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Note

Kinetics of drug release from a hyaluronan–steroid conjugate investigated by NMR spectroscopy

Anna Taglienti [a,*], Paolo Sequi [b], Massimiliano Valentini [a,*]

[a] Agricultural Research Council, Research Centre for Soil–Plant Systems, Instrumental Center of Tor Mancina, S. P. Pascolarese-Strada della Neve Km 1, 00015 Monterotondo, Rome, Italy
[b] Agricultural Research Council, Research Centre for Soil–Plant Systems, Via della Navicella 2-4, 00184 Rome, Italy

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ABSTRACT

The methylprednisolone steroid ester of hyaluronan was hydrolyzed under physiological conditions in vitro, and the kinetics of drug release was investigated by NMR spectroscopy. Transverse relaxation times are correlated with the molecular rotational freedom, which undergoes large changes for methylprednisolone when released. Multi-exponential decays were observed, which together with the corresponding population gave valuable insights into the conformational changes that occur in the biopolymer during hydrolysis. The biomaterial exists in aqueous solution in two conformations, 'collapsed' and 'water-exposed', in equilibrium. Under physiological conditions, the methylprednisolone is completely released within 48 h. Transverse relaxation times proved to be an appropriate tool for monitoring the drug release in vitro.

Hyaluronic acid[1,2] (HA) is a natural polysaccharide belonging to the class of glycosaminoglycans. It is a major component of the extracellular matrix and of liquid connective tissues (synovial fluid, eye vitreous body). The biological relevance[3] and the versatility of the hyaluronan structure make this molecule an attractive building block for the preparation of polymer conjugates[4–6] with potential biomedical applications.[7–11] One of the most promising polymeric prodrugs based on HA is obtained from the combination of hyaluronan with the anti-inflammatory steroid methylprednisolone (HYC).[12] The latter is covalently bound to HA by esterification of the hydroxyl groups in the C-21 position of the steroid with the carboxyl groups of the glucuronic acid moieties of hyaluronan.[13]

A notable feature of the HYC system is that an anti-inflammatory drug of proven efficiency is linked to a polymer that is used in the treatment of inflammatory joint diseases itself.[14–18] The ester bond should allow for slow release of the drug, and once hydrolyzed, the free hyaluronan could exert its viscosupplementation effect. The ester bond between the drug and the carrier has been also chosen considering that the covalent bond is capable of affording stability to the product with time, though being labile enough under physiological conditions, for example, temperature and pH.

HYC has been already tested in vitro for its resistance to proteins and cell adhesion,[19] to evaluate one of the critical aspects of its use as a biomaterial. The kinetics of methylprednisolone release from HYC is another key point for potential biomedical applications. In fact, such studies assess whether this system is able to deliver an anti-inflammatory pharmacological activity, and the hydrolysis time scale suggests the adjustment of some side parameters in the treatment to make the biomaterial more efficient. Moreover, the kinetics of drug release affects the frequency of the inoculations, and, if the delivery should result too slow, one could think of possible strategies to speed it up, for example, local warming of the inoculation site by infrared radiation. Hyaluronan is quickly degraded in an inflamed synovial fluid, with a residence time 6–8 h, and so a slow release completed in a period of around 24 h from the injection is desired.

Drug release has been investigated by means of several techniques, including NMR spectroscopy.[20] ^{1}H NMR spectroscopy has been used for tracking molecular transformations occurring in solution; drug release and oxidation can be detected in the NMR spectrum by monitoring changes of selected peaks (shape and intensity).[21] Nevertheless, these parameters are not at all informative in case of signal overlap between the bound and released drug. Here, we propose T_2 measurements as a possible tool for investigating the drug release kinetics of a biomaterial having NMR peaks of free and bound drug superimposed.

Figure 1 shows the ^{1}H NMR spectrum of HYCp45 in D_2O obtained with a water suppression pulse sequence, which allows the removal of the intense HDO peak at ca. 4.70 ppm. One can clearly see three resonances between 6.0 and 6.5 ppm

* Corresponding authors. Tel.: +39 6 90627203; fax: +39 06 9068309 (M.V.).
E-mail addresses: anna.taglienti@entecra.it (A. Taglienti), massimiliano.valentini@entecra.it (M. Valentini).

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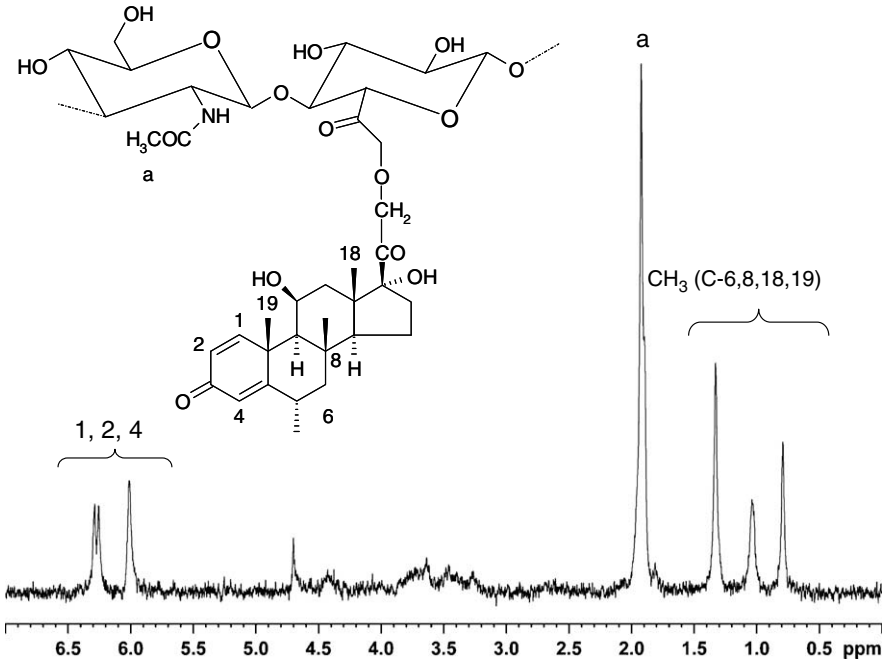


Figure 1. ^{1}H NMR spectrum, acquired with a spectrometer with an operating frequency of 400.13 MHz, of a HYCp45 0.5% w/V solution in D_2O.

corresponding to the methylprednisolone olefinic protons (1, 2, and 4 of cyclo A). The intense peak at $\delta = 1.95$ ppm arises from the methyl of the acetamido function of hyaluronan, and the remaining four signals (the two at ca. 1.00 ppm are barely separable) in the aliphatic region, that is, 0.7–1.3 ppm, belong to the methyl protons of cyclo B and C of the steroidic fragment (positions 6, 8, 18, and 19). Furthermore, one can barely see some broad and weak peaks in the range 3.0–4.5 ppm, which can be assigned to the remaining protons of methylprednisolone and the sugar moiety. The featureless structure of these signals is due to the very short transverse relaxation times, which occur for a drastic reduction of the local mobility. In fact, the full-width at half-height of an NMR signal, Δv, depends on the transverse relaxation time T_2, according to Eq. 1

$$\frac{1}{\pi T_2} = \Delta v \tag{1}$$

In turn, T_2 is inversely proportional with the correlation time, τ_c, which is defined for an isotropic molecule as the average time taken for the molecule to rotate through one radian. In practical terms, rapidly tumbling protons are characterized by short correlation times, thus long transverse relaxation times and hence narrow linewidths. On the contrary, slowly tumbling protons have longer τ_c, corresponding to short T_2 and broad NMR signals.[22] T_2, and therefore τ_c, refers to the local mobility, and it is worthwhile to point out that the τ_c, or the T_2, for a portion of a molecule can be shorter, or longer, than one would expect considering the total molecular volume, due to a locally higher, or lower, degree of freedom. This is particularly important for large molecules, for example, proteins and polymers, and specifically in our case for hyaluronan derivates. This implies that some specific protons can have shorter or longer τ_c than others within the same molecule.

We have investigated the kinetics of HYCp45 hydrolysis by measuring the transverse relaxation times of two selected protons of the biomaterial, one belonging to the methylprednisolone fragment at $\delta = 1.95$ ppm and the other to the hyaluronan backbone at $\delta = 0.80$ ppm. The first resonance is used as the parameter, which is directly related to the drug-release kinetic and the second plays the role of the control.

In the case of partially hydrolyzed HYCp45, methylprednisolone is in part released, and the rest is still bound to the hyaluronan backbone. The former tumbles faster than the latter, therefore the first has shorter τ_c and longer T_2 than the second. Because the NMR signals of free methylprednisolone and bound methylprednisolone are superimposed, one would expect, and this was the case, to observe for these resonances a double-exponential decay for the T_2s. The slow-relaxing component corresponds to the fast moving, that is, free methylprednisolone, while the faster one to the bound drug.

Figure 2 shows the intensity of the NMR signal at 0.80 ppm (methylprednisolone) which is measured with a Carr–Purcell–Meiboom–Gill sequence at different echo times, that is, $n2\tau$, for partially hydrolyzed HYCp45 samples. The fitting of the points requires a double-exponential decay function, Eq. 2

$$A(t) = A_f \exp(-t/T_{2_f}) + A_s \exp(-t/T_{2_s}) \tag{2}$$

which contains a T_{2f} and a T_{2s} term, representing fast and slow transverse relaxation time, respectively. The two components have

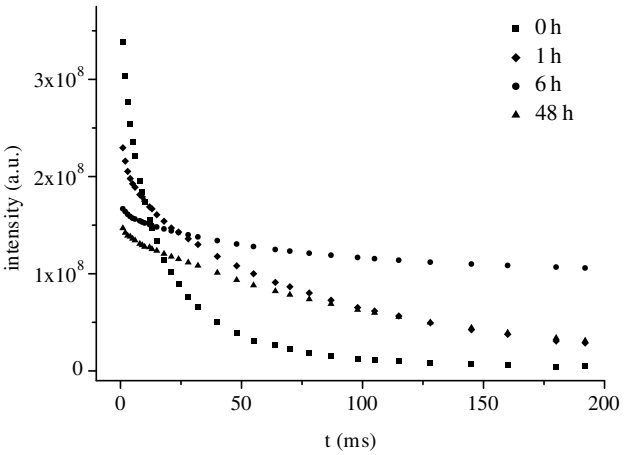


Figure 2. Intensities of the signal at 0.80 ppm versus t, at different hydrolysis times, of HYCp45 (i.e., 0, 1, 6, and 48 h) at 37 °C and pH 7.0.

Table 1
T_{2f}, T_{2s}, A_f, and A_s values for the protons at 0.80 ppm (methylprednisolone) and 1.95 ppm (acetamido group of polysaccharidic backbone), calculated with Eq. 2 at different hydrolysis times of HYCp45 at 37 °C and pH 7.0

t (h)	δ = 1.95 ppm				δ = 0.80 ppm			
	T_{2f} (ms)	T_{2s} (ms)	A_f (%)	A_s (%)	T_{2f} (ms)	T_{2s} (ms)	A_f (%)	A_s (%)
0	4.1 ± 0.2	38.0 ± 1.0	59	41	7.1 ± 0.5	35.2 ± 1.9	55	45
1	4.8 ± 0.2	46.1 ± 1.0	50	50	2.1 ± 0.5	66.0 ± 1.4	23	77
3	4.5 ± 0.2	44.2 ± 1.0	49	51	3.1 ± 0.6	59.5 ± 1.0	21	79
6	4.4 ± 0.2	46.0 ± 1.0	50	50	2.4 ± 0.5	59.4 ± 0.8	17	83
9	5.1 ± 0.2	46.7 ± 1.0	52	48	3.0 ± 0.5	60.2 ± 1.0	20	80
18	5.9 ± 0.3	49.0 ± 1.0	47	53	0.9 ± 0.5	65.6 ± 1.2	12	88
32	5.2 ± 0.2	46.9 ± 1.0	45	52	1.9 ± 0.7	77.1 ± 1.2	11	88
48	4.9 ± 0.2	47.1 ± 1.0	45	55	2.4 ± 0.5	103 ± 4	8	92

different pre-exponential coefficients, that is, A_f and A_s, proportional to the percentage of the corresponding component. Table 1 reports the calculated values for T_{2f}, T_{2s}, A_f, and A_s for the methylprednisolone and for the acetamido group, at 0.80 and 1.95 ppm, respectively.

For the hyaluronan backbone proton, the fast-relaxing component has a T_2 value of approximately 4 ms, and it can be attributed to a fraction of HYCp45 molecules in a 'collapsed' conformation, as depicted in Scheme 1, characterized by a hydrophobic core with very low mobility, and thus long τ_c, of the hyaluronan atoms. Such a HYCp45 conformation was already assessed to be predominant in aqueous solution in the absence of hydrolyzing conditions.[23] The slow-relaxing component of the hyaluronan backbone protons has a T_2 of approximately 40 ms, which remains almost constant with ongoing hydrolysis. We attributed this component to HYCp45 molecules in an 'open' conformation (see Scheme 1), which allows extended contact with the water solvent, explaining thus the shorter correlation time.

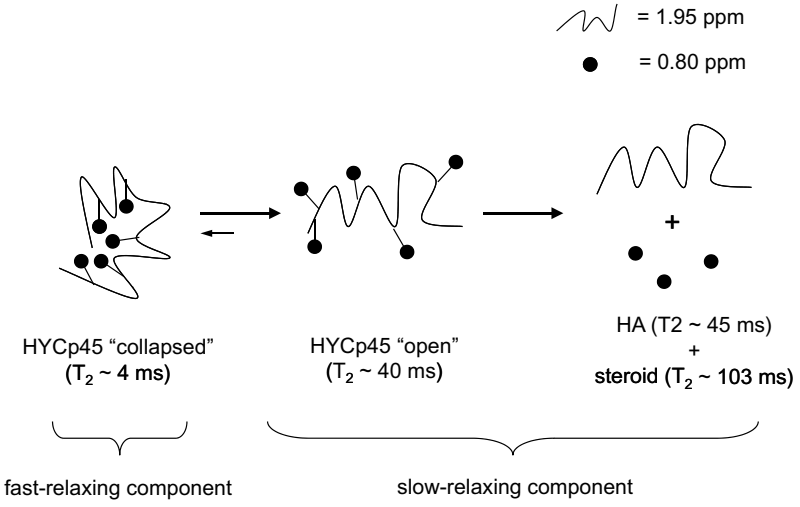
For methylprednisolone protons, that is, δ = 0.80 ppm, we observed at a hydrolysis time equal to 0 that the fast-relaxing component has T_2 values very similar to those found for the acetamido group of the backbone, which was attributed to HYCp45 'collapsed'. By increasing the hydrolysis time, T_{2f} becomes shorter until the component almost disappeared after 48 h. The slow-relaxing component increased its value from ca. 40 ms, the methylprednisolone in HYCp45 'open' conformation, up to 103 ms, which we attributed to the released methylprednisolone (see Scheme 1). This T_2 value is consistent with the one measured for a 0.1% w/V D_2O solution of 6α-methylprednisolone, which was equal to 114 ms.

Pre-exponential coefficients are as informative as T_2, they are expressed as a percentage of the corresponding component, and one can see that for the signal at δ = 1.95 ppm the fast-relaxing component A_f, which corresponds to the HYCp45 'collapsed', slightly decreased with hydrolysis, from 59% to 45%. Consequently, the slow-relaxing component, assigned to the 'open' conformation, increased up to 55%. For the peak at δ = 0.80 ppm A_f, corresponding to methylprednisolone of 'collapsed' HYCp45, decreased from 55% to 8%, and accordingly the slow-relaxing coefficient, belonging to released methylprednisolone, increased up to 92%.

The kinetics obtained from the T_2 data, although qualitative, is shown in Figure 3. In the left panel, the slow component of T_2s, for both hyaluronan and methylprednisolone protons, is plotted versus hydrolysis time. The signal at δ = 0.80 ppm, that is, methylprednisolone, is representative for the evolution of hydrolysis, while the other resonance, at δ = 1.95 ppm, is the control. In the right panel, the evolution of A_s for both measured signals is reported. It is worth mentioning that during T_2 measurements, the solution became slightly turbid, due to the precipitation of released methylprednisolone, which is poorly soluble in water. Nevertheless, the precipitation did not influence the T_2 measurement, which gave the same results on samples in which the precipitate was removed by filtration.

On the basis of obtained NMR data, we propose the existence of three components in solution during the hydrolysis reaction: the first is HYCp45 'collapsed', which slightly decreases with the ongoing hydrolysis and is characterized by a short T_2, that is, ~4 ms. The second is HYCp45 'open', having methylprednisolone and polysaccharide backbone exposed to water solvent and a T_2 of ~40 ms. Finally, the released steroid with transverse relaxation time of 103 ms is present. At t = 0, all the methylprednisolone is covalently bound to hyaluronan,[13] and only the 'collapsed' and 'open' conformations are present. However, after 48 h hydrolysis time the methylprednisolone proton accounts for the released state (T_2 = 103 ms), and the backbone proton's T_{2f} and T_{2s} represent the 'collapsed' and 'open' conformations. These data are in agreement with a previously reported study on a very similar system, that is, HYCp15.[12] The methylprednisolone release was investigated under different conditions, and it was found that at 37 °C and pH equal to 7.0 the $T_{50\%}$ was 6 h.

The analysis of the different populations as a function of hydrolysis time, Figure 3, right panel, is informative concerning



Scheme 1. The different species present in solution during hydrolysis characterized by their approximate T_2 value: the 'collapsed' HYCp45, 'open' HYCp45, HA, and released methylprednisolone.

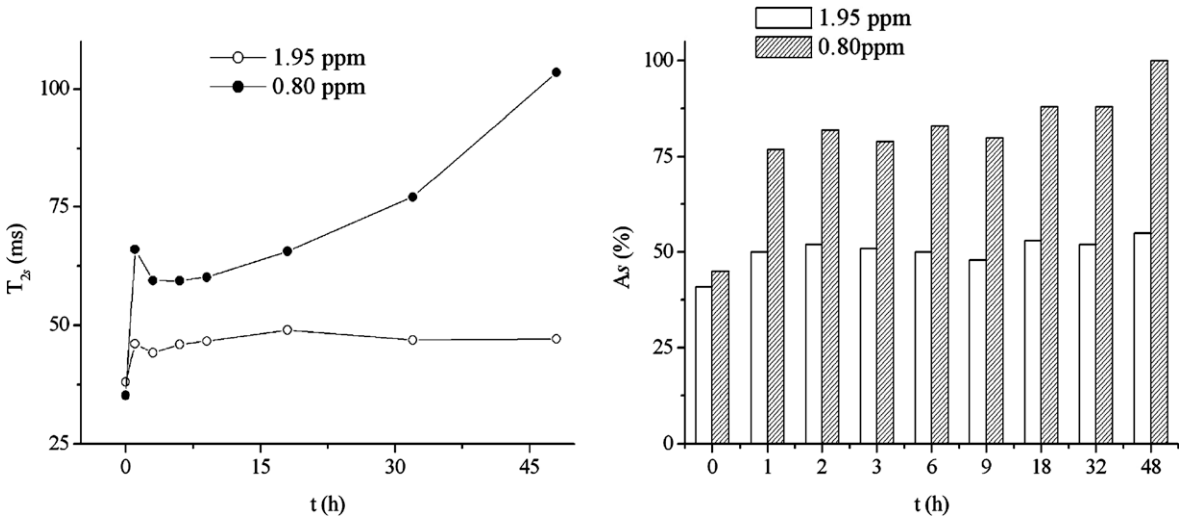


Figure 3. Evolution of T_2 (left panel) and A_s (right panel) with hydrolysis time. Data for protons resonating at 0.80 ppm (methylprednisolone) and 1.95 ppm (acetamido group of polysaccharidic backbone) are reported.

the relationships between 'collapsed' and 'open' conformations of HYCp45, and between bound methylprednisolone and released methylprednisolone. The populations A_f and A_s changed for both of the monitored protons with ongoing hydrolysis. The A_f decrease for the hyaluronan backbone is related to the conformational change of HYCp45 from 'collapsed' to 'open'.

The populations of the methylprednisolone protons, that is, 0.80 ppm, changed significantly upon hydrolysis. At $t = 0$, the populations represent the two HYCp45 conformations, which are in similar quantities. The 'open' conformation with bound methylprednisolone decreased markedly upon hydrolysis until disappearing completely. The reaction is particularly fast in the first hour, during which the free methylprednisolone amount almost doubles. The drug release also leads to the formation of HA, which has an extended coil conformation and a T_2 of ~45 ms. The T_2s of the signal at 1.95 ppm accounts for this species. Therefore, at the end of hydrolysis, only 'collapsed' HYCp45, HA, and released drug are present in solution. The 'open' HYCp45 is quickly hydrolysed in the first hours (Fig. 3, left panel) also for a mass action effect law: the addition of reactant drives the reaction towards products. The 'collapsed' HYCp45 amount reaches a plateau after 18 h, and is stable in neutral pH conditions. The remaining HYCp45 in the 'open' conformation continues to be hydrolyzed with a lower rate, probably because the reaction is under kinetic control and the amount of substrate is decreasing.

In conclusion, we have investigated the release of the anti-inflammatory drug methylprednisolone in vitro from the hyaluronan–methylprednisolone chemical conjugate HYCp45. The kinetics of hydrolysis of the ester bond under physiological conditions was elucidated using NMR spectroscopy by monitoring the transversal relaxation times T_2 of protons belonging to the carrier and the drug. All transverse relaxation profiles are double-exponential functions with a fast and a slow component. For the hyaluronan backbone, the fast component accounts for a 'collapsed' form and the slow for the 'open' one, the latter characterized by protons exposed to water solvent. For methylprednisolone protons, the long T_2 corresponds to the released drug and the short to the bound one. Populations of each component have been estimated from the pre-exponential factors of the fitting functions, and were informative on the relative amounts and on the equilibrium in solution. The release is completed in 48 h, and already after 1–2 h most of the steroid is available in solution. At the end of hydrolysis, the HYCp45 'open' is no longer present in solution, where only 'collapsed' HYCp45, HA, and free methylprednisolone can be found. We thus report a novel application of transverse relaxation times measured by NMR spectroscopy to study drug release from biomaterials. This approach offers a powerful tool for analogous investigations.

1. Experimental

1.1. Materials

D_2O was purchased from Acros Organics, and was used without further purification. Hyaluronan–methylprednisolone ester, HYC, was supplied by Fidia Farmaceutici S.p.A (Abano Terme, PD, Italy) as a freeze-dried powder, and was used without further purification. It was dissolved in D_2O at the desired concentration before use. The HYC derivative used, HYCp45, was synthesized from hyaluronan fraction M.W. = 600,000 and had 45% of the carboxylic groups of glucuronic acid functionalized with the steroid (by HPLC, data not shown), the remaining carboxylic groups being in the sodium salt form.

1.2. NMR experiments

NMR measurements were performed on a Bruker AVANCE 400 MHz spectrometer equipped with a microprocessor-controlled gradient unit and a multinuclear probe with an actively shielded Z-gradient coil. All samples were measured in D_2O, which was used for the reconstitution of the powder. The HYC derivative is fully

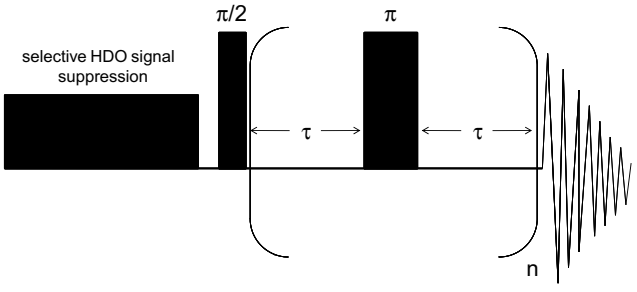
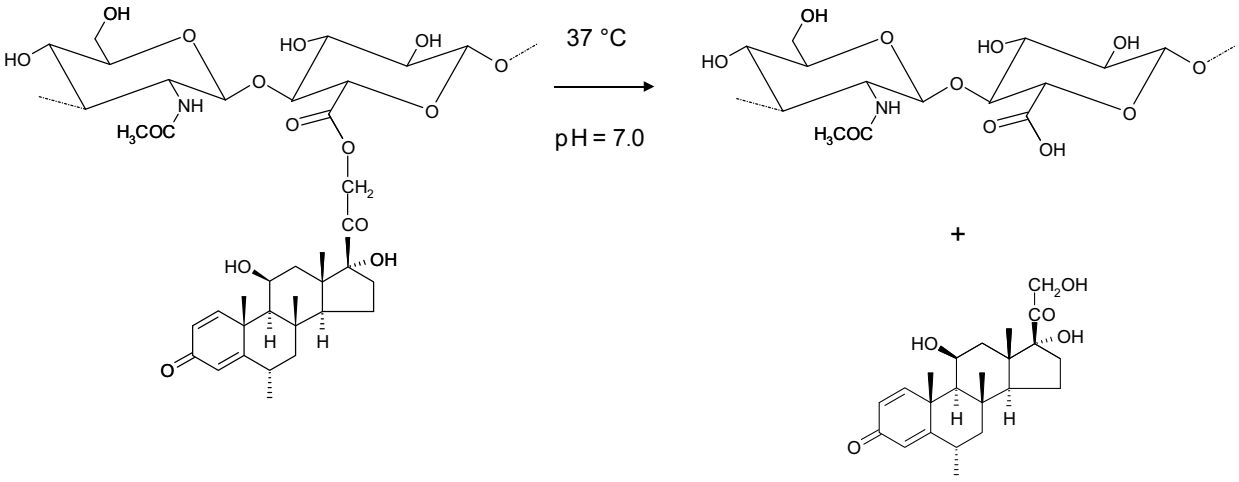


Figure 4. Sequence used for T_2 measurement. The echo is generated at $t = n2\tau$.



Scheme 2. Hydrolysis reaction of the ester bond of HYCp45 for the release of methylprednisolone at 37 °C and pH 7.0. The reaction was initiated by heating the solution.

soluble in water, and the solution shows nearly the same viscosity of pure water, as already shown in a previous work.[23]

^{1}H NMR spectra were recorded by using the selective water suppression according to a standard procedure.[24] HDO signal was saturated using a 3 s pulse having 45 dB intensity. Chemical shifts are reported using TSP as reference. ^{1}H NMR T_2 measurements were recorded using the Carr–Purcell–Meiboom–Gill[25,26]-based pulse sequence containing a water suppression part as described in Figure 4. Measurements were performed with 32 scans, 16 dummy scans, time domain of 1k, delay between scans of 10 s, $\pi/2$ pulse length was 4.90 μs and its amplitude 0 dB (Bruker library), τ was set equal to 500 μs; water suppression was achieved with a 5 s continuous irradiation of the water frequency at power level of 60 dB (Bruker library). The values of n used were 2, 4, 6, 8, 10, 12, 16, 18, 20, 24, 26, 30, 36, 42, 48, 56, 64, 80, 96, 110, 128, 140, 156, 174, 196, 210, 230, 256, 290, 320, 360, and 384, leading to delays between the first 90° pulse and beginning of acquisition equal to 1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 13, 15, 18, 21, 24, 32, 40, 48, 55, 64, 70, 78, 87, 98, 105, 115, 128, 145, 160, 180, and 192 ms. The data were handled as a 2D experiment, where the 32 experiments with different n values are stored as rows in the 2D matrix. Prior to being Fourier transformed, the FID was multiplied by a line-broadening (lb) factor equal to 0.30. We monitored the signal at 0.80 ppm, corresponding to the methylprednisolone moiety, and the peak at 1.95 ppm, the acetamido group of the polysaccharidic backbone of HYC.

1.3. Hydrolysis

The hydrolysis of the ester bond, which occurs according to Scheme 2, was activated on HYC samples dissolved in D_2O at 0.5% w/V concentration by adjusting the pH to 7.0 with 0.02 M NaOH, and heating the solution at 37 °C for periods ranging from 1 up to 48 h; the hydrolysis reaction was in this case initiated by heat. The hydrolysis product was then immediately quenched in melting ice and transferred in the NMR tube for T_2 measurement. All measurements and experiments were repeated twice, and data reported in Table 1 are the averages with the calculated standard deviations. The latter were obtained from the classical error propagation combining the experimental errors of the measurements.

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