecular weight cut-off 12 kDa; dialysis tubings, cellulose membrane; Sigma, St. Louis, MO) for 3 days in total at 10 °C in the dark. In detail they were dialysed one time against 5 mM HCl, then two times against the same medium but containing 1% NaCl to quench ionic interactions between the cationic polymer and the anionic sulfhydryl compound. Then, the samples were dialysed exhaustively two times against 1 mM HCl to adjust the pH of the polymer to 4. Thereafter, samples and controls were lyophilised by drying frozen aqueous polymer solutions at −30 °C and 0.01 mbar (Christ Beta 1-8K; Germany) and stored at 4 °C until further use.

2.2. Determination of thiol group content

The degree of modification, i.e. the amount of thiol groups immobilised on chitosan, was determined spectrophotometrically with Ellman's reagent quantifying the thiol groups. First, 0.500 mg conjugate was hydrated in 250 μl of demineralised water. Then, 250 μl 0.5 M phosphate buffer (pH 8.0) and 500 μl Ellman's reagent (3 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma, St. Louis, MO) in 10 ml of 0.5 M phosphate buffer (pH 8.0)) were added. The samples were incubated for 3 h at room temperature. The supernatant was separated from the precipitated polymer by centrifugation (24,000 × *g*, 5 min). Thereafter, 300 μl of the supernatant was transferred to a microtitration plate and the absorbency was measured at a wavelength of 450/620 (nm) with a microtitration plate reader (Anthos Reader 2001; Salzburg, Austria). TGA standards were used to calculate the amount of thiol groups immobilised on the polymer.

2.3. Formation of disulphide bonds

Chitosan-TGA conjugates were hydrated in demineralised water in a final concentration of 1.5% (m/v). The pH was adjusted with 500 mM acetate buffer at pH 5.5. Samples were incubated at 37 °C under permanent shaking. At predetermined time points aliquots of 50 mg were withdrawn and 50 μl of 1 M HCl was added in order to quench any further reaction. The amount of remaining thiol groups was determined spectrophotometrically with Ellman's reagent as described earlier.

2.4. Rheological studies

The viscoelastic properties of chitosan-TGA conjugates were determined with a cone-plate rheometer (RotoVisco RT20; Haake GmbH). Chitosan-TGA conjugates and controls were hydrated in demineralised water. After a hydration time of 1 h, 500 mM acetate buffer (pH 5.5) was added to obtain a final concentration of the polymer of 1.5% (m/v). The samples were incubated at 37 °C. At predetermined time points dynamic oscillatory tests within the linear viscoelasticity region were performed for 1 ml aliquots of the samples at 20 ± 0.5 °C. The parameters obtained thereby were the complex modulus (G^*) and the phase angle (δ). The elastic modulus (G'), the viscous modulus (G'') and the dynamic viscosity (η^*) were calculated by:

$$G' = G^* \sin \delta$$

$$G'' = G^* \cos \delta$$

$$\eta^* = \frac{G''}{\omega}$$

where ω is the angular frequency which is related to the oscillatory frequency ν by the relationship $\omega = 2\pi\nu$. Loss tangent ($\tan \delta$), a parameter that represents the ratio between the viscous and elastic properties of the polymer, was also calculated. Parallel, the formation of disulphide bonds was monitored by determining the decrease of free thiol groups with Ellman's reagent as described earlier.

2.5. Preparation of scaffolds

The resulting lyophilised chitosan-TGA conjugate was divided into two equal parts. One part was rehydrated with demineralised water to obtain a gel of chitosan-TGA conjugate of 3% (w/v). The obtained gel was sterilised by autoclaving (10 min, 121 °C) and stored at room temperature under sterile conditions until further use.

The other lyophilised part of the chitosan-TGA conjugate was sterilised in following manner: small sheets were incubated with 96% (v/v) EtOH for 1 h followed by an incubation with 70% (v/v) isopropanol for 48 h. For further storage 70% (v/v) EtOH was used.

2.6. Cell culture

Cell culture studies were conducted in 24-well culture plates. Untreated culture wells served as control. Sheets of chitosan-TGA were cut into 5 mm × 5 mm pieces, air dried in the culture vessel and rinsed three times with phosphate buffered saline (PBS, pH 7.4; Life Technologies®) to adjust the pH to 7.4. Gels were liquefied and drops (approximately 5 mm in diameter) were transferred into the culture plates. L-929 cells (mouse fibroblasts) were resuspended in 10 μl DMEM (Life Technologies®) plus 10% FCS (Life Technologies®) and 1×10^4 cells were seeded onto each chitosan-TGA containing vessel as well as onto control vessels. After 2 h of incubation, 750 μl of medium was added. Phase contrast microscopic images of the cells were taken digitally every 2 days. Morphometric analyses were conducted using image analysis software.

2.7. Statistical data analysis

Statistical data analysis were performed using the *t*-test with $P < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Synthesis of the chitosan-TGA conjugate

TGA was attached covalently to the primary amino groups of chitosan under the formation of amide bonds (Fig. 1). The efficacy of the purification method for the resulting polymer-TGA conjugates was verified by controls which were prepared in exactly the same way as the polymer conjugates but omitting EDAC during the coupling reaction, exhibiting a negligible

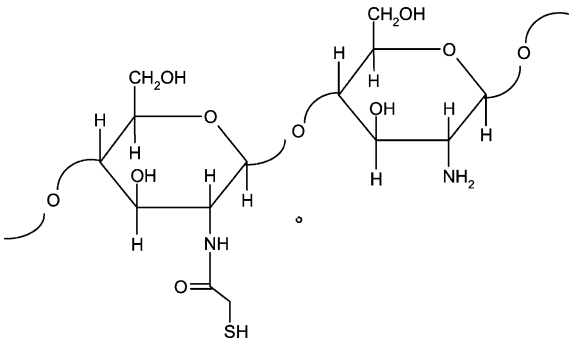

Fig. 1. Presumptive substructure of chitosan-TGA conjugate; covalent attachment was achieved by the formation of amide bonds between the primary amino groups of the polymer and the carboxylic acid groups of TGA mediated by a carbodiimide (EDAC).

amount of thiol groups. The obtained chitosan-TGA displayed 240 ± 30 μmol thiol groups per gram polymer. Due to this relatively high degree of modification, the lyophilised chitosan-TGA conjugate appeared as white, odourless fibre. It easily swelled in aqueous solutions at a pH below 5 and formed transparent gels of high viscosity. Furthermore, the cutting in small pieces utilising a scalpel was easy to perform.

As already described in former studies, the introduction of thiol groups leads to a controllable degradation of the polymer (Kast and Bernkop-Schnürch, 2001). Because of this feature, chitosan-TGA is an interesting candidate as scaffold material. The enzymatic breakdown of the polymeric network will be controlled exactly according to the demands of the tissue being restored. This gives the possibility to design well-defined scaffolds.

3.2. Formation of disulphide bonds and in situ gelling

Comparative studies with unmodified chitosan showed a significant decrease in viscosity during the sterilisation process (data not shown). In contrast, the solution of thiolated chitosan resulted in a gel of very high viscosity which was impracticable to measure. To outline the effect of the temperature on the polymer network in general, the change in the amount of thiol groups at 37 °C was determined and rheological studies were carried out. As depicted in Fig. 2, the thiol groups were decreasing constantly during an observation time of 6 h at pH 5.5. This result was in

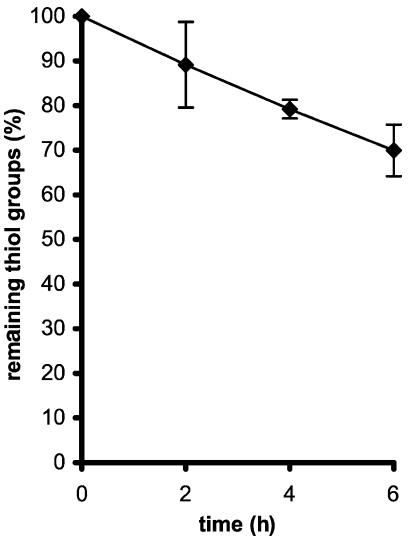

Fig. 2. Decrease of the thiol group content within a gel of 1.5% (m/v) chitosan-TGA conjugate (pH 5.5) with time of hydration at 37 °C. Indicated values are means (±S.D.) of at least three experiments.

good correlation with former studies on chitosan-TGA conjugates (Kast and Bernkop-Schnürch, 2001). After 24 h a small amount of thiol groups could be still determined (data not shown). It is likely that the thiol groups which are close to each other form disulphide bonds resulting in an increase of viscosity, whereas isolated thiol groups remain stable.

To evaluate the correlation between formation of disulphide bonds and the change in viscosity, oscillatory measurement were carried out simultaneously. These measurements give information about the dynamic viscosity (η^*), the elastic or storage modulus G' and the viscous or loss modulus G''. In order to simplify the comparison of the obtained results, the indicated values of G', G'' and η^* at a single representative frequency (1 Hz) within the linear viscoelasticity range are plotted as a function of time. Fig. 3A shows the increase in dynamic viscosity which resulted in a 4.3-fold increase after 6 h of incubation. The changes in the viscoelastic properties of the conjugate are illustrated in Fig. 3B. It shows the different increase in G' and G'' which indicates that the elastic properties prevail within chitosan-TGA conjugate gels. The ratio between the G' and the G'' is described by $\tan\delta$. The significant increase of the elastic properties

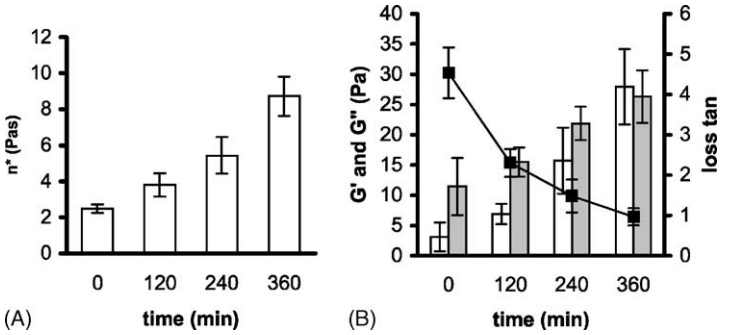

Fig. 3. (A) Increase in dynamic viscosity (η^*) at pH 5.5 with time of hydration at 37 °C. Indicated values are means (±S.D., $n = 3$). (B) Comparison of the elastic modulus G' (white bars) and viscous modulus G'' (grey bars) of chitosan-TGA conjugate solution at pH 5.5, respectively, and the decrease in loss tan δ (■). Indicated values are means (±S.D., $n = 3$).

of chitosan-TGA conjugate is evidenced by a decrease in loss tan δ (Fig. 3B).

These results indicate the in situ gelling property of chitosan-TGA conjugate. It might be very useful in tissue engineering. Since the in situ gelling depends mostly on the amount of thiol groups immobilised to the polymer and on the pH (data not shown), gel-like scaffolds with a specific shape being similar to the native tissue may be formed at a physiological pH and at 37 °C.

3.3. In vitro studies

In vitro studies with L-929 mouse fibroblasts were conducted for 4 weeks. The medium was changed three times a week to maintain ideal incubation conditions for the cells. The structure of the lyophilised chitosan-TGA remained stable despite incubating with different kinds of alcohol. The porosity of this material was achieved by freezing the original chitosan-TGA solution at −80 °C and lyophilising it immediately afterwards. Furthermore, during the incubation with the cell culture medium the thiol groups could be oxidised and additional pores may be formed. Consequently, the support of essential substances may be provided in three dimensions within the scaffold. During incubation with the culture medium, the sheets started to hydrate to a certain extent but kept its shape within the whole period of examination.

In comparison, the structure of the gel-like conjugate changed. The amount of thiol groups decreased because of sterilising at 121 °C (data not shown). Nevertheless, thiol groups could still be determined afterwards. During the incubation of the cells on the polymer at 37 °C, disulphide bonds could be formed and stabilise the scaffold material additionally. This will provide a semi-solid surface for the cells which allows the cells to migrate also into the scaffold without loosing contact to essential proteins and nutrients. As shown in Fig. 4A-D, the cells grew better on the chitosan-TGA sheet compared to the gel as scaffold. This observation may be explained by the different surface structure of these two kinds of scaffolds. The porous chitosan-TGA sheets showed a rough and uneven surface, whereas the gels were of soft consistency with a smooth surface. The more porous structure of the chitosan-TGA sheet seems to be advantageous for the supply of essential nutrients for the cells and results in a faster and more dense growth of the cells on the scaffold. Concerning the viability of the used L-929 cells, it seemed that the introduction of thiol groups on chitosan had no toxic effect on the cells.

3.4. Future trends

Due to the in situ gelling properties and the controllable biodegradation, it might be possible to design a certain shape of scaffold material mimicking the tissue being restored with a stated breakdown. First, attempts have already been undertaken with amphiphilic block copolymers of poloxamer. They are liquid at room temperature and start to gel above 25 °C (Jeong et al., 1997, 1999). Chenite et al. (2000) described a new in situ gelling system based on chitosan. By the combination of chitosan with polyol salts a new thermally sensitive gelling system could be developed (Chenite

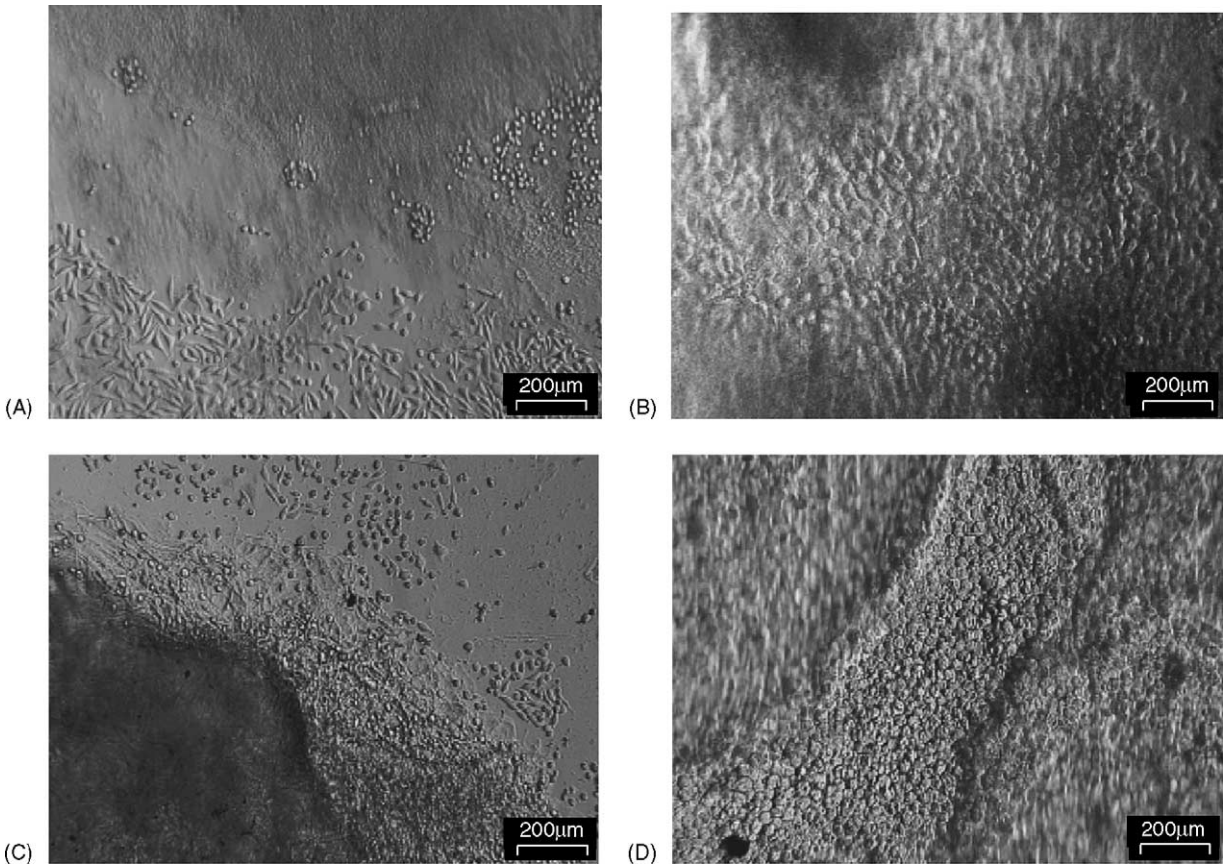

Fig. 4. (A) L-929 mouse fibroblasts seeded onto chitosan-TGA gel after 6 and (B) after 24 days of incubation. (C) L-929 mouse fibroblasts seeded onto chitosan-TGA sheet after 6 and (D) after 24 days of incubation.

et al., 2000). Furthermore, a polymer solution may be applied by injection forming semi-solid scaffolds at the site of tissue damage. Since chitosan-TGA conjugate hydrated in water, is liquid at room temperature and is rapidly gelling at 37 °C, it seems to be a promising candidate for such application. This system might also be used for a sustained drug delivery from a gel-like depot located subcutaneously (Ruel-Gariepy et al., 2000).

4. Conclusion

The introduction of thiol groups to chitosan led to a new polymer exhibiting various useful properties for tissue engineering. The controlled biodegradability and the in situ gelling property may allow the design of specified scaffolds with defined parameters. Hence, chitosan-TGA conjugate represents a new class of scaffold materials for bio-artificial tissues and organs.

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