Crosslinking of Chitosan with Dialdehyde Derivatives of Nucleosides and Nucleotides. Mechanism and Comparison with Glutaraldehyde

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ABSTRACT
In medical and pharmaceutical applications, chitosan is used as a component of hydrogels—macromolecular networks swollen in water. Chemical hydrogels are formed by covalent links between the crosslinking reagents and amino functionalities of chitosan. To date, the most commonly used chitosan crosslinkers are dialdehydes, such as glutaraldehyde (GA). We have developed novel GA like crosslinkers with additional functional groups—dialdehyde derivatives of uridine (oUrd) and nucleotides (oUMP and oAMP)—leading to chitosan-based biomaterials with new properties. The process of chitosan crosslinking was investigated in details and compared to crosslinking with GA. The rates of crosslinking with oUMP, oAMP, and GA were essentially the same, though much higher than in the case of oUrd. The remarkable difference in the crosslinking properties of nucleoside and nucleotide dialdehydes can be clearly attributed to the presence of the phosphate group in nucleotides that participates in the gelation process through ionic interactions with the amino groups of chitosan. Using NMR spectroscopy, we have not observed the formation of aldimine bonds. It can be concluded that the real number of crosslinks needed to cause gelation of chitosan chains may be less than 1%.

Introduction
Chitosan is prepared from chitin, the second most abundant natural polymer in the world after cellulose. Chitosan is a copolymer of $\beta$-(1$\rightarrow$4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose and...
Figure 1. Proposed structure of chitosan crosslinked with GA.

can be obtained by deacetylation of chitin, which is isolated from shells of crustaceans, insects, and other sources. The presence of reactive amino groups in chitosan is highly beneficial for chemical modifications to construct sophisticated molecular architectures. Due to these properties, chitosan is considered as a biofunctional polymer having much higher potential than cellulose. Chitosan is a nontoxic, biodegradable, and biocompatible natural polymer and can be used in a wide range of applications, such as biomedicine, membranes, drug delivery systems, hydrogels, water treatment, food packaging, etc.\[1–5\]

Crosslinking reagents, such as dialdehydes, genipin, tripolyphosphate, glycerol-1-phosphate diglycidyl ether, and diisocyanate\[6–10\] are widely used for the preparation of microspheres, films, gels, absorbents, immobilization of proteins and so on. Probably the mildest and the most straightforward route for crosslinking of materials based on primary amines is the reaction with an aldehyde to form the corresponding imine. Due to the solubility of chitosan in water at slightly acidic conditions (pH 4–6), this reaction proceeds rather fast. To date, the most commonly used crosslinkers are dialdehydes, and the most effective and reliable one is glutaraldehyde (GA). The main drawback of GA crosslinking, however, is the formation of irregular products due to the aldol condensation of GA.\[11–14\] The crosslinking of chitosan by GA is a complex process. Chitosan accelerates the polymerization of GA to form inhomogeneous products. In the crosslinked chitosan, the length and the composition of the oligomeric GA chains are dependent on the crosslinking conditions (the concentration of GA and pH of the reaction medium).\[14\] This fact should be taken into consideration if the reaction of chitosan with GA is used to create materials for medical or pharmaceutical applications. When excess of GA is used under neutral or slightly alkaline conditions, a co-polymer of poly-GA and chitosan may be formed (Figure 1).\[14,15\]

Another useful crosslinking reagent is genipin, which is believed to be less toxic than GA and might replace GA with the advantage of biocompatibility of the crosslinked products. As in the case of GA, irregular products are formed when genipin reacts with chitosan, and their structure is highly dependent on the reaction conditions.\[6,16,17\] This might be a serious limitation for the use of genipin crosslinked chitosan for medical applications. Moreover, the rate of crosslinking is much lower compared with GA,\[18,19\] so there is still a room for design of new crosslinking reagents and further improvement of existing ones.
To conclude, at the moment none of the covalent crosslinking reagents may be considered “perfect” in terms of the overall properties: low toxicity, high rate of crosslinking reaction and regular structure of the products. In addition, the structure of the crosslinking reagent may be capable of introducing additional functional groups (simultaneous crosslinking and functionalization). That is why the ionic crosslinkers, such as sodium tripolyphosphate, are increasingly used.\textsuperscript{[3,9]} However, when using these, it is difficult to achieve uniform gelation, as electrostatic interactions are established rather fast.\textsuperscript{[20]} To improve the characteristics of chitosan-based materials, we have recently proposed a series of novel effective reagents.\textsuperscript{[21–24]} We have noted a striking similarity between GA and oxidized nucleosides and nucleotides (Figure 2). Dialdehyde derivatives of nucleosides and 5'-nucleotides can be readily prepared by reaction of the parent natural compounds with an equimolar amount of NaIO\textsubscript{4} or HIO\textsubscript{4} in water at room temperature.\textsuperscript{[25–29]} These dialdehydes may be considered as derivatives of 3-oxa-glutaraldehyde and have been previously used for crosslinking of proteins.\textsuperscript{[30]}

The general scheme of aldol reaction (i) and aldol condensation (ii) is as follows (Scheme 1). The first step (i) requires at least one hydrogen on the $\alpha$-carbon atom, and the formation of aldol is reversible. The next condensation step (ii) requires the presence of two protons in the starting aldehyde, and the equilibrium is shifted to the right. Conjugation of the newly formed double bond with the carbonyl group stabilizes the product and provides the thermodynamic driving force for the dehydration process. The absence of CH\textsubscript{2} groups in $\alpha$-position to the carbonyl groups in the crosslinking reagents oUrd, oUMP, and oAMP prevents aldol condensation and, as a consequence, the formation of irregular co-polymer as in the case of GA.
Herein, we report the further investigation on the use of these reagents for chitosan crosslinking along with an in-depth study of the crosslinking mechanism and comparison with GA.

**Experimental**

**Materials**

High molecular weight chitosan (avg. MW 190000, deacetylation degree 87%) was provided by Roeper GmbH (Germany). Low molecular weight chitosan (avg. MW 12200, deacetylation degree 98%) was kindly supplied by Dr. V.E. Tikhonov (Nesmeyanov Institute of Organoelement Compounds of the Russian Academy of Sciences).

Uridine, uridine-5′-monophosphate disodium salt, adenosine-5′-monophosphate disodium salt, sodium metaperiodate, periodic acid were Aldrich (USA) products, and glutaraldehyde (50% aqueous solution) was purchased from Merck (Germany). Ion-exchange resin AG 1-X8 was obtained from Bio-Rad Laboratories (USA) and was used as HCO$_3^-$-form. All chemicals were reagent grade and were used without further purification.

**UV spectroscopy**

UV-Spectra were recorded on Cary 50 Bio spectrophotometer equipped with the temperature control system. The samples were placed into a 1 cm quartz cell and sealed with a parafilm tape. UV-Spectra were measured every 30 min at 200–300 nm (1 nm resolution). Difference spectra were obtained by subtracting the initial spectrum from other spectra obtained.

Solutions of oUrd, oUMP, and oAMP (1.4 × 10$^{-5}$–1.8 × 10$^{-5}$ M) in 0.05 M potassium phosphate buffer (pH 4.1 and 5.6) were used for studying the stability of dialdehydes in buffer solutions at 20°C and 37°C. When studying the crosslinking reaction between oUrd, oUMP, oAMP and low molecular weight chitosan (avg. MW 12200) at 20°C and pH 5.6, the concentration of chitosan NH$_2$ groups in the reaction mixture was 1.0 × 10$^{-3}$ M and the NH$_2$ group/crosslinking reagent ratio was 10.0 mol/mol.

**NMR spectroscopy**

$^1$H, $^{13}$C, and $^{31}$P NMR spectra were recorded on Bruker AMX400 and AV600 spectrometers. Chemical shifts ($\delta$, ppm) in $^1$H and $^{13}$C NMR spectra were measured relative to methanol as the internal standard (3.34 and 49.50 ppm for $^1$H and $^{13}$C, respectively). Coupling constants were measured in Hz. $^{13}$C NMR spectra were recorded with suppression of proton coupling by the waltz-16 method; the relaxation delay was 4 s. FIDs were processed by exponential weighing with coefficient of
1.5 Hz in order to increase the signal/noise ratio. The information regarding $^1$H-$^1$H-coupling was obtained from COSY spectra (pulsed field gradients with the magnitude presentation of data were used). Single bond $^1$H-$^{13}$C correlations were recorded by the phase-sensitive impulse HSQC using pulsed field gradients. Long-range $^1$H-$^{13}$C couplings were recorded by HMBC using pulsed field gradients, optimized for the registration of $J_{^1H,^{13}C}$ close to 8 Hz.

In the studies of oAMP stability NaIO$_4$ (5.4 mg, 0.0253 mmol) was added to 0.5 mL of 5.0 × 10$^{-2}$ M solution of AMP in D$_2$O and the mixture was kept at 20°C for 30 min, then $^1$H and $^{31}$P NMR spectra were recorded every 30 min at 32°C (305 K) and pH 7.0.

In the study of the reaction of low molecular weight chitosan (average MW 12200) with oAMP the concentration of NH$_2$ groups of chitosan in the reaction mixture was 2.0 × 10$^{-2}$ M, concentration of oAMP was 1.0 × 10$^{-2}$ M. The spectra were recorded in D$_2$O every 30 min at 52°C (325 K) and pH 4.1.

**Preparation of nucleoside and nucleotide dialdehydes**

**Uridine dialdehyde (oUrd).** Uridine (2.44 g, 10 mmol) was dissolved in 10 mL of water and 10 mL of 1.5 M solution of HIO$_4$ was added. Reaction mixture was incubated in dark at 20°C for 1 h. TLC in 4:1 chloroform/ethanol: $R_f$ Urd 0.35, oUrd (extending spot) 0.68. Ion-exchange resin (50 mL, HCO$_3^-$-form) was added to the mixture. After 30 min the resin was filtered and washed with water (2 × 50 mL). Water was evaporated in vacuo to dryness and the residue was co-evaporated with methanol (2 × 10 mL) at 30 °C. The obtained white foam was dried in vacuo over P$_2$O$_5$. Yield 2.35 g (97%). $^1$H NMR spectrum of oUrd is shown on Figure 6–7 (supporting Info).

**Uridine-5‘-monophosphate dialdehyde (oUMP).** Disodium salt of UMP (0.736 g, 2 mmol) was dissolved in 2.5 mL of water and 4.2 mL of 0.5 M solution of HIO$_4$ was added. The reaction mixture was incubated in dark at 20°C for 1 h. TLC in 9:1 methanol/sat. Na$_2$B$_4$O$_7$·10H$_2$O in 1 M CH$_3$COONH$_4$: $R_f$ UMP 0.01, oUMP (extending spot) 0.65. The reaction mixture was left at 0°C for 24 h. The precipitated inorganic salts were filtered and 0.3 M solution of oUMP was obtained. The resulting solution may be kept for at least 1 month at 0°C without notable decrease of crosslinking capacity.

**Adenosine-5‘-monophosphate dialdehyde (oAMP).** Disodium salt of AMP (0.782 g, 2 mmol) was dissolved in 2.5 mL of water and 4.2 mL of 0.5 M solution of HIO$_4$ was added. The reaction mixture was incubated in dark at 20°C for 1 h. TLC in 9:1 methanol/sat. Na$_2$B$_4$O$_7$·10H$_2$O in 1 M CH$_3$COONH$_4$: $R_f$ AMP 0.01, oAMP (extending spot) 0.65. The reaction mixture was left at 4°C for 24 h. The precipitated inorganic salts were filtered and 0.3 M solution of oAMP was obtained. $^1$H NMR spectrum of oAMP is shown on Figure 4. The resulted solution may be kept for at least 1 month at 0°C without notable decrease of crosslinking capacity.
**Chitosan solutions and gelation experiments**

A mixture of 2 g of high molecular weight chitosan (avg. MW 190000, deacetylation degree 87%) in 2% acetic acid (100 mL) was left to swell in the solvent for 3 h at 20 °C. Further dissolving was carried out with gentle stirring. The resulting solution was kept in the fridge for 2–4 weeks without notable decrease of viscosity. The 2% chitosan solution in 2% acetic acid (pH 4.1) was used for gelation experiments.

The above prepared solution was carefully adjusted to 5.6 by adding of 5 M NaOH under gentle stirring. The resulting solution (pH 5.6) was kept in the fridge and was used for gelation experiments.

Chitosan gels were prepared by mixing 2% chitosan solution (2 g) with 0.5 ml of aqueous solution of GA, oUrd, oUMP, or oAMP. The NH$_2$/CH = O groups ratio was varied by changing the concentration of the crosslinking reagent. The time after which the system lost the ability to flow was used as the gelation point. Gelation time was determined as time when the reaction mixture in tube with diameter of 1 cm stopped flowing when being turned upside down.

![GEL](image)

**Results and discussion**

We have studied chitosan gelation in the presence of novel crosslinking reagents and compared the results with those for GA. Hydrogels are three-dimensional networks that swell in water and aqueous solutions. These materials, based on both natural and synthetic polymers, are of great interest as bioactive molecules and in tissue engineering. Among natural biopolymers of interest, chitosan stands out due to its unique properties. Chitosan hydrogels can be divided into two classes: physical and chemical. Chemical hydrogels are formed by covalent links, whereas physical hydrogels are formed by electrostatic interactions and hydrogen bonds.$^{[3,20]}$
The kinetics of gelation of chitosan solutions in the presence of crosslinkers and the growth of gel viscosity were investigated. Solubility of chitosan in water is determined by the degree of protonation of its amino groups (Figure 1, Supporting info). The pH of a 2% solution of chitosan with average molecular weight 190000 (190 kDa) and degree of deacetylation 90% in 2% acetic acid is 4.1. Careful addition of NaOH to this solution up to pH 5.6 (which is 0.3 lower than the pH of chitosan’s cloud point) increases the number of deprotonated amino groups up to 30% with retention of the homogeneity of the solution.

The time interval after which the system lost the ability to flow was used as the gelation point. At pH 5.6, the rate of chitosan gelation in the presence of all studied crosslinking reagents was much higher than at pH 4.1. The time of gelation may be decreased when using increased amounts of crosslinkers. At pH 5.6, the rate of gelation in the presence of oUrd was much lower than in the case of GA. The rates of gelation with GA, oUMP, and oAMP under different concentrations were similar (Figure 3).

**Properties of oUrd, oUMP, and oAMP**

To gain insight into these effects, we examined the physicochemical properties of oUrd, oUMP and oAMP in details and compared them with those of GA. NMR spectroscopy was used for structure elucidation and examination of the conformational equilibrium in the solutions. In aqueous solutions, GA exists as a mixture of free aldehyde, mono- and dihydrate, as well as cyclic cis and trans isomers (Figure 5, Supporting Info).\[^{14}\] It was also reported that the ratio of different forms depends on the GA concentration\[^{14}\] and is primarily determined by temperature: an increase in temperature leads to a substantial increase in the amount of free aldehyde. Approximately 20–30% of the free aldehyde groups were detected depending on the temperature and pH.\[^{14}\] A typical \(^1\)H NMR spectrum of GA may be found in Supporting Info (Figure 3). In contrast, the free aldehyde groups were not detected in D\(_2\)O solutions of oUrd. The \(^1\)H NMR spectra of nucleoside dialdehydes are substantially more complex than those of the starting nucleosides. This is due to the existence

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**Figure 3.** Gelation time at different ratio of crosslinking reagents and NH\(_2\) groups of chitosan (20°C, pH 5.6). 1. oUrd; 2. oAMP; 3. GA.

**Gelation of chitosan**

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Comparison of the phosphate group β-elimination reactions in oUMP and oAMP with and without low molecular weight chitosan (pH 5.6).

| Dialdehyde | T (°C) | Reaction time (h) | Dialdehyde/NH$_2$-groups ratio mol/mol | $\lambda_{\text{max}}$ (ε) H$_2$C = C–C = O |
|------------|--------|------------------|----------------------------------------|---------------------------------------------|
| oUMP       | 37     | 46               | –                                      | 236 (4900)                                  |
| oUMP       | 20     | 20               | 1:10                                   | 236 (6200)                                  |
| oAMP       | 37     | 40               | –                                      | 236 (3800)                                  |
| oAMP       | 20     | 20               | 1:10                                   | 235 (6000)                                  |

of dialdehyde derivatives in aqueous solutions as hydrates that also form internal hemiacetals and diastereomeric dioxane derivatives.[31] In the downfield region of the $^1$H NMR spectrum of oUrd, six major doublets ($J = 8$ Hz) of H-6 of the heterocyclic base together with some minor signals are observed (Figure 7, Supporting Info).

The analysis of the $^1$H NMR spectrum of oAMP solution (Figure 4) allowed us to conclude that oAMP exists as a mixture of four hydrated forms (Supporting Info, Figures 9–11 and Table 1), including the fully hydrated form and three of the four possible diastereomers of the dioxane derivatives; these data are quite similar to the previously reported for periodate oxidized ATP.[32] $^1$H NMR spectrum of oUrd is even more complex because of the presence of 5’-hydroxyl group that may be involved in the formation of hemiacetals (Figure 8, Supporting Info).[31]

The striking difference in gelation properties of aldehyde derivatives of nucleosides and nucleotides can be clearly attributed to the presence of the phosphate group that may be involved in additional ionic interactions. There are two main types of hydrogel, the first one being formed by covalent bonds as with GA, and in the second one two polymeric chains are crosslinked using ionic interactions and hydrogen bonds.[20] The formation of physical gels with glycerophosphate is well documented in the literature.[3] The additional phosphate group in oAMP and oUMP clearly participates in ionic interactions with chitosan and these compounds may be considered as novel crosslinking reagents combining covalent and ionic interactions. It should be noted that we have failed to use adenosine 5′-phosphate as an ionic crosslinker.

**β-Elimination of the phosphate group**

Dialdehyde derivatives of nucleotides are rather unstable due to the β-elimination of the phosphate group (Scheme 2).[25,33,34] This process may be readily envisaged using NMR spectroscopy. The reaction of chitosan with oAMP was completed after

![Scheme 2. β-Elimination of the phosphate group in oAMP.](image)
15 h at 32°C and pH 7.0. As a result, conjugated H2C = CH-CH = O system was formed with a non-hydrated aldehyde proton in 1H NMR spectrum around 9 ppm (Figure 4).

Elimination of the phosphate group in oUMP can be monitored by UV spectroscopy (Supporting Info). A new peak with $\lambda_{\text{max}}$ 236 nm ($\varepsilon$ 4900) can be observed in the spectra and may be attributed to the absorption of a double bond conjugated with an aldehyde group. Essentially the same results were obtained in the case of oAMP: $\lambda_{\text{max}}$ was 236 nm ($\varepsilon$ 3800). The spectroscopic characteristics of en-oAdo (Scheme 2) are in good agreement with those obtained from periodate oxidized ATP.[34] A difference UV spectrum for the compound en-oAdo minus ATP revealed two peaks at 235 nm ($\varepsilon$ 3700) and 280 nm ($\varepsilon$ 350).[34] A larger extinction coefficient (232 nm, $\varepsilon$ 6000) was determined in another instance.[35]

Encouraged by the obtained results, we decided to investigate the elimination of the phosphate group in the presence of chitosan. For this purpose, we have used low molecular weight chitosan (average MW 12200 with degree of deacetylation 98%). According to the UV spectra, at room temperature the reaction was completed after 18 h (half time of conversion 3 h), and the increase of absorption at 236 nm was somewhat higher ($\varepsilon$ 5700) than those without chitosan. In this case, a larger extinction may be attributed partly to the absorption of a double bond conjugated with an aldimine group. Under the same conditions, the elimination of phosphate group in oUMP proceeds much slower with estimated half time of conversion around 40 h. It may be concluded that the presence of chitosan significantly accelerates the elimination of the phosphate group (Table 1).
The interaction between chitosan and oAMP was also studied by NMR spectroscopy. In the concentration range used for the NMR experiments \((10^{-2} \text{M})\) at pH 5.6, the crosslinking reaction was fast and a precipitate was formed within several minutes. Therefore, the reaction was conducted at pH 4.1 and an equimolar ratio of oAMP and NH\(_2\) groups of chitosan was used. Under these conditions, no precipitation and gel formation were observed.

In the \(^{31}\)P NMR spectrum of the reaction mixture (with proton decoupling), four signals were observed immediately after mixing (Figure 5A). Over time, their intensity changed (Figure 5B), and after 5 h at 52°C the signals were converted into two singlets at 1.4 ppm and 0.8 ppm (Figure 5C). When \(^{31}\)P NMR spectra were recorded at room temperature without proton decoupling, a singlet at 0.01 ppm was observed, which could be attributed to the inorganic phosphate. The other triplet signal at 0.65 ppm with \(J = 4.8\) Hz corresponded to the monoester of phosphoric acid (Figure 6). These \(J\) values are typical for natural 5′-nucleotides.\(^{[36]}\) Thus, in the reaction between chitosan and oAMP under these conditions most of the oAMP undergoes the β-elimination of phosphate, however, some 5′-phosphate is still present. It should be mentioned that maximum stability of oAMP at different pH was observed at pH 3–4.\(^{[33]}\)

We have also examined the gelation of high molecular weight chitosan with average molecular weight 190000 with GA and oUrd directly in the NMR tube at pH 4.1. The gel formation was accompanied by a significant broadening of NMR signals, owing to both a faster relaxation of macromolecule-bound crosslinking agent.
and an overall decrease in homogeneity of the sample. This, together with a low concentration of the crosslinked fragments, led to the absence of the aldimine group signals as well as the cross-peaks between nuclei of chitosan and the crosslinking reagents in 1D and 2D NMR spectra.

**How many covalent linkages are involved in the crosslinking process?**

The striking difference in the crosslinking properties of GA and oUrd may be attributed to the different concentration of free aldehydes and, consequently, the rate of the Schiff base formation. It is clear from the obtained results that the rate of gelation depends also on the concentration of the unprotonated amino groups of chitosan. It is believed that at neutral pH the dehydration of the hemiaminal addition product is a rate determining step.[37] The crosslinking requires the formation of two covalent bonds between two different chains of chitosan with one molecule of dialdehyde (formation of a Schiff base or a hemiaminal product) (Scheme 3).

![Scheme 3. General scheme of aldimine formation.](image)

Recently, the formation of aldimines in aqueous solutions of different aldehydes and amines was studied using $^1$H NMR spectroscopy.[38] It was demonstrated that the equilibrium of this reaction markedly depended on the structure of the reacting compounds. When using aromatic aldehydes and basic amines, the equilibrium is nearly completely shifted towards imine formation. On the other hand, hydrated aliphatic aldehydes do not form visible amounts of products.[38] Imine
exchange (transimination) experiments involving the addition of another amine or aldehyde to the solution of a given amine–aldehyde pair indicated that exchange and re-equilibration occurred instantaneously, i.e. within the time needed to perform the NMR measurement. The equilibrium of this reaction also depends on the pH of aqueous solutions: at neutral pH, the formation of imine is more pronounced than in a slightly acidic medium (pH 3–5). Even with aromatic aldehydes, the aldimine formation on chitosan backbone in aqueous solution proceeds in low yield, and its efficiency is significantly increased after drying or in solid films.

Crosslinking of chitosan with GA was previously studied with different physico-chemical methods. It is rather clear that the previously observed formation of aldimine conjugated with double bond (−N = CH−HC = CH−) in solid $^{13}$C NMR is a result of drying during which the equilibrium depicted on Scheme 3 is shifted to the right. The preparation of the samples for solid $^{13}$C NMR included the neutralization of the formed gel with sodium hydroxide to neutral pH and drying in vacuo. Moreover, under these conditions the aldol condensation of GA may occur. We have not observed measurable formation of aldimine bonds in solution when using NMR spectroscopy, so the concentration of these bonds may be less than 1–2%.

Taking into consideration the above results, it may be concluded that during the reaction of chitosan with aliphatic dialdehydes only a few covalent crosslinks are formed. It is a rather challenging problem to determine how many crosslinks are needed to cause gelation. Strictly speaking, dialdehyde derivatives are not “pure” covalent crosslinking reagents, especially in aqueous solution, because the equilibrium of these reactions in the case of aliphatic aldehydes and amines is shifted towards the starting compounds.

To shed some light on crosslinking processes, we investigated gelation of chitosan using small amounts of dialdehyde reagents. From the results shown on Figure 3B it is possible to calculate the number of crosslinks needed for gelation of chitosan: 5 equivalents of oAMP or 12 equivalents of GA for 1 equivalent of chitosan. Given that dialdehyde should link two polysaccharide chains of chitosan, the theoretical amounts of crosslinks should be 10 and 24 for high molecular weight chitosan with average MW 190000, which contains approximately 1000 glucosamine residues. This calculation is made under the assumption that all crosslinking reagents react with different chains of chitosan with both aldehyde groups being involved in the formation of aldimine bonds. Using NMR spectroscopy we have not observed the formation of aldimine bonds. It may be concluded that the real number of crosslinks needed to cause gelation of chitosan chains may be less than 1%.

**Mechanism of the crosslinking reaction**

The remarkable difference in crosslinking properties between dialdehyde derivatives of nucleosides and nucleotides may be clearly attributed to the presence of
Scheme 4. Crosslinking of chitosan with nucleotide dialdehydes oUMP (B = Ura) and oAMP (B = Ade).

the phosphate group that participates in the gelation process through ionic interactions with the amino group of chitosan followed by the formation of an aldimine bond (Scheme 4). The next slow pH sensitive process resulted in \( \beta \)-elimination of the phosphate group with the formation of a double bond conjugated with the aldimine functionality. We have not observed substantial polymerization of the crosslinking reagents as in the case of GA and genipin, which will be advantageous for using crosslinked chitosan materials for medical and pharmaceutical applications. These crosslinking reagents may be easily prepared by periodate oxidation of 5'-nucleotides and may be considered as ionically assisted covalent crosslinkers. The use of dialdehyde derivatives of nucleotides allows the effective crosslinking of chitosan chains with simultaneous modification such as introduction of heterocyclic bases and leads to chitosan based biomaterials with novel properties.

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References

1. Muzzarelli, R.A.A.; Muzzarelli, C. Chitosan chemistry: relevance to the biomedical sciences. Adv. Poly. Sci. 2005, 186, 151–209.

2. Kurita, K. Chitin and chitosan: functional biopolymers from marine crustaceans. Mar. Biotechnol. 2006, 8, 203–226.

3. Berger, J.; Reist, M.; Mayer, J.M.; Felt, O.; Peppas, N.A.; Gurny, R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. Eur. J. Pharm. Biopharm. 2004, 57, 19–34.

4. Azuma, K.; Izumi, R.; Osaki, T.; Ifuku, S.; Morimoto, M.; Saimoto, H.; Minami, S.; Okamoto, Y. Chitin, Chitosan, and Its Derivatives for Wound Healing: Old and New Materials. J. Funct. Biomater. 2015, 6, 104–142.

5. Kyzas, G.Z.; Bikiaris; D.N. Recent modifications of chitosan for adsorption applications: A critical and systematic review. Mar. Drugs. 2015, 13, 312–337.

6. Muzzarelli, R.A.A. Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. Carbohydr. Polym. 2009, 77, 1–9.

7. Yi, H.; Wu, L.-Q.; Bentley, W.E.; Ghodssi, R.; Rubloff, G.W.; Culver, J.N.; et al. Biofabrication with Chitosan. Biomacromolecules. 2005, 6, 2881–2894.

8. Hennink, W.E.; van Nostrum, C.F. Novel crosslinking methods to design hydrogels. Adv. Drug Deliv. Rev. 2002, 54, 13–36.

9. Anitha, A.; Rejinold, N.S.; Bumgardner, J.D.; Nair, S.V.; Jayakumar, R., in Chitosan-Based Systems for Biopharmaceuticals: Delivery, Targeting and Polymer Therapeutics, eds. B. Sarmento, & J. Neves, Chichester, West Sussex: John Wiley & Sons, 2012, pp.107–124.

10. Johnson, S.B.; Dunstan, D.E.; Franks, G.V. A novel thermally-activated crosslinking agent for chitosan in aqueous solution: a rheological investigation. Colloid Polym. Sci. 2004, 282, 602–612.

11. Monteiro, O.A.C.; Airoldi, C. Some studies of crosslinking chitosan -glutaraldehyde interaction in a homogeneous system. Int. J. Biol. Macromol. 1999, 26, 119–128.

12. Crescenzi, V.; Francescangeli, A.; Taglienti, A.; Capitani, D.; Mannina, L. Synthesis and partial characterization of hydrogels obtained via glutaraldehyde crosslinking of acetylated chitosan and of hyaluronan derivatives. Biomacromolecules. 2003, 4, 1045–1054.

13. Migneault, I.; Dartigueneave, C.; Bertrand, M.J.; Waldron, K.C. Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. Biotechniques. 2004, 37, 790–802.

14. Kildeeva, N.R.; Perminov, P.A.; Vladimirov, L.V.; Novikov, V.V.; Mikhailov, S.N. About mechanism of chitosan crosslinking with glutaraldehyde. Russian J. Bioorg. Chem.. 2009, 35, 360–369.

15. Margel, S.; Rembaum, A. Synthesis and characterization of poly(glutaraldehyde). A potential reagent for protein immobilization and cell separation. Macromolecules. 1980, 13, 19–24.

16. Butler, M.F.; Ng, Y.F.; Pudney, P.D. Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. J. Polym. Sci. Part A: Polym. Chem. 2003, 41, 3941–3953.

17. Mi, F.-L.; Shyu, S.-S.; Peng, C.-K. Characterization of Ring-Opening Polymerization of Genipin and pH-Dependent Cross-Linking Reactions Between Chitosan and Genipin. J. Polym. Sci. Part A: Polym. Chem. 2005, 43, 1985–2000.

18. Slusarewicz, P.; Zhu, K.; Hedman, T. Kinetic characterization and comparison of various protein crosslinking reagents for matrix modification. J. Mat. Sci: Mat. Med.. 2010, 21, 1175–1181.
19. Dimida, S.; Demitri, C.; De Benedictis, V. M.; Scalera, F.; Gervaso, F.; Sannino, A. Genipin-
cross-linked chitosan-based hydrogels: Reaction kinetics and structure-related characteris-
tics. J. Appl. Polym. Sci. 2015, 132, DOI: 10.1002/APP.42256
20. Maitra, J.; Shukla, V. K. Cross-linking in Hydrogels-A Review. Am. J. Polymer Sci. 2014,
4, 25–31.
21. Mikhailov, S. N.; Kildeeva, N. R.; Perminov, P. A.; Zakharova, A. N.; Nikonorov, V. V.; Donets-
skaja, A. A. Novel crosslinking reagents for producing chitosan-based biocompatible materi-
als, in Russian Patent 2408618.
22. Kildeeva, N. R.; Nikonorov, V. V.; Perminov, P. A.; Zakharova, A. N.; Mikhailov, S. N. Periodate
oxidized derivatives of nucleosides and nucleotides as novel crosslinking reagents. Collection
Symp. Ser. 2008, 10, 407–409.
23. Zakharova, A. N.; Novikov, V. V.; Donetskaya, A. I.; Perminov, P. A.; Kildeeva, N. R.; Mikhailov,
S. N. Dialdehyde derivatives of nucleosides and nucleotides: novel effective crosslinking reagents. In Advances in chitin science; Rustichelli, F.; Caramella, C.; Senel, S.; Vaarum, K. M., Eds.; 2009, Vol. XI, pp 407–474.
24. Azarova, A. I.; Perminov, P. A.; Kildeeva, N. R.; Mikhailov, S. N.; Vladimirov, L. V. Gel for-
mation in polymeric composites for modification of fibrous materials. Fibre Chem. 2011, 43,
129–133.
25. Ermolinsky, B. S.; Mikhailov, S. N. Periodate Oxidation in chemistry of nucleic acids: Dia-
dehyde Derivatives of Nucleosides, Nucleotides, and Oligonucleotides. Russian J. Bioorg. Chem.
2000, 26, 483–504.
26. Perlin, A. S. Glycol-Cleavage Oxidation. Adv Carbohydr Chem Biochem.. 2006, 60, 183–250.
27. Mikhailov, S. N.; Yakovlev, G. I. The use of periodate oxidation reaction in combination with
PMR spectroscopy for the determination of nucleoside, monosaccharide and their analogs
structure. Khimiya Prirodnykh Soedinenii (USSR), 1987, 30–33.
28. Mikhailov, S. N.; Florentiev, V. L.; Pfeiderer, W. Convenient synthesis of partially blocked
oxidized-reduced nucleosides. Synthesis. 1985, 4 399–400.
29. Efremova, A. S.; Zakharenko, A. L.; Shram, S. I.; Kulikova, I. V.; Drenichev, M. S.; Sukhanova,
M. V.; et al. Disaccharide pyrimidine nucleosides and their derivatives: a novel group of cell-
penetrating inhibitors of poly(ADP-ribose) polymerase-1. Nucleosides, Nucleotides Nucleic
Acids. 2013, 32, 510–528.
30. Senapathy, P.; Ali, M. A.; Jacob, M. T. Mechanism of coupling periodate-oxidized nucleosides
to proteins. FEBS Lett. 1985, 190, 337–341.
31. Howarth, O.; Jones, A. S.; Walker, R. T.; Wyatt, P. G. Solution structures of some uridine dialde-
yhde derivatives. Journal of the Chemical Society, Perkin Transactions. 1984, 2, 261–265.
32. Lowe, P. N.; Beechy, R. B. Preparation, structure and properties of periodate-oxidised ATP,
a potential affinity-labelling reagent. Bioorg. Chem. 1982, 11, 55–71.
33. Hansske, E.; Sprinzl, M.; Cramer, E. Reaction of the ribose moiety of adenosine and AMP
with periodate and carboxylic acid hydrazides. Bioorg. Chem. 1974, 3, 367–376.
34. Lowe, P. N.; Beechy, R. B. Interactions between the mitochondrial adenosine triphos-
phatase and periodate-oxidized adenosine 5′-triphosphate, an affinity label for adenosine
5′-triphosphate binding sites. Biochemistry. 1982, 21, 4073–4082.
35. Grant, A. J.; Lerner, L. M. Heat-induced formation of α,β unsaturated nucleoside dialdehydes
and their activity with adenosine deaminase. J. Med. Chem. 1980, 23, 795–798.
36. Davies D. B. Conformations of nucleosides and nucleotides. Prog. Nucl. Magn. Resonance
Spectroscopy. 1978, 12, 135–225.
37. Cordes, E. H.; Jencks, W. P. On the mechanism of Schiff base formation and hydrolysis. J.
Am. Chem. Soc. 1962, 84, 832–837.
38. Godoy-Alcántar, C.; Yatsimirsky, A. K.; Lehn, J. M. Structure-stability correlations for imine
formation in aqueous solution. J. Phys. Org. Chem. 2005, 18, 979–985.
39. García Del Vado, M.A.; Donoso, J.; Muñoz, F.; Echevarría, G.R.; Blanco, F.G. Kinetic and thermodynamic parameters for Schiff’s base formation between pyridoxal 5′-phosphate and n-hexylamine. *J. Chem. Soc., Perkin Transactions 2*. **1987**, *4*, 445–448.

40. Marin, L.; Simionescu, B.; Barboiu, M. Imino-chitosan biodynamers. *Chem. Commun.* **2012**, *48*, 8778–8780.