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Characterisation of a new bioadhesive system based on polysaccharides with the potential to be used as bone glue

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Abstract Although gluing bone is in theory a very attractive alternative to classical fracture treatment, this method is not yet clinically established due to the lack of an adhesive which would meet all the necessary requirements. We therefore developed a novel two-component bioadhesive system with the potential to be used as a bone adhesive based on biocompatible and degradable biopolymers (chitosan, oxidised dextran or starch). After mixing in water, the two components covalently cross-link by forming a Schiff's base. By the same mechanism, the glue binds to any other exposed amino group such as for example those exposed in fractured bone, even in the presence of water. Modified chitosan was synthesised from commercially available chitosan by deacetylation and was then reduced in molecular weight by heating in acid. The amount of free amino groups was analysed by IR. The molecular weight was determined by viscosimetry. Starch or dextran were oxidised with periodic acid to generate aldehyde groups, which were quantified by titration. L-Dopa was conjugated to oxidised dextran or starch in analogy to the gluing mechanism of mussels. Biomechanical studies revealed that the new glue is superior to fibrin glue, but has less adhesive strength than cyanoacrylates. In vitro cell testing demonstrated excellent biocompatibility, rendering this glue a potential candidate for clinical use.

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1 Introduction

The method usually employed to repair bone fractures is mechanical fixation with nails and screws. In theory, bone adhesives provide an attractive alternative to classical fixation methods, especially when it comes to small bone fragments, which experience only minor mechanical stress. However, a bone adhesive designed for clinical application has to meet a long list of requirements (for review see [1]). First of all, it has to react chemically with the surface of the tissue and meanwhile has to establish a strong cross-linking network within the glue. By the same time, it has to provide high bonding strength in the presence of physiological body fluids. In addition, it should be biocompatible and resorbable, and its degradation products must not be toxic. Ideally, it should favour the healing process of the damaged tissue and it should not cause an immune response. Although a number of biological glues based on gelatine [2–5], collagen [6, 7], polysaccharide [4, 8], reuterin [9], glutardialdehyde [5, 10], cyanoacrylate or methacrylate [11–13] have been developed, no satisfactory bone glue is yet established for clinical practice.

Cyanoacrylate adhesives, which yield the highest bonding strength are currently being used to glue skin wounds.

Nonetheless, besides having toxic side effects [14], cyanoacrylates are not resorbable and thus inhibit endogenous bone repair. Fibrin glue, consisting of fibrinogen and thrombin, is biodegradable and therefore it is the most commonly used adhesive in clinical practice. However, its bonding strength is relatively weak and its degradation is fast. For this reason, it has predominantly been used to stop bleeding from parenchymatous organs such as the spleen or the liver. In addition, because of its origin from human blood, it bears the risk of infectious contamination. Problems with other tissue glues mostly arise from inadequate bonding strength in a watery milieu and toxic side effects, which are the two most important issues when designing bone and tissue adhesives for use in the human body.

Here, we report the synthesis as well as the chemical, biological and biomechanical characterisation of a two-component bioadhesive with the potential to be used as a bone adhesive based on degradable polysaccharides. The bonding mechanism employs the reaction of aldehyde groups with amino groups in the presence of water, which covalently bind to each other in a Schiff's base reaction. Because of its naturally available amino groups, chitosan was selected as the amino-group carrier. Chitosan has successfully been used in wound dressing [15] and tissue engineering [15, 16] applications, and a positive effect on cell attachment and on the spreading of osteoblasts has been shown [17]. Starch, a non-toxic biopolymer, was oxidised with periodic acid to provide the aldehyde groups. Alternatively, dextran may be used as the aldehyde-group carrier. The aldehyde groups will react with amino groups exposed by surrounding tissues on the one hand and with the chitosan component on the other. This enables a strong bonding to the tissue and a cross-linking within the adhesive, even in the presence of watery solutions. To further increase the bonding strength, the starch or dextran compound was additionally conjugated with L-Dopa. It has been found that L-Dopa is an important element of mussel adhesives [18–20], enabling the mussel to tightly attach onto rocks and other underwater surfaces.

Although gluing bone fragments are considered to be an attractive alternative to classical bone fixation methods, to date no bone adhesive has been successfully introduced into clinical practice. The purpose of this study was therefore to develop and characterise a two-component bone glue for clinical use based on resorbable, biocompatible polysaccharides.

2 Experimental

2.1 Materials

Chitosan of low molecular weight (Mol. wt.: 50.000–190.000; degree of deacetylation: 75–85%) and of medium molecular weight (Mol. wt.: 190.000–310.000; degree of deacetylation: 75–85%) was purchased from Sigma-Aldrich. Dextran (MW wt. = 15.000–20.000) was supplied from Fluka and starch (soluble, MW wt. = 30.000) from Merck. L-Dopa was obtained from Fluka. Ortho-periodic acid was purchased from Riedel-de Haën. Fibrin glue (Tissucol Duo S) and cyanoacrylate (Dermabond, 2-octyl cyanoacrylate) were used as reference and were purchased from Baxter (Unterschleissheim, Germany) and Ethicon (Somerville, New Jersey). The molecular weight of deacetylated chitosan was measured with an Ubbelohde viscosimeter at 25°C by using a Kap Ic capillary. The solvent used was a mixture of 0.2 M AcONa and 0.3 M AcOH. FT-IR–ATR measurements were carried out with an instrument of Thermo Nicolet (AVATAR 370 FT-IR). The pyrolysis temperature was measured by thermogravimetry (TG) under air atmosphere at a heating rate of 10 K/min (Linseis L81/1550). The pyrolysis gas was analysed by an MS-spectrometer (ThermoStar, Pfeiffer Vacuum).

2.2 Preparation and characterisation of deacetylated chitosan

Low molecular weight chitosan (1.5 g) was heated under argon atmosphere for 1, 14, 24, 48 or 72 h in 150 ml of 0.1 N HCl solution. After a certain reaction time, the pH-value was set to 5.8 and the mixture dialysed (Visking Dialysis tubing, regenerated cellulose, SERVA) against distilled water for an additional 72 h to remove products with smaller molecular weight. Finally, the solution was lyophilised. The molecular weight of chitosan was determined from intrinsic viscosity using the relationship reported by Rinaudo et al. [21]: $[\eta] = K \times \mathrm{MW}^a$ ($K = 0.082$, $a = 0.76$). The relationship of NH_2/NHAc was calculated by pH-titration [15]. The pH-titration showed two jumping points of the pH-value. The distance between these two points give the degree of NH_2/NHAc.

2.3 Preparation and characterisation of oxidised dextran and starch

Dextran or starch (1 g in 30 ml solution) were oxidised with 5 wt% periodic acid for different time spans (1, 14, 24, 48 or 72 h) at room temperature under stirring. The resulting products were dialysed against distilled water for 72 h to remove periodic acid and low molecular weight products. Finally, the product was freeze-dried. For qualitative analysis, the IR-spectroscopy was employed. IR-spectra show an additional band at 1644 cm^{-1}, indicating a C=O valence vibration of the CHO group of the oxidised species [22]. The amount of aldehyde groups introduced into dextran or starch was determined by titration. Five

grams of the sample (oxidised starch or dextran) were placed in a 100 ml Erlenmeyer flask and 50 ml of a solution (17.5 g hydroxyamino hydrochloride in 50 ml water and 200 ml propanol, which contained 3′,3″,5′,5″-tetrabromo-phenolsulfonaphthalein as indicator) were added. After stirring for 30 min, the solution turns yellow. Finally, the solution was titrated with NaOH until the solution became blue. The amount of NaOH corresponded to the concentration of aldehyde groups.

2.4 Measurement of adhesive strength

Because adhesive force has been tested on differing surfaces such as porcine thigh [6], porcine skin [3, 5, 23], porcine pericardia [2], or inorganic substrates [18, 24], it is difficult to compare the bonding strength of the various glues that have been developed and described up to date. We therefore chose a model, which would be as close to the intended use as possible. The adhesive strength was thus measured in a tear-off test on bovine cortical bone specimens. To this end, bovine femora were obtained from the local abattoir and cut into rectangular cuboids measuring 40 mm by 8 mm by 8 mm using a diamond-coated precision saw (Exact System, Norderstedt, Germany). At both ends of the cuboid, 4 mm diameter holes were drilled with a box column drill. Care was taken to keep the drill axes parallel. Finally, the predrilled cuboid was cut in half at the center of its longitudinal axis and the edges were chamfered. Thus prepared, the bone cuboids were soaked in 0.9% NaCl solution and stored at −20°C until further use.

The adhesive strength was tested for oxidised dextran and chitosan mixed at a ratio of 1:1. The chitosan constituent, which was hydrolysed for 8 or 24 h, was dissolved at 7 wt% in aqueous solution. The dextran constituent used was oxidised for 24 h either with or without the addition of L-Dopa and was dissolved at 20 wt% in aqueous solution. As a reference to the newly developed adhesives, cyanoacrylate glue (Dermabond, 2-octyl cyanoacrylate, Ethicon, Somerville, New Jersey) and fibrin glue (Tissucol Duo S, Baxter, Unterschleissheim, Germany) were also mechanically tested. The bone cuboids were thawed for 4 h in physiologic saline solution at 37°C. The contact surface was determined by measuring their individual edge lengths with a caliper. The contact surfaces were swabbed dry and both surfaces were then coated with a thin layer of either one of the adhesive's compounds or the control glues, respectively. The two counterparts of the cuboids were pressed together with a constant force of 10 N for 3 h while completely stored in physiologic saline solution at 37°C. After 3 h the glued cuboids were placed in a material testing machine (Zwick, Einsingen Germany) while still stored in saline solution. The two parts of the cuboids were strained at a constant velocity of 2 mm/min until tear-off under continuous registration of the load displacement curve using the testExpert software (Zwick, Einsingen, Germany). Adhesive strength was calculated from the ratio of force at tear-off and contact surface area. For each adhesive, a total of 10 specimens was employed. Differences in adhesive strength were assessed for statistical significance by the Wilcoxon signed rank test.

2.5 Cell culture

MC3T3 mouse fibroblasts were expanded in T225 flasks (Nunc, Wiesbaden, Germany) in minimum essential medium (MEM Alpha Medium with Glutamax, Gibco/Invitrogen, Karlsruhe, Germany) containing 10% fetal bovine serum supplemented with 2 ml penicillin/streptomycin per 500 ml. They were kept in a 5% CO_2/95% air incubator with 95% humidity at 37°C. Medium was changed three times per week. After 3 days of expansion culture cells were trypsinised and seeded for cytotoxicity testing at a density of 2×10^4 cells/cm^2.

2.6 Cytotoxicity testing

The cytotoxicity of the bone glue was examined by in vitro cell tests using MC3T3 cells according to ISO 10993 (EN ISO 10993 standard. Biological evaluation of medical devices—Part 5: Test for cytotoxicity: in vitro methods, 1999). A minimum of two experimental runs was performed, all experiments were done in triplicates. Prior to testing, cylindrical glass slides of 15 mm in diameter and 2 mm of thickness were coated with modified chitosan and oxidised starch in a layer-to-layer technique, resulting in a total of 20 layers. The immobilisation was carried out analogously to the functionalisation of hydroxyapatite powder described in detail elsewhere [22]. In brief, the first step of functionalisation is the silanisation with 3-aminopropyltriethoxysilane. The generated amino groups at the surface are used to immobilise oxidised starch and chitosan alternately on the surface. To this end, the glass slides were incubated with a 1 wt% solution of either polysaccharide for 2 h in an alternating fashion until a total of 20 layers was achieved. Prior to cytotoxicity-testing, the glue-coated glass slides were gamma-sterilized. Viability testing was started by seeding the cells on the glass cylinders in regular tissue culture wells. For each testing run, a negative control (uncoated glass slide) and a positive control (uncoated glass slide treated with 0.5% Triton X 100 prior to evaluation) were included. The cells were incubated at standard conditions for 1, 3 or 6 days and evaluated by live-dead-assay (see below).

2.7 Live-dead-assay

To assess survival of cells cultured on the new bone adhesive, cells were incubated with fluoresceindiacetate (FDA) and propidium iodide (PI) and evaluated by fluorescence microscopy. Using this assay, viable cells fluoresce bright green, while dead cells are bright red. FDA and PI were purchased from Fluka (Sigma-Aldrich, Munich, Germany). Prior to testing, FDA was freshly dissolved in acetone according to manufacturer's instructions and diluted 1:500 in phosphate buffered saline (PBS). To obtain the final FDA/PI staining solution, diluted FDA was mixed at a ratio of 1:1 with PI.

For testing, the medium was removed from the wells and cells were washed with PBS. The positive control well was incubated with 0.5% Triton X 100 for 1 min. The other control well was left untreated and served as negative control. Each well was then incubated for 1 min with 500 µl FDA/PI staining solution. Cells were then washed with PBS and subsequently analysed by fluorescence microscopy using an Axioskop 2 microscope equipped with a 75 W mercury lamp (Zeiss, Munich, Germany). To detect red and green fluorescence, the Zeiss filter sets #10 and #15 were used. Pictures were taken with a Zeiss black and white digital camera (AxioCam MRm), processed with the Zeiss Axiovision software and saved as zvi-files (Zeiss). Dead and alive cells were counted per 3 random high power fields (HPFs) and averaged. The percent vitality of each sample was calculated. Percent vitality of cells in two separate experimental runs were averaged and given as final results. Statistical analysis was performed using SigmaPlot version 8 (SPSS, Munich, Germany). Significances were calculated using student's *t*-test. A value of $P < 0.05$ was considered significant.

3 Results and discussion

3.1 Characterisation of the polysaccharide compounds

In the presence of water, aldehyde groups react with amino groups forming imine groups (Schiff's bases) at body temperature. Oxidised dextran or starch has several aldehyde groups in one molecule. Therefore, they can cross-link the amino-group-containing chitosan component and react with exposed amino groups of bone or any other tissue. Chitosan, which is a commercial product, must be transformed into a water soluble form. Consequently, chitosan was hydrolysed with hydrochloride for different periods of time. Depending on the reaction time, the molecular weight and the relationships between amino and aminoacetyl groups changes. Figure 1 shows the molecular weight depending on the hydrolysis time of low and medium molecular weight chitosan. Low molecular weight chitosan shows a great reduction of chain length after a reaction time of 1 h. After 14 h of hydrolysis, only a slow further decrease of the average molecular weight is noted. After 20 h of hydrolysis, both kinds of chitosan (low and medium molecular weight) reached the same molecular weight.

To determine the relationship between amino and aminoacetyl groups pH-titration [15] was carried out (Table. 1). There is no dependence of the amino/aminoacetyl ratio on the hydrolysis time. The low molecular chitosan and its hydrolysis derivatives possess an amino group amount of 74% up to 84%, whereas the medium molecular chitosan and its derivatives vary between 60% and 71%.

Polysaccharides can be oxidised with periodic acid to open their sugar units at two vicinal hydroxy groups. This method has previously been used to prepare polyaldehyde derivatives [4, 22, 25, 26]. Oxidised dextran and starch are soluble in water. Figure 2 shows the oxidation rate of dextran and starch depending on the reaction time. The glucose units of dextran and soluble starch (mainly

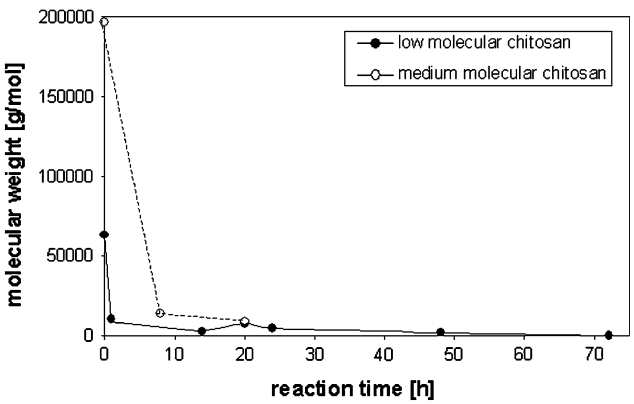

Fig. 1 Molecular weight of chitosan (low and medium molecular) depending on the time of hydrolysis

Table 1 Degree of acetylation (NH_2/NHAc) of chitosan after different times of acid hydrolysis determined by pH-titration

Hydrolysis time, h	NH_2/NHAc
Low molecular chitosan	
0	74/26
24	85/15
48	84/16
72	76/24
Medium molecular chitosan	
0	71/29
8	60/40
20	66/34

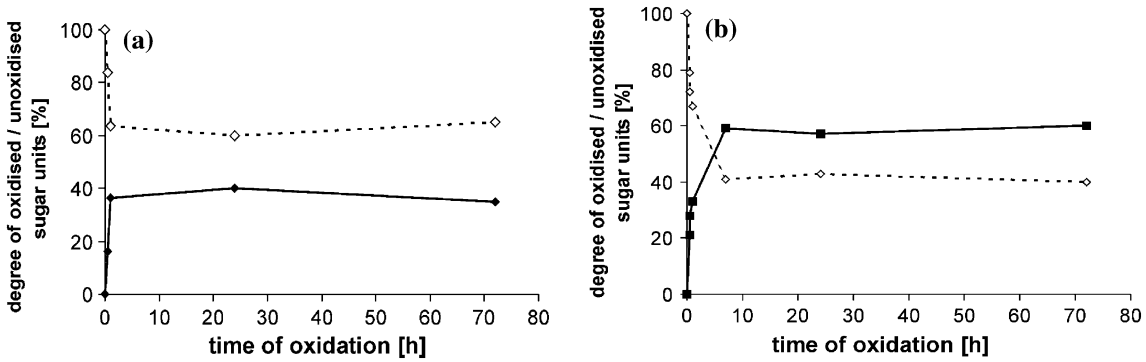

Fig. 2 Degree of oxidised sugar units depending on oxidation time with periodic acid of dextran (**a**) and starch (**b**) (oxidised sugar units: full line, unoxidised sugar units: *dotted line*)

α-amylose) are linked in different ways. Dextran consists of α-(1,6)-linked D-glucopyranose units in the main chain, while soluble starch (mainly α-amylose) is a linear molecule with α-(1,4) glycosidically linked glucose units. This difference in linking leads to differently oxidised end products. Soluble starch requires one periodate molecule to open the sugar ring and to generate two aldehyde groups. Dextran, in turn, requires one periodate molecule to open the sugar ring and another one is used in a second oxidation step to remove one carbon atom as formic acid from the sugar ring [4]. This difference in oxidation mechanism may explain the differing ratio of oxidised to unoxidised sugar units at equal oxidation parameters (amount of periodic acid, temperature and reaction time). Using identical reaction conditions, only 40% of the sugar units of dextran were oxidised while 60% of the sugar units of soluble starch were oxidised (Fig. 2). To additionally link L-Dopa to the polysaccharide backbone, the polysaccharide was oxidised for ½ h with periodate to generate aldehyde groups, which reacted with the amino group of L-Dopa in a subsequent step. After this procedure, the substance was oxidised with periodate for a second time to generate aldehyde groups and to oxidise L-Dopa to quinone. The amino groups of chitosan can react with either the aldehyde groups of the oxidised dextran or with those of starch. The quinone structure of L-Dopa, which is covalently bound to the polyaldehyde, can also react with amino groups by an imine formation or a Michael adduct formation [19]. All of these reactions result in a strong adhesive force within the glue. The adhesion to the tissue or bone can be established by the same mechanism: collagen, which is a main compound of bone, exhibits the amino groups necessary for covalent attachment of the glue to the bony surface.

The average molecular weight and the $NHAc/NH_2$-ratio of chitosan decrease during deacetylation, corresponding to a decrease in pyrolysis temperature (Fig. 3a). The TG curves (mass loss dependent on temperature) of chitosan and their derivatives are shown in Fig. 3a. The first part of the thermogram, below 150°C, shows a small decrease of weight, which is due to the loss of water. This was verified by MS-measurements of the pyrolysis gas. Above 200°C two separate pyrolysis steps were observed. The first stage of pyrolysis, with a maximum degradation rate at 292, 241, 250 and 233°C for unprocessed chitosan, chitosan deacetylated for 24, 48 and 72 h, respectively, corresponds to a weight loss of about 50%. The reduction of the pyrolysis temperature with increasing deacetylation time is due to the smaller average molecular weight of deacetylated chitosan and the degree of acetylation. The trend that chitosan is shifted towards a lower pyroloysis temperature [27] in comparison to chitin may be explained by the lower thermal stability as a consequence of decreased acetyl content [28]. Similarly to chitosan, oxidised dextran (Fig. 3b) and starch (Fig. 3c) show a first decrease of weight in the thermogram below 150°C caused by the loss of water. In general, there is a decrease of the pyrolysis temperature of dextran (328°C; Fig. 3b) and starch (298°C; Fig. 3c) by oxidation of the polysaccharide. The oxidation of the sugar units leads to the opening of sugar rings and thereby the thermostability of the polysaccharide is lowered. Furthermore, the pyrolysis sequence changes upon conversion of dextran/starch to their oxidised species. Dextran and starch show a two-step pyrolysis, where the first pyrolysis temperature of 328°C (dextran) or 298°C (starch) corresponds to a weight loss of about 70% (dextran) and 65% (starch), respectively. The second step of weight loss is a continuous process up to 550°C. The oxidised species show one additional step (all species about 200, 280 and 410°C) and their loss in weight is more steady with increasing temperature. Apparently, an additional binding of L-Dopa (oxidised to quinone) to the oxidised starch or dextran backbone leads to a lower pyroloysis temperature. The thermograms of the lyophilised glue (Fig. 3d) show a constant decrease of weight with increasing temperature. The three step pyrolysis profile found for oxidised starch/dextran was not detected.

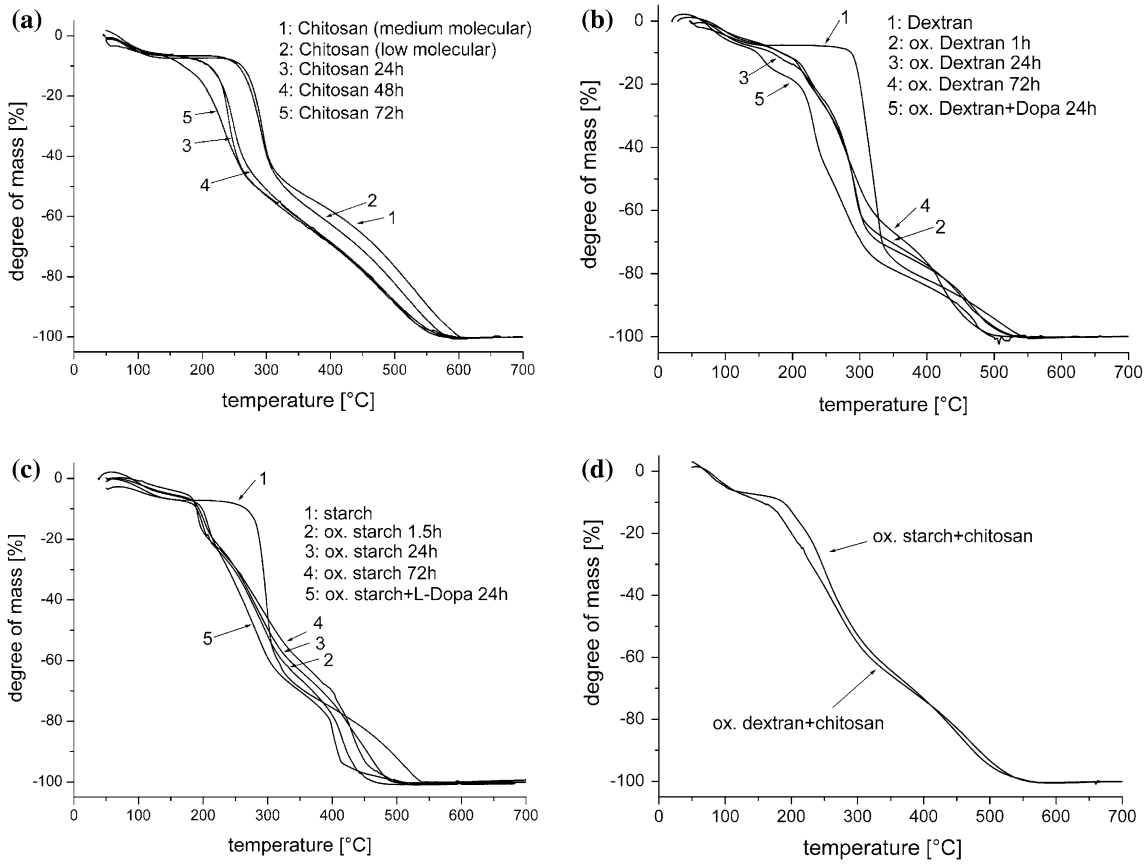

Fig. 3 Thermogram of chitosan (**b**), dextran and oxidised dextran (**b**), starch and oxidised starch (**c**) and lyophilised glue (**d**). Mass loss (%) is in correspondence with the temperature (°C). Numbers correspond to the deacetylation or oxidation time in hours

3.2 Adhesive strength

Given that different times of deacetylation and oxidation resulted in different chain lengths and different amounts of amino and aldehyde groups, respectively, we expected the modified components to differ in adhesive strength as well. We, therefore, tested the adhesive strength of different combinations of basic components. Because preliminary tests showed that the adhesive strength of chitosan in combination with oxidised dextran was three to seven times higher than that of chitosan in combination with oxidised starch, we only assessed different combinations of oxidised dextran and chitosan (Table 2). Two chitosan modifications, which differ in molecular weight, were tested. Firstly, low molecular weight chitosan was tested after 24 h of deacetylation in combination with oxidised dextran functionalised with L-Dopa after 24 h of oxidation [chitosan (24 h) + dextran + L-Dopa (24 h)]. Secondly, low molecular weight chitosan was tested after 24 h of deacetylation in combination with oxidised dextran after 24 h of oxidation [chitosan (24 h) + dextran (24 h)]. Thirdly, medium molecular weight chitosan was tested

Table 2 Adhesive strength of different bone glues

	Adhesive strength, MPa	Quantile (10% … 90%)	P_{cyano}	P_{fibrin}
Cyanoacrylate	1.53	1.05…1.96	–	0.001
Fibrin	0.14	0.05…0.18	0.001	–
Chitosan (24 h) + ox. dextran + L-Dopa (24 h)	0.19	0.01…0.36	0.002	0.17
Chitosan (24 h) + ox. dextran (24 h)	0.18	0.08…0.47	0.008	0.16
Chitosan (8 h) + ox. dextran + L-Dopa (24 h)	0.31	0.04…1.22	0.004	0.02
Chitosan (8 h) + ox. dextran (24 h)	0.41	0.07…0.71	0.01	0.03

Median and quantile range (10%…90%). Differences between groups were assessed with the Wilcoxon signed rank test. P_{cyano}: *P*-value for comparison with cyanoacrylate glue. P_{fibrin}: *P*-value for comparison with fibrin glue

after 8 h of deacetylation in combination with oxidised dextran functionalised with L-Dopa after 24 h of oxidation [chitosan (8 h) + dextran + L-Dopa (24 h)]. And finally, medium molecular weight chitosan was tested after 8 h of deacetylation in combination with oxidised dextran after 24 h of oxidation [chitosan (8 h) + dextran (24 h)].

This last combination reached the highest adhesive strength, which was about three times higher than that of fibrin glue ($P = 0.01$). However, the adhesive strength of acrylate glue was not reached ($P = 0.03$). The adhesive strengths were lower for the low molecular weight chitosan (average molecular weight after 24 h deacetylation: 5100 g/mol) compared to the medium molecular weight chitosan (average molecular weight after 8 h deacetylation: 13500 g/mol). These results indicate that the adhesive strength depends upon the chain length of the chitosan. Because the low molecular chitosan exhibits more amino groups (85%) than the medium molecular chitosan (60%), one would expect a higher cross-linking ability and thus an increased adhesive strength. In contrast to this anticipation, our results showed that the glue combinations with the higher molecular weight chitosan displayed the upmost bonding strengths. This may be due to the fact that the molecular weight has a greater influence on the final adhesive strength than the amount of amino groups. This would also explain the finding that the addition of L-Dopa, which acts by increasing the number of cross-linking possibilities, did not significantly enhance the bonding strength of the glue.

3.3 Cytotoxicity testing

To assess cytotoxicity, MC3T3 cells were directly seeded on glue-coated glass slides and cultured under standard conditions. After 1, 3, and 6 days, one set of samples was stained with a cell viability stain and evaluated by

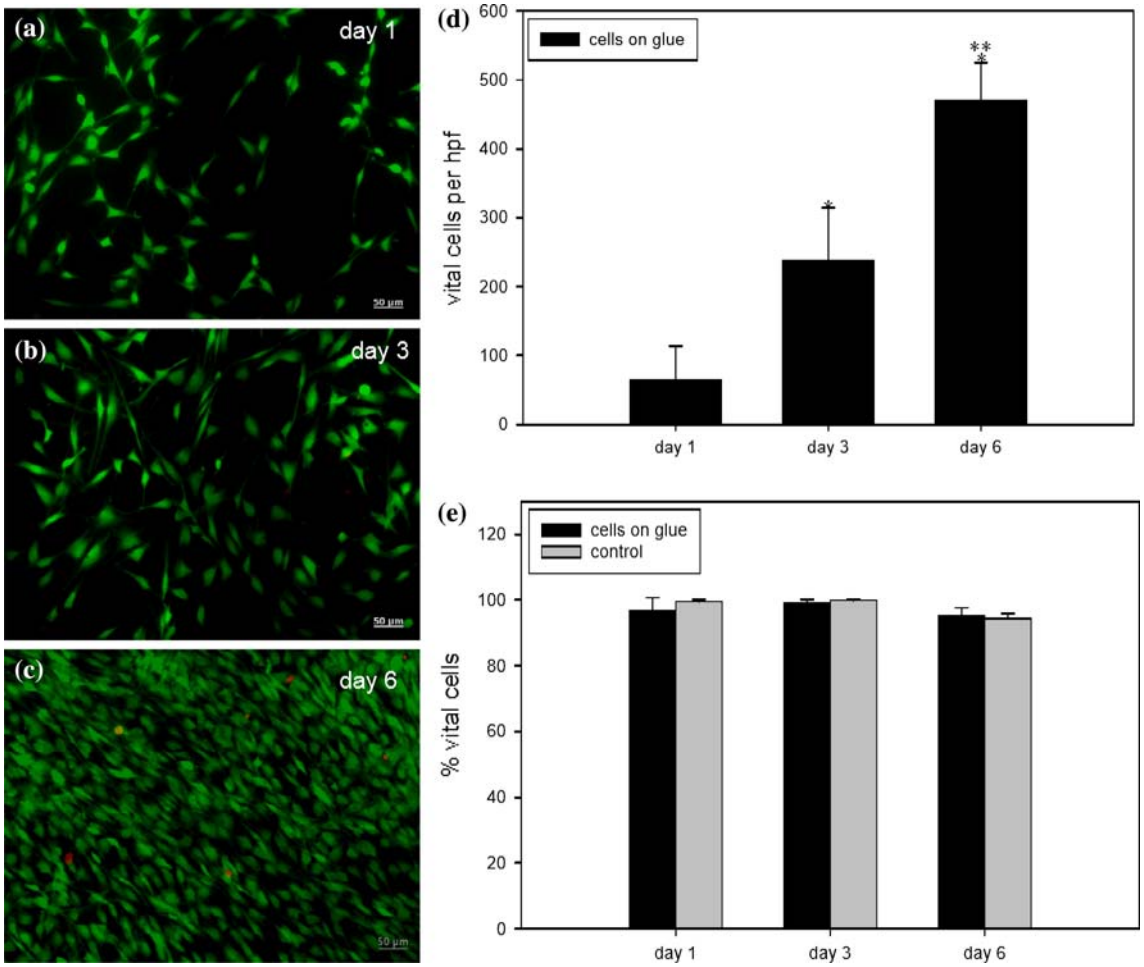

Fig. 4 The $2 \times 10^4/cm^2$ MC3T3 cells were directly seeded on glass cylinders covered with the new bone adhesive. Live-dead-assay was performed after 1, 3, or 6 days. Fluorescence microscopy (**a**–**c**) reveals predominantly vital cells (*green*) at all time points. Proliferation (**d**) and viability (**e**) were assessed by analysis of three random high power fields (hpf) per sample. Separate experimental runs were averaged and given as final results. One asterisk indicates $P < 0.05$ relative to day 1, two asterisks indicate $P < 0.05$ relative to day 3

fluorescence microscopy. The pictures demonstrate that most of the cells seeded on the new bone adhesive are alive (green) after 1, 3, and 6 days (Fig. 4a–c). Cells spread out on the surface of the material, indicating good cell–surface interaction. Furthermore, cells proliferated well on the glue demonstrating excellent biocompatibility. The total number of cells was significantly ($P < 0.05$) increased over time (Fig. 4d). The vitality of MC3T3 cells was largely unaffected with a vitality score equal to that of the negative controls (Fig. 4e). In sum, the new glue does not have major cytotoxic effects and it displays a high degree of biocompatibility.

4 Summary

We developed an attractive new bioadhesive system consisting of chitosan and oxidised dextran without any cytotoxic effects. Because both components are natural, biodegradable polysaccharides, this bioadhesive seems to be a good candidate for bone or soft tissue gluing applications in surgery. Although the new bio-glue displays a three times higher bonding strength than fibrin glue, it has a lower bonding strength than cyanoacrylates. Therefore, investigations to increase its bonding strength are now warranted.

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