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Phosphorylation of Hyaluronic Acid †

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Abstract: Chemical phosphorylation of hyaluronic acid (HA) remains an unresolved problem for the chemistry of this unique polysaccharide, since convenient phosphorylating reagents are not reactive enough to obtain HA phosphates (HA-P) with a satisfactory degree of esterification of hydroxyl groups. The synthesis of phosphates of low-molecular-weight (43 kDa) and high-molecular-weight (0.5–0.7 MDa) HA was undertaken using such reagents as sodium trimetaphosphate $Na_3P_3O_9$, H_3PO_4, NaH_2PO_4/Na_2HPO_4, and anhydride P_2O_5. Solid-phase HA esterification with P_2O_5 was found to be the most convenient and efficient method. The HA-P samples were characterized by XRF and NMR spectroscopy (^{31}P and ^{1}H-^{31}P) and contained, depending on the HA/P_2O_5 ratio, 0.30–6.25% P wt., in the form of disubstituted mono-, di-, and polyphosphates.

Keywords: hyaluronic acid; dry phosphorylation; oxide phosphorus (V); polyphosphates

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1. Introduction

Chemical phosphorylation of hyaluronic acid (HA) with several phosphorylating reagents was recently undertaken by Bojarski et al. [1]. Trimetaphosphate sodium salt $Na_3P_3O_9$ (STMP), $P_2O_5/H_3PO_4/Et_3PO_4$ (in hexanol), polyphosphoric acid/tributylamine (in DMSO), $POCl_3$/DMF (in DMSO), and P_2O_5/methanesulfonic acid in diethyl ether were used. However, all these reagents, including STMP, were found to be insufficiently effective, and obtaining HA phosphates (HA-P) with a satisfactory degree of esterification of hydroxyl groups was not possible. Interestingly, the STMP method was previously patented and characterized as effective [2]. Taking into account the contradictory data regarding STMP's reactivity, we decided to repeat the experiment with it once again. In addition to STMP, orthophosphoric acid H_3PO_4 (85%), a mixture of NaH_2PO_4/Na_2HPO_4 salts [3], and P_2O_5 were tested as HA-phosphorylating agents. Reactions with salts and anhydride were carried out in the solid phase.

2. Materials and Methods

Samples of low-molecular-weight (LMW, 43 kDa, Leko Style, St.-Petersburg) and high-molecular-weight (HMW, 0.5–0.7 MDa, Contipro, Czech Republic) HA were used. Phosphorous (V) oxide was purchased from Acros Organics. D_2O was bought from Eurisotop. Other chemicals were of analytical reagent grade.

NMR spectra (^{31}P and ^{1}H-^{31}P HMBC) were recorded on a Bruker Avance II 400 MHz (400.13 MHz for ^{1}H and 161.90 MHz for ^{31}P) spectrometer. Samples were analyzed as solutions in D_2O (5–20 mg/mL) at room temperature (δ 0 ppm for H_3PO_4). The total content of P in HA-P samples was analyzed with the help of an XRF spectrometer EDX-7000 (Shimadzu, Kyoto, Japan).

3. Results and Discussion

The reactions of LMW and HMW HA with STMP were carried out under conditions close to those of the patent [2], at various HA concentrations, HA/STMP ratios, alkaline

reagents (K_2CO_3 or NaOH), and reaction times (Table 1). Samples were purified by dialysis for three days. However, even after such purification, the ^{31}P NMR spectra of all samples contained a signal at (−21)–(−21.5) ppm characteristic for STMP, as well as signals at 2.44, −5.43, −5.54, and −6.47 ppm, most likely corresponding to the decomposition products of STMP under the action of alkali. Evidence for the covalent binding of P to HA was obtained using two-dimensional ^{1}H-^{31}P HMBC spectra, in which cross-peaks were detected at 3.84/0.02 (C-6 in GlcNAc), 3.77/0.02 (C-6 in GlcNAc), and 3.46/0.02 ppm (C-3 in GlcA, minor intensity) and corresponded to disubstituted monophosphates of HA (dMP); phosphate residue was connected mainly with the HA primary hydroxyl groups. As can be seen from Table 1, the conditions of protocols 2 (0.3% P, K_2CO_3, 48 h) and 3 (0.27% P, NaOH, 3 h) were found to be the best. The long reaction time in protocol 4 apparently caused the hydrolytic elimination of phosphate groups. HMW HA reacted with STMP (entry 7) only under the conditions of experience 2, but less efficiently than LMW HA.

Phosphorylation of LMW HA with H_3PO_4 and NaH_2PO_4/Na_2HPO_4 was found to be unsuccessful (Table 1, entries 8, 9).

Table 1. Conditions for HA reactions with $Na_3P_3O_9$, H_3PO_4 (85%), and NaH_2PO_4/Na_2HPO_4 and characteristics of the products.

No.	HA Concentration in Aqueous Solution (Entries 1–7), mg/mL; Ratio of Reagents (Calculated per HA Disaccharide Unit; Temperature; Reaction Time	Content of P, % wt.
entry 1	[LMW HA] = 33.3; HA:$Na_3P_3O_9$:K_2CO_3 = 1:5:1; 20 °C; 3 h	0
entry 2	[LMW HA] = 33.3; HA:$Na_3P_3O_9$:K_2CO_3 = 1:5:1; 20 °C; 48 h	0.30
entry 3	[LMW HA] = 33.3; HA:$Na_3P_3O_9$:NaOH = 1:5:1; 20 °C; 3 h	0.27
entry 4	[LMW HA] = 33.3; HA:$Na_3P_3O_9$:NaOH = 1:5:1; 20 °C; 48 h	0
entry 5	[LMW HA] = 66.7; HA:$Na_3P_3O_9$:NaOH = 1:2.5:1; 20 °C; 3 h	0
entry 6	[LMW HA] = 66.7; HA:$Na_3P_3O_9$:NaOH = 1:2.5:1; 20 °C; 48 h	0
entry 7	[HMW HA] = 33.3; HA:$Na_3P_3O_9$:K_2CO_3 = 1:5:1; 20 °C; 48 h	0.09
entry 8 *	LMW HA:H_3PO_4 = 1:40; 20 °C; 24 and 48 h	0
entry 9 **	LMW HA + NaH_2PO_4/Na_2HPO_4 (2.5:1 ratio); 55 °C (24 h) and 105 °C (3 h)	0

* LMW HA (1 g, 2.45 mmol) was dissolved in 5 mL of H_3PO_4 (85%), kept for 24 h and 48 h, then purified as in [1] and dried. ** Reaction was carried out according to ref. [3] with some modifications.

The reactions of LMW HA and HMW HA with P_2O_5 at different ratios were carried out by intensive grinding of dry HA and P_2O_5 in a porcelain mortar at room temperature for 20–30 min. Next, the samples were kept for ~2 h, and then purified and dried. The total P content found by XRF analysis was 1.28–6.25% for LMW HA-P and 0.30–2.55% for HMW HA-P and varied depending on the HA/P_2O_5 ratio (Table 2). As can be seen from these data, dry HA phosphorylation under the action of P_2O_5 is much more efficient than other methods of obtaining HA-P [1].

Table 2. Characteristics of LMW HA-P (entries 1–4) and HMW HA-P (entries 5–8) samples depending on the HA/P_2O_5 ratio.

No.	HA/P_2O_5	Total P, % wt.	*P* in dMP, % wt.	*αP* in dDP + dPP *, % wt.	-$(P)_n$- in dPP **, % wt.
entry 1	1:0.2	1.39	0.63	0.60	0.16
entry 2	1:0.5	1.28	0.07	1.00	0.21
entry 3	1:1	1.74	0.06	1.34	0.30
entry 4	1:2	6.25	0.10	5.60	0.30
entry 5 ***	1:0.2	0.30	-	-	-
entry 6 ***	1:0.5	0.75	-	-	-
entry 7	1:1	2.55	0.07	0.57	1.91
entry 8	1:2	2.25	0	0.55	1.70

* In the ^{31}P NMR spectra, the signals of the αP in dDP and dPP (see Figure 1) are in the same range of chemical shifts, (−10)–(−11.5) ppm; therefore, the total content of αP in (dDP + dPP) is given. ** In the dPP, $n \geq 1$. The P content is given only for the middle -$(P)_n$-. *** The spectra of these two samples were not recorded due to the low P content and the high viscosity of their solutions in D_2O.

It is known from the literature that various types of phosphate residues can be formed in the process of phosphorylation of polysaccharides (PSs): mono- and disubstituted monophosphates (mMPs and dMPs), mono- and disubstituted diphosphates (mDPs and dDPs), polyphosphates (PPs) both with a terminal phosphate group (mPPs) and in the form of disubstituted derivatives (dPPs). The characteristic signals in the ^{31}P NMR spectra for each of the listed structures are shown in Table 3.

Table 3. Characteristic signals of atom P in ^{31}P NMR spectra depending on the structure of phosphates.

Structure of Phosphat		Characteristic Signals, ppm
mMP	PS-***P***	2.2–5.3
dMP	PS-***P***-PS	(−1.0)–1.0
mDP	PS-α***P***-β***P***	α***P***: (−10.0)–(−11.5) β***P***: (−4.5)–(−6.0)
dDP	PS-***P***-***P***-PS	(−10.0)–(−11.5)
mPP	PS-α***P***-(***P***)$_n$-ω***P***	α***P***: (−10.0)–(−11.5) (***P***)$_n$: (−19.0)–(−24.0) ω***P***: (−4.5)–(−6.0)
dPP	PS-α***P***-(***P***)$_n$-α***P***-PS	α***P***: (−10.0)–(−11.5) (***P***)$_n$: (−19.0)–(−24.0)

According to these data (Table 3), the obtained HA-P samples contain only three types of phosphate residues: dMF (0.02 ppm), dDP (−10.8 ppm), and dPP ((−22.2)–(−23.8) ppm). Their distribution in HA-P, calculated from the XRF data and the integral intensity of each of the corresponding signals in the ^{31}P NMR spectra, is represented by the P content and is given in Table 2.

It is interesting to note the high content of polyphosphate sequences in samples 7 and 8. The ^{31}P NMR spectrum of sample 8 is shown in Figure 1.

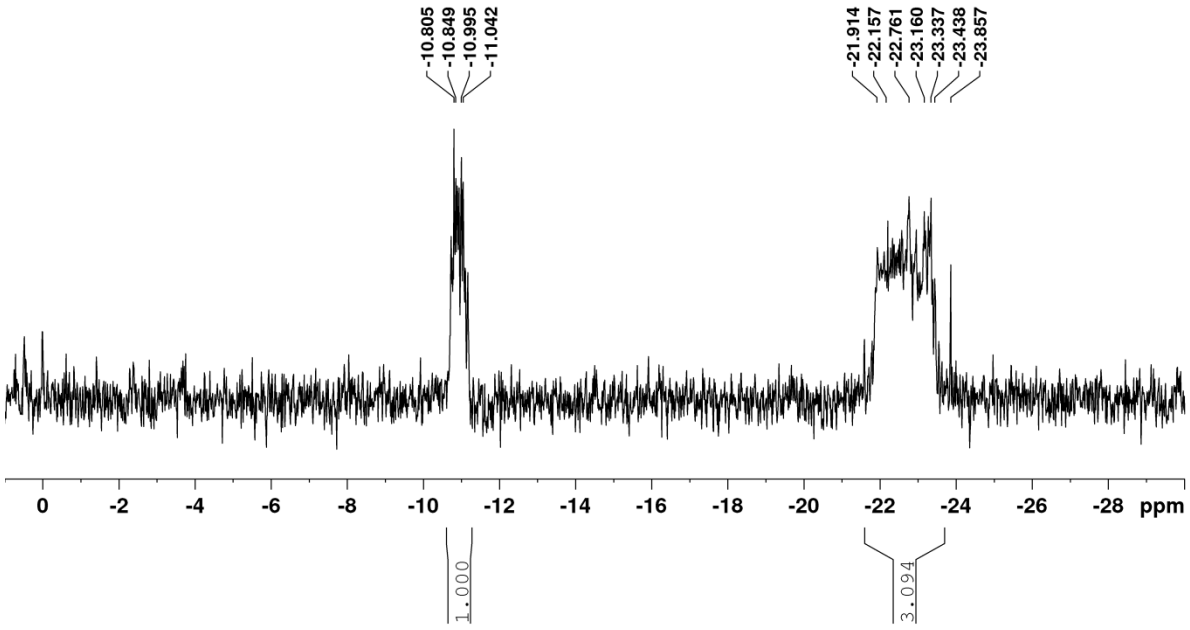

Figure 1. ^{31}P NMR spectrum of sample 8 (Table 3).

Polyphosphates (PPs) can be considered as inorganic fragments included in the HA macromolecular chains. According to modern concepts, inorganic PPs are a source of

phosphate in the process of bone mineralization. PPs can also be used in regenerative medicine. Firstly, they have shown morphogenetic activity, i.e., take part in cell differentiation through gene induction; secondly, they act as an accumulator and energy donor in the intercellular space. In addition, adenosine diphosphate and adenosine triphosphate (ADP/ATP) are formed from PPs under the combined action of alkaline phosphatase and adenylate kinase. For example, inorganic PPs added externally to mammalian cells lead to a 3-fold increase in ATP [4].

4. Conclusions

HMW and LMW HA phosphorylated derivatives with a high content of phosphate residues were first obtained by solid-phase reaction with P_2O_5. One feature of the phosphorylation process was formation of disubstituted mono-, di-, and polyphosphates in the structure of HA macromolecules.

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