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The use of bifunctional polyethyleneglycol derivatives for coupling of proteins to and cross-linking of collagen matrices

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The realization of three-dimensional (3D) degradable matrices which slowly release bioactive components represents a major challenge in the field of tissue engineering. In this paper we report on the usage of commercially available bifunctional agents for both the covalent coupling of proteins to and the cross-linking of collagen matrices. Proteins – horse radish peroxidase (HRP) was used as a model protein – were cross-linked with either a homobifunctional (disuccinimidyldisuccinatepolyethylene-glycol) or a heterobifunctional (N-hydroxysuccinimidylvinylsulfonepolyethyleneglycol) agent. In the case of the heterobifunctional cross-linking agent the collagen matrices were previously modified with succinimidylacetylthioacetate in order to introduce sulfhydryl groups. As compared with control experiments a 10-fold and 50-fold increase of immobilized proteins were achieved with the homobifunctional and heterobifunctional cross-linker resp. The HRP–PEG conjugates demonstrated a better long-term stability as compared to the non-treated HRP. The effects of the cross-linking agents and the thiolation reagent succinimidylacetylthio acetate on the *in vitro* degradation of the collagen matrices by collagenase were also investigated. In particular the reaction with succinimidylacetylthio acetate appears to offer interesting opportunities both for coupling active proteins and modulating the degradation times of collagen matrices.

1. Introduction

One of the aims of the interdisciplinary field of research termed tissue engineering is the regeneration or replacement of lost cells and tissue by applying modern bioengineering techniques [1–3]. Regeneration of desired functional tissue requires regulation of cellular adhesion, modulation of the direction and speed of cell migration, encouragement of cell proliferation, maintenance of cell viability and the differentiated function [1, 4].

Soluble signaling proteins, such as growth factors, have proven to be valuable tools for the control of many of the cellular behaviors associated with tissue regeneration and formation [5].

The great advances in biotechnology, such as recombinant technologies, have made it possible to produce large quantities of highly purified polypeptides and proteins. However, in general, direct injection of bioactive proteins such as growth factors – even with extremely high doses – into the target site is ineffective, since the *in vivo* stability of many of these agents are short, normally of the order of minutes, a time scale which is shorter than the time usually required for a tissue response [6].

The main goal of the present study is to develop a protein delivery system, which releases bioactive proteins in a controlled manner at the site of action over a longer period of time. A controlled and sustained release may be achieved by many different approaches [1]. One of the approaches comprises the tethering of proteins to natural or synthetic matrices via a specific linkage strategy. The advantage of this procedure is that covalently bound proteins will be released simultaneously with the degradation of matrix, whereas admixed and non-cross-linked proteins will be washed out on a much faster time scale. In the present study, matrices made of collagen were chosen as the carriers of the bioactive proteins. Collagen represents a suitable substrate for cell attachment, it is biocompatible and degrades into harmless products, that are metabolized or excreted [7]. Collagen can be formed into three-dimensional (3D) matrices which may be applied as a tissue substitutes and scaffolds for tissue regeneration. Therefore, the main focus of the

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present study has been on the immobilization of proteins in 3D collagen matrices, which will release their protein load on a time scale, and this process is controlled by the degradation of the matrix.

Since growth factors, in spite of the recombinant technologies, are still rather expensive we started our investigations with a model protein, horse radish peroxidase (HRP). HRP was coupled to the collagen matrix along two procedures with two different, commercially available cross-linking agents. One procedure, which involves the introduction of thiol functions into the collagen matrix [8], also leads to an internal cross-linking of the collagen and thus to a concomitant change of the *in vitro* degradation [9]. This procedure thus enables us to potentially modulate the *in vivo* degradation of the collagen matrix and as a consequence the release rates of the immobilized proteins.

In this publication we present results, which demonstrate that the use of bifunctional coupling agents, in combination with the introduction of sulfhydryl groups, may be useful for the development of 3D matrices releasing bioactive proteins in a controlled and sustained manner.

2. Materials and methods

2.1. Three-dimensional collagen matrices

Collagen sponges were manufactured according to a directional freeze-drying procedure for producing matrices with a uniform porous microstructure [10, 11]. The basic material is a suspension of fibrillar collagen isolated from bovine skin, which contains 1.8 wt % of type I collagen (a generous gift of Dr Suwelack Skin & Health Care AG, Billerbeck, Germany to the Helmholtz Institute for Biomedical Engineering Aachen, Germany). In order to adjust the pore size of the collagen sponge, 1.5 wt % acetic acid was added to the collagen suspension resulting in pH = 2.8. The suspension was then directionally solidified under thermally constant freezing conditions succeeded by a freeze-drying process. Thus, collagen sponges with a uniform microstructure and a defined pore size of about 20 µm were obtained [10].

2.2. Preparation of HRP–PEG_{3400}–SS or HRP–PEG_{3400}–VS conjugates

The commercially available cross-linkers disuccinimidyldisuccinatepolyethyleneglycol (SS–PEG_{3400}–SS) and succinimidylvinylsulfonepolyethyleneglycol (NHS–PEG_{3400}–VS) (Shearwater Polymers Inc., Huntsville, USA) were used to covalently couple HRP to collagen. The covalent cross-linking was achieved through a multi-step process. The first step reaction (conjugation reaction) leads to the synthesis of protein–PEG_{3400}–SS or protein–PEG_{3400}–VS conjugates. 5 µg HRP are dissolved in 250 µl phosphate buffer saline buffer (PBS) and allowed to react with 20 µg SS–PEG_{3400}–SS or NHS–PEG_{3400}–VS for 10 min at room temperature. One of the two SS groups of the homobifunctional cross-linker or the NHS group of the heterobifunctional cross-linker reacts with amino groups of the protein to form SS–PEG_{3400}–protein resp. VS–PEG_{3400}–protein conjugates.

The reactions can be described as:

$$\text{HRP–NH}_2\text{–SS–PEG}_{3400}\text{–SS} \longrightarrow \text{HRP–PEG}_{3400}\text{–SS (conjugate)}$$
$$\text{HRP–NH}_2\text{–NHS–PEG}_{3400}\text{–VS} \longrightarrow \text{HRP–PEG}_{3400}\text{–VS (conjugate)}$$

Subsequently the entire reaction mixtures are applied to a desalting column (NAP™ 25 column, Pharmacia Biotech, Uppsala, Sweden) to stop the reaction and to separate the conjugates from excess cross-linker. The conjugates are now ready for being coupled to the collagen matrices.

2.3. Introduction of SH-groups into collagen matrices

Three-dimensional collagen matrices ($0.5 * 0.5 * 0.2$ cm, 2 mg) were immersed in 250 µl of 50 mM sodium phosphate solution (pH 7.5, containing 1 mM EDTA) and allowed to react with 150 µg succinimidylacetylthioacetate (SATA, Pierce) for 30 min at room temperature. The reaction was then stopped by an extensive washing procedure with the sodium phosphate buffer. The acetylated SH groups were deprotected by adding 250 µl of 0.5 M hydroxylamine hydrochloride ($NH_2OH \cdot HCl$, Pierce) in 50 mM sodium phosphate (pH 7.5), the hydrolysis was assumed to be complete after 2 h at room temperature. Subsequently the collagen sponges were extensively washed with PBS in order to remove the excess of hydroxylamine hydrochloride. To test the degree of SH-modification, the sponges which had been treated with SATA and NH_2OH were allowed to react with 200 µg Ellman's reagent (5,5′-dithio-bis-[2-nitrobenzoicacid], Pierce) in 2500 µl of sodium phosphate buffer for 15 min. After determination of the absorbance at 412 nm, the sulfhydryl contents of the collagen matrices were deduced from standard curves obtained with cysteine.

2.4. Coupling of HRP–PEG–SS and HRP–PEG–VS conjugates to collagen

Covalent binding of SS–PEG_{3400}–HRP conjugates to collagen was achieved by adding 5 µg of the conjugates to collagen matrices ($5 \times 5 \times 2$ mm) in 250 µl PBS, the reaction was performed overnight at room temperature.

Cross-linking of HRP–PEG_{3400}–VS was performed by adding 5 µg of HRP–PEG_{3400}–VS, which previously had been unmasked by NH_2OH, to collagen matrices with SH-groups introduced as described in Section 2.3.

2.5. Determination of HRP-activity

To determine the HRP-activity 10 µl of a solution of 10 mg tetramethylbenzidine (TMB, Merck, Darmstadt, Germany) in 1 ml dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) and 10 µl 0.3% H_2O_2 (Merck, Darmstadt, Germany) were added to 1 ml samples and to 1 ml of the buffer solution for the reference. The reaction was allowed to proceed for 15 min and stopped with 1 ml 10% H_2SO_4. The HRP activity is obtained by determining the absorbance at 450 nm and comparing the data with standard curves.

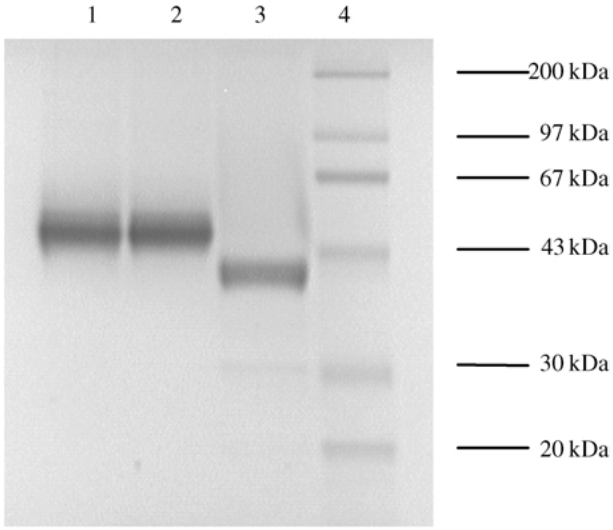

Figure 1 SDS-polyacrylamidegel electrophoresis of native HRP and HRP–PEG conjugates. Lane 1: HRP–PEG_{3400}–VS conjugate; Lane 2: HRP–PEG_{3400}–SS conjugate; Lane 3: and HRP–PEG_{3400}–VS conjugate; Lane 4: marker proteins.

Prior to the testing of the covalent coupling efficiency of HRP–PEG_{3400}–SS or HRP–PEG_{3400}–VS conjugates binding to collagen sponges, a washing procedure was performed. After being exposed in an overnight period to HRP, HRP–PEG_{3400}–SS or HRP–PEG_{3400}–VS conjugates, the collagen sponge specimens were extensively washed with 1 ml PBS (6 times), the sponges were then degraded with collagenase. The released HRP is now available for quantitative analysis.

2.6. SDS-Polyacrylamidegelelectrophoresis

Sodiumdodecylsulfate-polyacrylamidegelelectrophoresis (SDS-PAGE) was performed to analyze the reaction of HRP with SS–PEG_{3400}–SS or VS–PEG_{3400}–NHS (Fig. 1). Samples were prepared as follows: To prepare the samples, 20 µg of HRP were allowed to react with 80 µg SS–PEG_{3400}–SS or NHS–PEG_{3400}–VS in PBS solution (20 µl, pH 7.2) for 10 min. Samples were diluted 1 : 1 with sample buffer (15% sucrose; 4% SDS; 62.5 mM Tris; 1% β-mercaptoethanol; pH 4.8) and incubated in a 95 °C water bath for 5 min. Standard HRP sample was treated in the same way. Samples were run on a 10% acrylamide gel overnight (150 V). The gel was stained with Coomassie Blue to visualize protein.

2.7. Reversed phase chromatography

Samples were prepared as follows: 20 µg HRP were reacted with 80 µg SS–PEG_{3400}–SS in 20 µl PBS solution (pH 7.2) for 10 min, the reaction was stopped by ultrafiltration (Millipore YM 10 000, Bedford, USA). HRP (20 µg) dissolved in 20 µl PBS solution was taken as the control. After the ultrafiltration procedure, samples were immediately acidified to pH 3.0 by dilution with 1 volume of 0.1% trifluoroacetic acid (TFA, Merck, Darmstadt, Germany) and chromatographed on a C18 reverse phase column (Vydac 218TP54, 0.46 ∗ 25 cm), connected to an HPLC system (Hewlett-Packard System 1050). Solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile) were mixed for obtaining a two-step gradient with plateaus at 38% and 52% solvent B. The column was run at room temperature and a flow rate of 1 ml/min.

2.8. Matrix assisted laser desorption ionization time-of-flight spectrometry

Molecular mass determination was performed on an MALDI-TOF mass spectrometer (BiflexTM III, Bruker-Franzen Analytik GmbH, Bremen, Germany) equipped with a multiprobe gridless delayed extraction ion source and an ion-mirror. The laser system consists of a pulsed nitrogen laser with 337 nm wavelength and 3 ns pulse width. Ion acceleration voltage is in the range of 20 kV and the ion-mirror voltage was 23.5 kV. For delayed extraction, a 6 kV potential difference between the probe and the extraction lens was applied with a time delay in the range of 150–200 ns after each laser pulse using high voltage switch. A micro-channel plate detector with 2 kV post acceleration was used for ion detection both in linear or reflection mode. Mass spectra were acquired as the sum of ion signals generated by irradiation of the target with 100–250 laser pulses.

For MALDI analysis, 50 µg of HRP (50 µg) were allowed to react with 200 µg SS–PEG_{3400}–SS or 200 µg NHS–PEG_{3400}–VS for 10 min in 500 µl PBS, pH 7.2. A sample – corresponding to 10 nmol – was then diluted 1 : 10 with water and mixed with an equal volume of a saturated solution of trans-3,5-dimethoxy-4-hydroxy-cinnamic acid. 0.5 µl of this mixture – corresponding to 250 pmol – were then applied to a stainless steel disk, washed several times with 0.1% trifluoroacetic acid and dried. Mass spectra were calibrated externally with carbonic anhydrase (Mr 29026) and bovine serum albumin (Mr 66431). A solution of 50 µg HRP in 500 µl water was taken as control.

2.9. Stability of the HRP–PEG–SS and HRP–PEG–VS conjugates

To determine the stability of HRP–PEG_{3400}–SS or HRP–PEG_{3400}–VS conjugates, the conjugation reaction was stopped after 10 min by removing the excess of cross-linker by gelfiltration on a desalting column (NAP™25). Solutions were incubated for 8 days at room temperature and HRP-activities were determined at time intervals of 24 h.

2.10. Degradation of collagen matrices by collagenase

Specimens of collagen sponges were immersed in 0.25 ml of a solution containing collagenase (from *Clostridium histolyticum* (type IA), Worthington Biochemical Corporation, Lakewood, USA) at a concentration of 40 U/ml. Specimens (0.5 × 0.5 × 0.2 cm) were incubated at 37 °C and observed at intervals of 5 min during incubation. Incubation times corresponding to the complete disappearance of the collagen matrices were monitored.

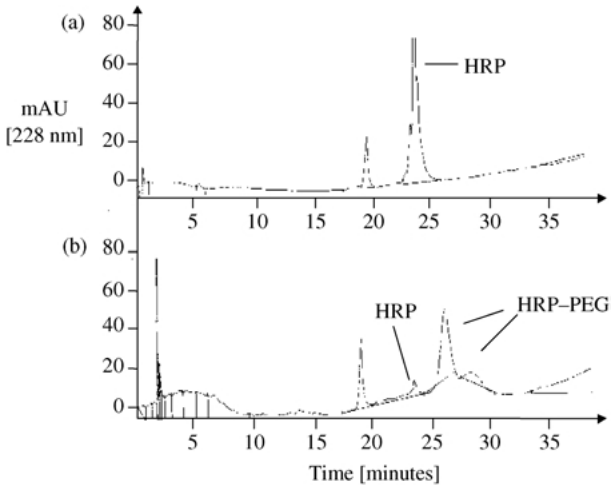

Figure 2 Reversed Phase-HPLC chromatograms of native HRP (a) and HRP–PEG_{3400}–SS conjugate (b). The conjugate was obtained after a reaction time of 10 min and the chromatographic procedure were performed as described in Materials and methods.

2.11. Determination of free amino groups

The primary amino group content of the collagen sponge samples was determined spectrophotometrically (492 nm wavelength) after reaction of primary amino groups with Sulfosuccinimidyl-4-O-(4,4′-dimethoxytrityl)butyrate (STDB, Sigma, St. Louis, USA), and subsequent hydrolysis of the reaction products by $HClO_4$. The amount of amino groups and the degree of cross-linking are calculated by using the equations:

$$A = (\text{Absorbance}_{427} - 0.01)/0.007$$

(A: concentration of amino groups in [mol/l])

$$\text{Cross-linking degree}(\%) = \frac{(A \text{ of control group} - \text{A of sample}) * 100}{(A \text{ of control group})}$$

3. Results and discussion

3.1.1. Conjugation of HRP with SS–PEG_{3400}–SS or NHS–PEG_{3400}–VS

The formation of HRP-PEG_{3400}–SS or HRP–PEG_{3400}–VS conjugates was monitored by three different methods SDS-PAGE, Reversed Phase HPLC and MALDI-TOF spectrometry.

Optimal reactions were obtained from a series of experiments in which the concentrations, the molar ratio of HRP and the cross-linking agents, the pH and the reaction time were varied. The following conditions were found to give the best: results: (a) a concentration of 1 mg HRP per ml and (b) a molar ratio of 1 molecule HRP per 50 molecules cross-linker; pH 7.2 and a reaction time of only 10 min.

The result of the polyacrylamidegel electrophoresis are shown in Fig. 1. The apparent molecular mass of the non-modified HRP corresponds to the theoretical mass deduced from the biochemical characteristics of this glycoprotein (mass of the protein moiety: 33 kD; mass of the carbohydrate moiety: 11 kD). After the reaction of HRP with SS–PEG_{3400}–SS and NHS–PEG_{3400}–VS resp. newly formed bands appear with apparent molecular masses of about 48 kDa. This molecular mass nicely corresponds to the sum of the mass of one molecule HRP and the mass of one molecule of the cross-linking agents. In addition to conjugate species which comprise one molecule of the PEG derivative also conjugate species which most likely comprise two PEG molecules are observed.

The same samples were also analyzed by Reversed Phase HPLC. In the experiment corresponding to Fig. 2(a), native HRP was injected. The peak appearing at a retention time of 24 min thus represents non-modified HRP. Fig. 2(b) correspond to an experiment in which HRP and SS–PEG_{3400}–SS were allowed to react for 10 min at the conditions described above. New peaks emerge at 26 and 28 min; these peaks most likely correspond to the newly formed HRP–PEG_{3400}–SS conjugates. As in the SDS-PAGE-electrophoresis experiment the original HRP has been transformed into two species of HRP–PEG_{3400} conjugates.

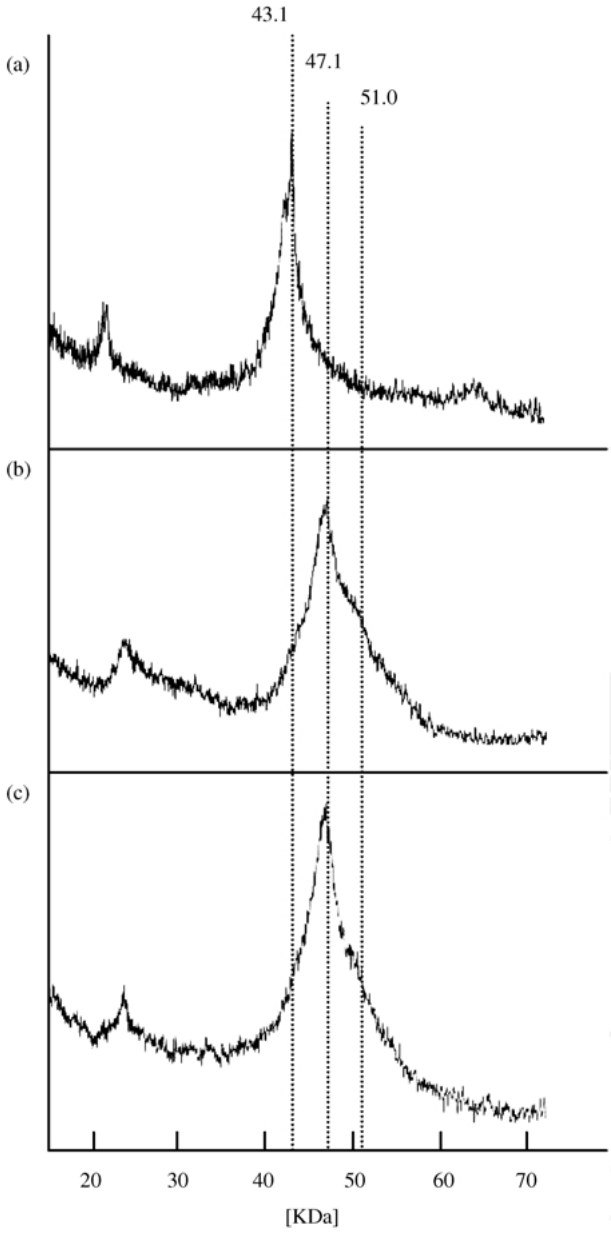

Figure 3 Maldi-tof mass spectra of native HRP (a), HRP–PEG_{3400}–SS conjugate (b) and HRP–PEG_{3400}–VS conjugate (c). The conjugates were obtained after a reaction time of 10 min and the spectra were obtained as described in Materials and methods.

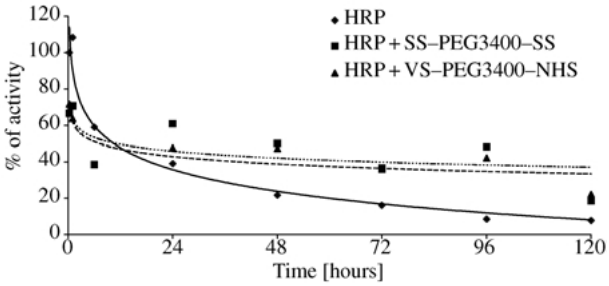

Figure 4 Activities of native HRP (◆), as well as HRP–PEG_{3400}–SS (■) and HRP–PEG_{3400}–VS (▲) conjugates as functions of time.

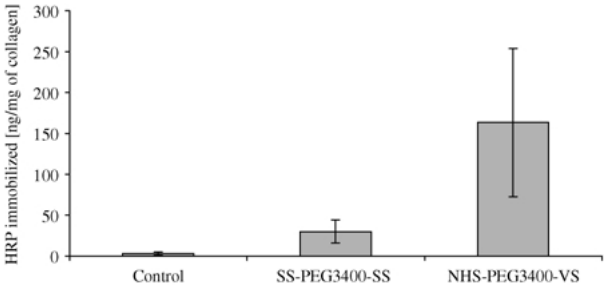

Figure 5 Binding of HRP to collagen matrices (2 mg; size: $0.5 * 0.5 * 0.2$ cm; volume: 0.05 cm^3) in the absence of cross-linking agents and in the presence of SS–PEG_{3400}–SS on NHS–PEG_{3400}–VS. Column heights correspond to the mean values and the error bars to the standard deviations ($n = 6$).

This result thus is in good agreement with the results of the SDS-PAGE experiment. Further evidence for the nature of the conjugates was obtained by MALDI-TOF spectrometry. This technique allows the determination of mass of the native and conjugated products. Fig. 3 shows the molecular mass of native HRP (43.4 kD) (a), the products of the reaction between HRP and SS–PEG_{3400}–SS (b) and between HRP and NHS–PEG_{3400}–VS (c). Deduced from the latter molecular mass, we can demonstrate the formation of HRP–PEG_{3400}–SS and HRP–PEG_{3400}–VS conjugates. The reaction parameters are the same as described for the RP–HPLC experiments. The calculated molecular mass of HRP–PEG_{3400}–SS and HRP–PEG_{3400}–VS conjugates (47 kD) is in good agreement with the mass experimentally determined.

3.1.2. *Stability of the* HRP–PEG_{3400}–SS *and* HRP–PEG_{3400}–VS *conjugates*

As a bio-active enzyme, HRP loses part of its activity as a function of time when incubated in PBS. Fig. 4 shows that after 24 h of incubation in PBS at room temperature, the activity has been reduced to less than 40% of the original HRP activity. Polyethyleneglycol is an uncharged hydrophilic polymer and typically increases solubility and stability of active enzyme molecules when attached to them [12, 13]. The HRP–PEG_{3400}–VS and HRP–PEG_{3400}–SS conjugates demonstrated – although their activities initially had been reduced to about 60% of the non-treated HRP – an obviously slower loss of activity (Fig. 4). This observation strongly suggests that conjugation of HRP with the PEG-derivatives, substantially increases the stability of the conjugated HRP. The initial loss of HRP activity after conjugation, may be due to the presence of the PEG spacer on or near the active site of HRP. It has been reported that modification of the amino groups of lysozyme by various acylating reagents resulted in complete loss of activity [14].

3.2. Coupling of conjugates to collagen matrices

The necessity of performing multi-step procedures was very well discussed by Hermanson [15]. Prior to the second step reaction, i.e. the coupling of the conjugate to the collagen matrix, the excess of the cross-linkers was removed through chromatographing the mixture on a desalting column by which the conjugation reaction was stopped. If the excess cross-linker molecules are not removed by the gel-filtration procedure, they may competitively block reactive amino-groups.

Fig. 5 shows that after the cross-linking reaction followed by an extensive washing procedure, a 10 times higher amount of HRP (about 30 ng/per mg collagen) was observed in the group that HRP was covalently coupled to collagen via the homobifunctional cross-linker SS–PEG_{3400}–SS, when compared with the control group in which HRP was loosely non-covalently adsorbed in the collagen sponges. By using the heterobifuctional cross-linker NHS–PEG_{3400}–VS – in combination with the introduction of sulfhydryl groups in the collagen matrix – a 50–60 times higher amount of HRP (about 160 ng/mg collagen) was immobilized as compared to the adsorptive binding in the absence of the cross-linking agents.

The lower cross-linking efficiency as observed in the case of the homobifunctional cross-linker SS–PEG_{3400}–SS may be due to the fact that succinimidylester bonds have half-lifes of only 10 min [16]. Thus, it may be assumed that many of the succinimidylester functions have already been hydrolyzed in the course of the first reaction step and that they are no longer available for cross-linking the HRP–PEG conjugate to the collagen. As shown by our data this problem can be overcome by using the heterobifunctional cross-linker NHS–PEG_{3400}–VS. In this case, the second functional group for cross-linking the HRP–PEG conjugate to collagen is a vinylsulfone group, which remains stable until being unmasked with hydroxylamine to yield sulfhydryl groups (Fig. 6).

A prerequisite for a satisfying cross-linking efficiency with the heterobifunctional agent is the availability of sufficient sulfhydryl groups. The sulfhydryl functions were introduced with the reagent succinimidylacetyl-thioacetate (SATA). The extent of the modification was tested with Ellman's reagent, the result of this investigation is shown in Fig. 7. The sulfhydryl content of the collagen matrices was determined as a function of the amount of the thiolating reagent SATA. Fig. 6 shows that (i) no thiol groups were detected in the non-treated collagen sponges and (ii) the number of the newly formed thiol groups clearly correlate with the amount of reagent used for the thiolating reaction. The absolute value of 15–30 μmol SH-groups per mg collagen represents a satisfying result, since it should allow the cross-linking of protein amounts in the order of 1 μg per mg of collagen.

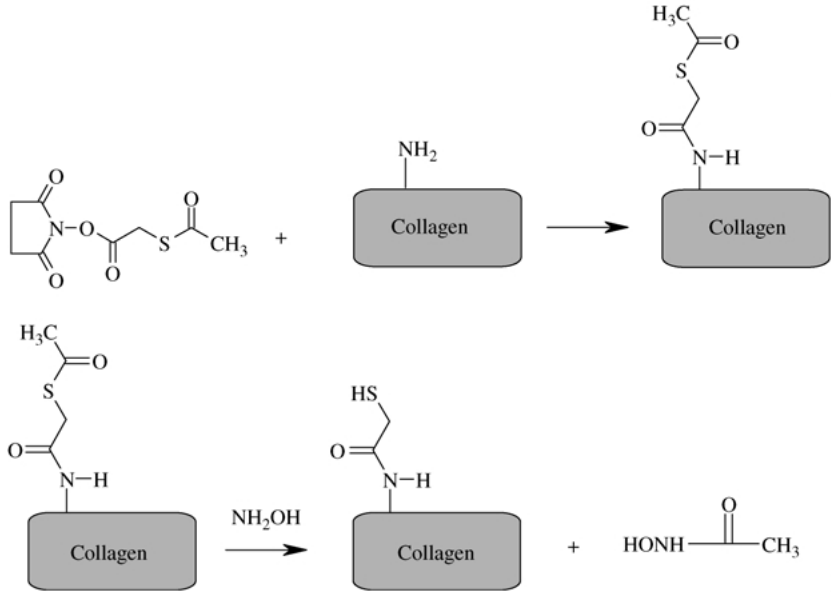

Figure 6 Introduction of sulfhydryl groups into collagen matrices with succinimidylthioacetate (SATA).

3.3. Cross-linking of collagen and *in vitro* degradation

Depending on the utilization of the matrix different degradation behaviors may be required [17–19]. For the application as a drug delivery system, an implant has to maintain its structural integrity and porosity, the degradation should allow in-growing cells to proliferate and to secrete their own extracellular matrix, thus being gradually replaced by endogenous collagen. In order to slow down the degradation process it may be necessary to introduce additional cross-links into the collagen network. Conventional cross-linking procedures often use homobifunctional reagents, which form bridges between amino acid side chains of adjacent collagen molecules. Among them, glutardialdehyde is widely used. However, the degradation products of matrices cross-linked with glutardialdehyde often induce cytotoxicity and inflammation [9, 20].

As mentioned in the introduction, also cross-linking agents like SS–PEG_{3400}–SS may be used to extra cross-link the collagen molecules within the collagen sponge. By this procedure collagen sponges may become more resistant against enzyme attack, leading to longer degradation times, which may be advantageous for controlled release systems.

The *in vitro* degradation of modified and non-modified collagen sponges was studied by exposing the materials to collagenase from *Clostridium histolyticum*. As shown in Fig. 8, the results show that at cross-linker con-centrations in excess of 4 mg/ml, the degradation of SS–PEG_{3400}–SS treated collagen sponges was substantially delayed as compared to non-treated collagen sponges.

As expected the cross-linking of collagen using SS–PEG_{3400}–SS results in a decrease of the amino groups. As shown in Fig. 8, collagen sponge cross-linking degree rises as the increase of SS–PEG_{3400}–SS concentration,

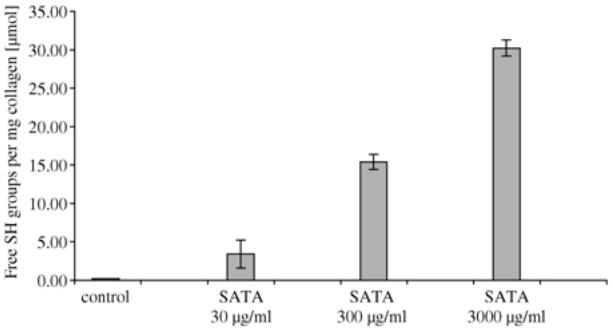

Figure 7 Dependence of the number of sulfhydrylgroups on collagen matrices as a function of the concentration of succinimidylthioacetate (SATA). Column heights correspond to the mean values and the error bars to the standard deviations $(n = 6)$.

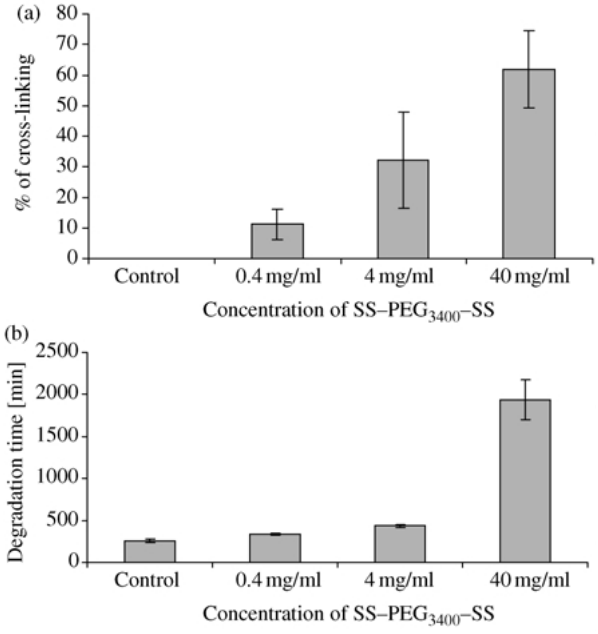

Figure 8 Dependence of the *in vitro* degradation time of collagen matrices cross-linked with varying concentrations SS-PEG_{3400}-SS (A) and dependence of the cross-linking degree on varying concentrations of SS-PEG_{3400}-SS (B). Column heights correspond to the mean values and the error bars to the standard deviations $(n = 6)$.

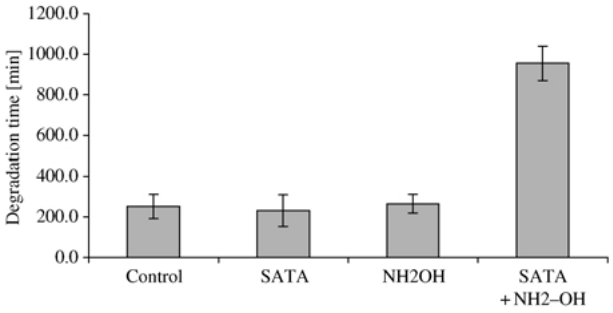

Figure 9 In vitro degradation times of collagen matrices under various conditions. Degradation times correspond to times in minutes required for complete dissolution of the sponges. Column heights correspond to the mean values and the error bars to the standard deviations ($n = 6$).

but it decreases when reaction lasts longer which is in good agreement with the results of *in vitro* degradation experiments.

The results of Fig. 9 show only minor differences between the degradation behavior of non-treated sponges and matrices treated with SATA, EDTA or NH_2OH alone. Only the specimens, which were treated with both SATA and NH_2OH showed a substantial increase of the resistance versus proteolysis by collagenase. We therefore assume that the formation of disulfide bridges upon air oxidation may explain the observed delay in the degradation. Similar results were observed earlier by Nicolas and Gagnieu [8, 9].

4. Conclusion

This investigation demonstrates that a covalent coupling of proteins – HRP was used as a model protein – to collagen matrices via the homobifunctional cross-linker SS–PEG_{3400}–SS and the heterobifunctional cross-linker NHS–PEG_{3400}–VS can be achieved. The application of SS–PEG_{3400}–SS resulted in a 5–6 times higher binding as compared to the adsorptive binding in the absence of the cross-linker. The use of the heterobifunctional cross-linker NHS–PEG_{3400}–VS leads to 50–60 times higher binding density as compared to the binding in the absence of the cross-linker.

The HRP–PEG conjugates have a more stable long-term bioactivity as compared to the non-modified HRP.

The cross-linking of collagen through either cross-linking agents or the formation of disulfide bridges introduced by succinimidylacetylthioacetate stabilize the collagen matrices and slow down their degradation *in vitro*.

Our results suggest that the procedures described are useful for manufacturing collagen matrices, which slowly release bioactive proteins.

Acknowledgments

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