Synthesis and Characterization of Chitosan/ Dextran-Based Hydrogels for Surgical Use

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Summary: A series of hydrogels were formed from the reaction between an amine functionalized succinyl chitosan and an oxidized dextran. The properties and rate of formation of the gel were related to both the amine and aldehyde levels of the precursors. These levels could be readily changed by altering the reaction conditions, and allowed good control of the gel properties. Oxidation of the dextran with periodate was accompanied by chain scission and a large reduction in molecular weight. The gel showed excellent haemostatic properties and reduction of adhesions in animal models.

Keywords: adhesions; chitosan; dextran; dextran; hydrogel; imine; oxidized periodate; succinylation

Introduction

Hydrogels are becoming an important adjunct to surgical procedures. Applied after the operation, they can help control bleeding, keep tissue in the right place during healing, and prevent the formation of adhesions. There are several medical hydrogels on the market, including Merogel and Spraygel products. Adhesions in particular are a very important post-operative problem in surgery, often requiring extra work to repair. For instance, in ear, nose and throat (ENT) operations serious adhesions will be found in 10–30% of post-operative patients.[1] The use of some types of surgical packings and haemostatic gels has been found to actually increase the rate of adhesion formation.[2,3] Any surgical gel that is used must have a stringent set of requirements: be sterile and non-toxic; non-immunogenic and non-irritating; have an appropriate setting time and physical characteristics (modulus, strength); stay swollen/wet under physiological conditions; control bleeding and adhesions; breakdown within a certain length of time (days to weeks) to give harmless products; be mucoadhesive; and be able to incorporate and release bioactives to help the healing process. Different types of surgery e.g. ENT or abdominal, may require quite different types of gels.

Chitosan is an amino-substituted polysaccharide produced from the chitin found in crustacean and insect shells, squid pens and some fungi. It is considered a potentially important ''green'' polymer, especially as it is derived from the portion of the animal which is usually discarded. It has also been intensively studied for use in biomedical applications[4] due to its biodegradability, haemostatic properties and very low toxicity. Chitin is known in two solid state crystalline forms, α- from insect and crustacean shells, and β- from squid pens.[5] The solid state packing arrangement in the chitin can carry over into the derived chitosan, and thus the source of the chitosan may well influence its reactivity. In particular, the β-form is less well packed and thus more reactive. In solution there should, in principle, be no difference between the differing forms of chitosan.

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Chitosan is soluble in dilute acid, but not at physiological pHs. It is necessary to substitute the chitosan with hydrophilic groups, usually negatively charged, in order to make it dissolve under neutral conditions. In particular, substitution of the amine groups with methylcarboxy,[6] ethylcarboxy,[7] and anhydride[8] groups is often used. Chitosan-based hydrogels have been studied by a variety of groups, using cross-linkers such as epoxy,[9] aldehyde,[10] and thiol[11] groups. In the present study, we chose to use a succinyl-substituted chitosan (SC) due to its simple synthesis, and suitability for large scale manufacture. After a trial of various aldehyde-containing cross-linking agents, we found that oxidized dextran (dextran aldehyde) gave the best results.

It was found that a mixture of succinylated chitosan and dextran aldehyde formed good gels at about 5% concentration in water in under a minute and was then tested as post-operative aids in-vivo in sheep nasal models developed in Adelaide.[12,13] Adhesions were almost completely eliminated[14] and control of bleeding was significantly improved.[15] With human trials planned, it was necessary to understand how the synthesis of the materials affected the gel formation and physical properties.

There are three main methods in the literature for making succinyl chitosan (SC). The first uses dipolar aprotic solvents to solubilise the chitosan before reacting with succinic anhydride.[16,17] However, only low molecular weight chitosan seems to be soluble in such solvents and so the applicability is limited. A better route appears to use a methanol/water/ acid mixture to solubilise the polymer and anhydride.[18,19] While in principle this is the most convenient process, the product that one gets is only soluble if all the residual acetyl groups (remaining from the chitin) have been completely removed from the chitosan first. This is a costly and time consuming process involving very strong base solutions, and to be avoided. It seems likely that the remaining acetyl groups in the new polymer are clumped together in blocks, forming insoluble domains even at low concentrations. A convenient route, especially at large scale, is heterogeneous acylation with anhydrides at high temperatures in DMF, followed by alkaline treatment.[20,21]

Experimental Part

Chitosan was supplied by Aldrich as practical grade and determined by GPC have a molecular weight (M_n of 480,000). Dextran was supplied by Sigma with a stated molecular weight 0f 60–90 K. DMF was reagent grade (Aldrich) and all other chemicals were bought from Aldrich

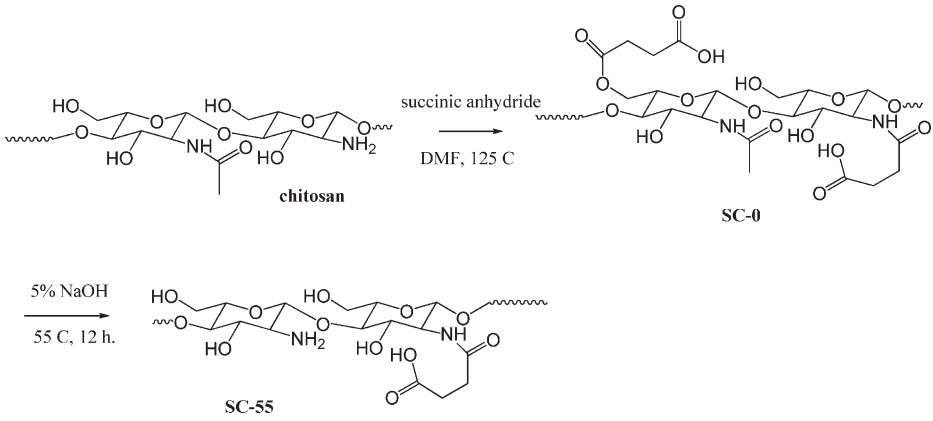
Dialysis was performed with a molecular weight cut-off of 2000 in 40 volumes distilled water changed daily for three days.

IR was performed on a PerkinElmer Spectrum BX FTIR spectrometer as KBr disks. NMR was performed in D_2O on a Varian Inova Spectrometer at 300 MHz. Viscometry was performed on a Haake RS1 with a 20 mm 1 °cone, 1 Hz frequency. GPC was performed in on a Polymer Laboratory PL 1 using 0.5 M sodium phosphate buffer pH 8.0 through a mixed C column at 1.0 ml min^{-1} with dextran standards. Gel times were taken as the point that a 2 mL solution failed to flow in an inverted 5 mL tube.

Aldehyde levels were measured by reaction with hydroxylamine hydrochloride and titration of the released acid.[22]

Synthesis of Succinyl Chitosan

Chitosan (3.6 g) and succinyl anhydride (4.2 g) was heated in DMF (100 mL) under nitrogen for 3 h at 125 °C. The mixture was cooled and the product collected by filtration and washed with methanol. The polymer was then dissolved by high speed stirring into sodium hydroxide solution (5% w/w, 150 mL), and heated to the required temperature (see text) overnight under nitrogen. The solution was filtered, and dialysed, and finally lyophilized to give the product as a white fibrous material, typical yields 3–4 g.



Scheme 1.
Synthesis of succinylated chitosan, with representative chain units shown.

Typical Synthesis of Oxidized Dextran
Dextran (3.0 g) was dissolved in water (40 ml), and sodium periodate (4.6 g, 1 mole eq.) was added in portions over 20 minutes with vigorous stirring, keeping the temperature under 30 °C by external cooling if necessary. The mixture was stirred for 2 h more, and then dialyzed and lyophilized to give the product as a white solid.

Discussion

Succinylation of the chitosan was carried out in DMF at 125 °C in a heterogeneous reaction. The chitosan swelled noticeably as the reaction progressed, though was still easily filterable after cooling. Not all chitosan samples gave soluble products though, and it was found that any acid or basic impurities left over from the conversion from chitin gave poor results. The resulting crude succinylated chitosan proved very soluble in aqueous base with only a small amount of insoluble gel material. However, NMR, microanalysis and IR analysis showed that there were no free amine groups left, and indeed a percentage of the free hydroxyl groups had also formed into succinyl esters, resulting in about 1.3 succinyl groups/chitosan repeat unit. As the cross-linking reaction needs free amines to be present, the crude succinylated chitosan was heated in aqueous base to hydrolyze some of the amide functionalities (Scheme 1). The extent of hydrolysis could be conveniently controlled by the reaction temperature, with temperatures of between 40–75 °C giving useful material (too much hydrolysis would of course lead back to chitosan). The succinyl esters were quickly hydrolyzed first, and the acetamide groups appeared a little more susceptible to cleavage than the succinyl amide groups. The degree of hydrolysis could be best measured by ^{1}H NMR. The majority of the saccharide ring protons lie between δ 3.2 and 4.0, the succinyl protons appear at δ 2.2–2.6, and the proton next to the free amine shows up at as a shoulder at δ 2.6 (Figure 1). At low base treatment temperatures it was hard to quantify the small peak at δ 2.6 properly as it was too close to the succinyl peaks, and in those cases it was found possible to derivatize the amino group in the succinyl chitosan with excess phthalic anhydride in methanol/water and integrate the resulting aromatic peaks.

Increasing the base treatment temperature between 35 and 75 °C gave increasing amounts of amine groups in the polymer (Table 1), but at temperatures over 85 °C mainly gel material was obtained as too many succinyl groups were removed to keep the material soluble. The molecular

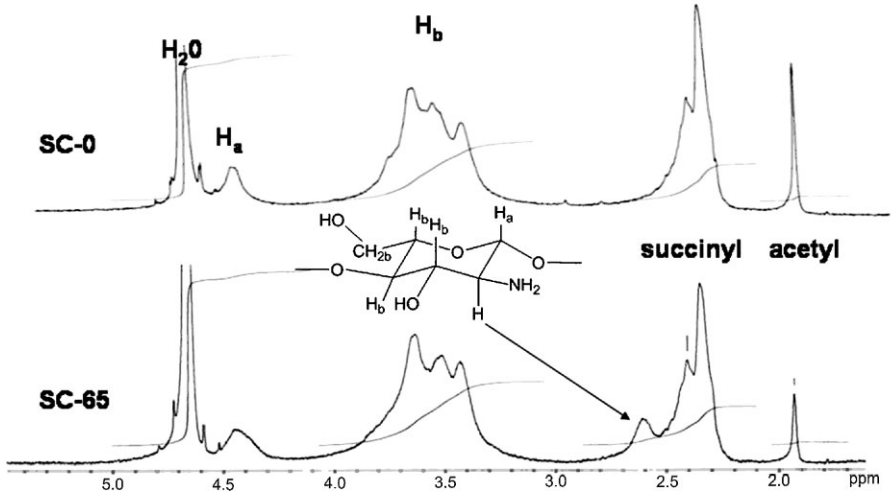


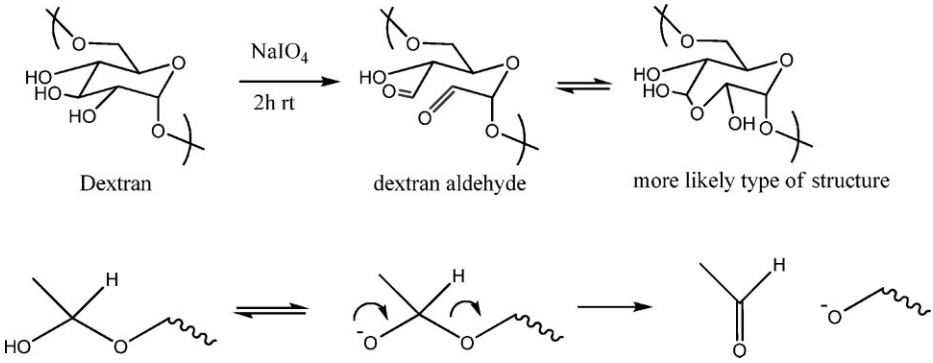
Figure 1.
^{1}H NMR in D_2O of succinyl chitosan before and after base treatment at 65 °C

weight of the material was not obviously affected by the treatments, as the starting chitosan had a measured M_n by GPC of around the same mark (480 KDa) as the resulting SC polymers. The resulting material formed viscous solutions in water at concentrations greater than 1%, but a 5% w/w aqueous solution proved fluid enough to be used in surgical syringe applicators in the animal and human trials. The succinyl chitosan showed typical polyelectrolyte behavior, with a much lower reduced viscosity (RV) in a salt solution (e.g. SC-55 had a RV of 3.5 dL/g in 0.5 M phosphate buffer pH 8, 0.1 g/dL) than in pure water (12 dL/g at 0.1 g/dL).

The dextran was readily oxidized by sodium periodate at room temperature, though the reaction was exothermic on a large scale and external cooling had to be used to keep the temperature under 30 °C. The sodium periodate is not very soluble so was added as a solid to slowly dissolve in the reaction mixture. The by-product sodium iodate is also not very soluble, and so partially crystallizes out if the reaction is left for too long before dialysis. The resultant dextran aldehyde after lyophilization is difficult to redissolve in pure water at high levels of oxidation, requiring extended (overnight) stirring or heating. It dissolves quickly if the pH is raised to above 8.0, though only slowly under acid (pH 4.0) conditions. From the absence of any aldehyde signals in the IR or NMR spectra, it is probable that the carbonyl groups are extensively acetalized,[23] and that in the solid inter-chain hemiacetals between form cross-links preventing dissolution. These hemiacetal linkages are quickly hydrolyzed in base (Scheme 2), but require much harsher acid conditions to break (any pH

Table 1.
Composition and gel time of succinyl chitosan after treatment with 5% NaOH.

Polymer sample	Temperature of base treatment	Mol % Acetyl groups[a]	Mol % Succ Groups[a]	Mol % free amine[a]	Gel Time[b] (s)
SC-0	no base treatment	16	93	0	–
SC-35	35 °C	15	91	trace	240
SC-55	55 °C	11	81	12	35
SC-65	65 °C	5	75	22	5
SC-75	75 °C	7	56	37	<5



Scheme 2.
Synthesis of the dextran aldehyde, and base-catalyzed hydrolysis of the interchain hemi-acetal cross-linkages.

conditions that was acidic enough to cleave the hemiacetals might also cleave the polysaccharide glycosidic linkages). This slow solubility of dextran aldehyde might be an issue in any commercialization. The level of oxidation of DA could be measured by reaction with hydroxylamine hydrochloride, followed by a titration to measure the released acid.[22] In general this method appeared reproducible, though we found that it took at least three days for the dextran to dissolve and the hydroxylamine to react fully before titration. The level of oxidation closely followed the amount of periodate used (with two moles of aldehyde groups expected per mole of periodate used). However, the molecular weight of the dextran dropped precipitously with increasing amounts of oxidation (Table 2), indicating an unwanted side-reaction occurring. The yields of the reaction were also good except at high levels of oxidation, which is probably due to loss of very low molecular weight material on dialysis.

The gel formation was studied by mixing equal volumes of aqueous solutions of the two components, cross-linking most probably occurring via imine formation. The ability of mixtures of SC and DA to form gels depended on their level of functionality as well as on their concentration. In general, chitosan solutions failed to gel at concentration much less than 5%, whereas the dextran concentration could be lowered to 0.5% before no gel formation failed to occur. The gel times reflected both the level of oxidation and the amount of amine in the polymer samples (Table 1 and 2). High oxidation level DA and high amine content SC gave a very fast setting harder gel, whereas a softer slower setting gel can be obtained from the use of less reactive components.

It is important to know something about the stability of the gels under conditions

Table 2.
Oxidation of dextran with sodium periodate.

Polymer sample	Mol % periodate[a]	Yield %	MW dextran[b] (M_n)	Mole % aldehyde groups[c]	Gel Time[d] (s)
DA-0	0	–	95,500	0	-
DA-25	25	95	20,270	32	220
DA-50	50	93	14,059	75	70
DA-75	75	86	10,010	118	45
DA-100	100	65	3700	165	35

(a) mol % periodate/mol repeat unit dextran, 2 h reaction at rt. (b) measured by GPC (c) measured by hydroxylamine assay (d) mixture of 5% w/v solution with SC-55 (5% w/v).

that might be found under surgery or in use in the body. The stability of the formed gels was evaluated by leaving them in sealed vials or by placing them in either excess water or 1% saline. In general, the gels were stable for months when sealed away. In water the softer gels (e.g. from SC-50 and CH-35) slowly dissolved over a couple of days in both saline and water. However the harder gels appeared more stable and kept their shape when placed in excess water over a period of weeks without swelling or dissolution. In saline they appeared to collapse somewhat, suggesting an influence of the electrolytic strength on the extension of the gel chains. This is certainly true of the SC component, where the reduced viscosity of SC-55 (as noted above) in pure water is much higher than in saline. The collapsed gels did not re-swell when placed back in pure water, and neither did the gels once dried; suggesting further cross-linking is taking place in the condensed state. This may be important in certain applications as ENT surgery, where the gels appear to dry out after a day or two in the nasal passages. In these cases the gel was found to have disappeared after two weeks, presumably by mechanical removal mechanisms rather than redissolution. The gels showed good adhesion to the nasal mucosal surfaces when applied, even with mild bleeding from the surfaces which it helped to stop. The harder gels from the very active succinyl-chitosan (SC 65 and SC-75) are more brittle, and though while they have not been tested in vivo yet, might be expected to be less adherent due to their greater tendency to fracture.

A scanning electron micrograph of a typical gel (SC-100/CH-55) is shown in Figure 2 before and after removal of the water by sublimation at low temperatures. It shows a well-connected, open cellular structure with submicron features. Work is ongoing in relating the gel structure under microscopy to its chemical composition and physical properties.

Sterilization of the gel components is going to be important in any commercial application. Samples of DA-100 and SC-55 were subjected to standard gamma irradiation protocols (25 kGy). The dextran appeared relatively unaffected and the molecular weight only dropped marginally, though it was low to begin with. More importantly its reactivity and gel forming ability remained acceptable after storage for a month or more. However the molecular weight of the chitosan dropped by 50%, and more importantly the gelling properties deteriorated slowly after irradiation. After 2–5 weeks post-irradiation the chitosan no longer was able to gel with the aldehyde solution. This was traced to oxidation of the amine groups after irradia-

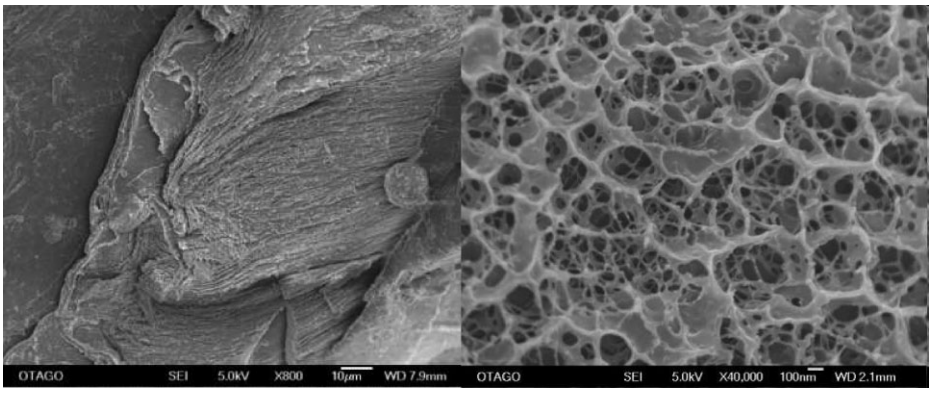


Figure 2.
A SEM micrograph of a DA-100/SC-55 gel frozen in liquid nitrogen before (left) and after (right) removal of water by sublimation.

tion, as shown by loss of the amine signals in the NMR. We are currently looking at ways of minimizing the oxidation damage to the chitosan product.

Conclusions

A new hydrogel product for surgery has been developed from dextran and chitosan. The cross-linking time and physical properties of the gel can be controlled over a wide range through changes in the synthesis conditions which impact on the level of cross-linking functionalities in the polymer.

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