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para-Hydrogenation of unsaturated moieties on poly(lysine) derived substrates for the development of novel hyperpolarized MRI contrast agents

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Four alkyne-functionalized poly(lysine) derivatives have been synthesized and characterized by ^{1}H and ^{13}C NMR spectroscopy. In the first poly(lysine) derivative, phenylpropiolate moieties are directly bound to the aminic arms, whereas in the other derivatives, propargylamine moieties are bound to the aminic poly(lysine) arms through glucaric acid and diethylene glycol (DG) chains, respectively. *para*-Hydrogenation of the alkyne-functionalized poly(lysine) compounds has been investigated and the results have been discussed in terms of spin lattice relaxation properties of the hydrogenated products. It is shown that the longer the mobile chain separating the unsaturation from the poly(lysine) backbone, the more intense the polarized signals when *para*-hydrogenation is carried out. This is due to (a) the maintenance of short reorientational times on the unsaturated ends, and therefore a sufficiently long T_1 of the protons added during hydrogenation, and (b) the minor effect of steric hindrance by the poly(lysine) backbone that decreases interaction of the unsaturation with the catalyst, allowing higher hydrogenation rates.

Introduction

Since *para*-H_2 effects in NMR spectra were discovered about fifteen years ago by Weitekamp and Eisenberg,[1–4] a number of systems have been investigated, mainly in order to better understand hydrogenation mechanisms.[5–8] In fact the magnetization transfer order from *para*-H_2 to the hydrogenated product yields extraordinary enhancements in the NMR signal which, in theory, may reach values as high as 10^5 times the signal intensity of the corresponding derivatives produced with normal H_2,[4] allowing the detection of species which are present in solution in very low concentrations.

The large enhancement of NMR signal due to the reaction with *para*-H_2 may also find an interesting application in the field of magnetic resonance imaging (MRI). The contrast in a MR image arises basically from differences in proton spin density and in their relaxation times. Therefore the search for contrast agents for MRI has been mainly focused on chemicals able to markedly enhance the relaxation rate of water protons.[9–11] Recently, Golman and coworkers[12] suggested a new exciting approach to the development of MRI contrast agents by exploiting the high signal/noise ratio of *para*-hydrogenated products. Actually, they set up a procedure aimed at transferring the spin-order of the *para*-hydrogen system to enhance the magnetization of a neighbouring ^{13}C atom. Thus they acquired an angiographic image obtained by monitoring the ^{13}C signal intensity of a *para*-hydrogenated molecule injected into the aorta of a rat. In this case the contrast agent was *para*-hydrogenated methyl acetylenedicarboxylate, and the angiogram of the rat was obtained by a single shot RARE sequence.[13]

The potential use of *para*-hydrogenated substrates in magnetic resonance imaging relies, first of all, on two main issues, namely an highly efficient hydrogenation step and the occurrence of long relaxation times of the two added hydrogens. Both issues can dramatically change between a small unsaturated molecule to its multimeric derivatives.[14] In particular, the relaxation time of the *para*-hydrogen derived nuclei is a critical parameter, as it should be as long as possible in order to allow the full exploitation of their polarization in the acquisition of the image.

Large-sized molecules containing hyperpolarized moieties may be of interest in several MRI applications. Longitudinal ^{1}H-relaxation time (T_1) in organic molecules is dominated by dipolar interactions and is inversely dependent upon the molecular reorientational time (τ_C),[14] which is directly related to the molecular size: the larger the molecule, the longer τ_C and the shorter T_1. Therefore, in order to keep τ_C short enough, one has to design large-sized systems in which the hyperpolarized moieties are linked to the macromolecular backbone by flexible chains that allow ample, local motions.

The long-term goal of our project is to synthesize systems containing many equivalent alkynyl moieties that, upon *para*-hydrogenation, act as hyperpolarized molecules whose distribution, as a consequence of the relatively high molecular weight, is confined to the intravascular space (blood pool agent). The substrate for the generation of multimeric *para*-hydrogenated systems is provided by poly(lysine) at different polymerization degrees (PD). This poly(amino acid) consists of a peptidic backbone with ε-butylamino groups pointing outward that may serve as anchoring sites for the conjugation of the unsaturated moieties susceptible to *para*-hydrogenation.

We surmise that it is possible to limit the decrease of the relaxation times of the olefinic protons if a proper spacer is introduced between the ε-amino groups of poly(lysine) and the unsaturated moiety. The spacer should provide the unsaturated moiety with a high mobility, as it is known that the dipolar relaxation mechanism responsible for the T_1 of the olefinic protons is dependent upon the reorientational time of the H–H magnetic vectors. The introduction of a long, flexible spacer would bring an additional advantage as far as the *para*-hydrogenation reaction is concerned. In fact, it is expected that the interaction between the unsaturated C≡C moiety and the hydrogenation catalyst will be favoured by increasing the length of the spacer.

Results

We address the study of the *para*-hydrogenation of phenylpropiolate, propargylamine and their multimeric derivatives, based on the conjugation of these monomers, with or without spacers, to poly(lysine) chains. In order to reduce H–H dipolar contributions to the observed relaxation times in hydrogenated propargylamine, the D/H replacement for the exchangeable protons has been carried out before hydrogenation, by dissolving the propargylamine-containing molecules in D_2O.

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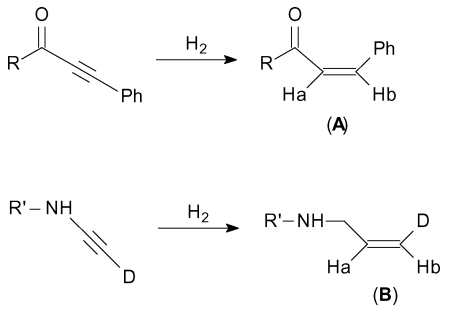
(1) *para*-Hydrogenation of phenylpropiolate (Ph–C≡C–COOEt) and deuterated propargylamine (D_2N–CH_2–C≡CD)

The *para*-hydrogenation reactions have been carried out directly in the NMR tube, in methanol solutions at ambient temperature, by using [Rh(COD)(dppb)][BF_4] (COD = cyclooctadiene, dppb = diphenylphosphinobutane)[15] as catalyst and 3 atm of *para*-H_2 (Scheme 1, R = OEt, R′ = D; experimental details are reported in the Experimental section). Relatively high yields (>90%) of the hydrogenated products have been obtained from both reactions. The expected *para*-hydrogen induced effects in the ^{1}H-NMR spectra of *para*-hydrogenated phenylpropiolate (**A**) and propargylamine (**B**) have been clearly detected in terms of large absorption and emission signals (Fig. 1—the corresponding spectra after *para*-H_2 relaxation do not show any signal for the hydrogenated products because at this stage of the hydrogenation reaction the products concentration is still too low to be detected, and have therefore been omitted). The estimated signal enhancement associated to the addition of the *p*-H_2 molecule is about 10^2 when the spectra are acquired immediately after the mixing of *p*-H_2 gas and the solutions containing the alkynes and the catalyst.



Scheme 1

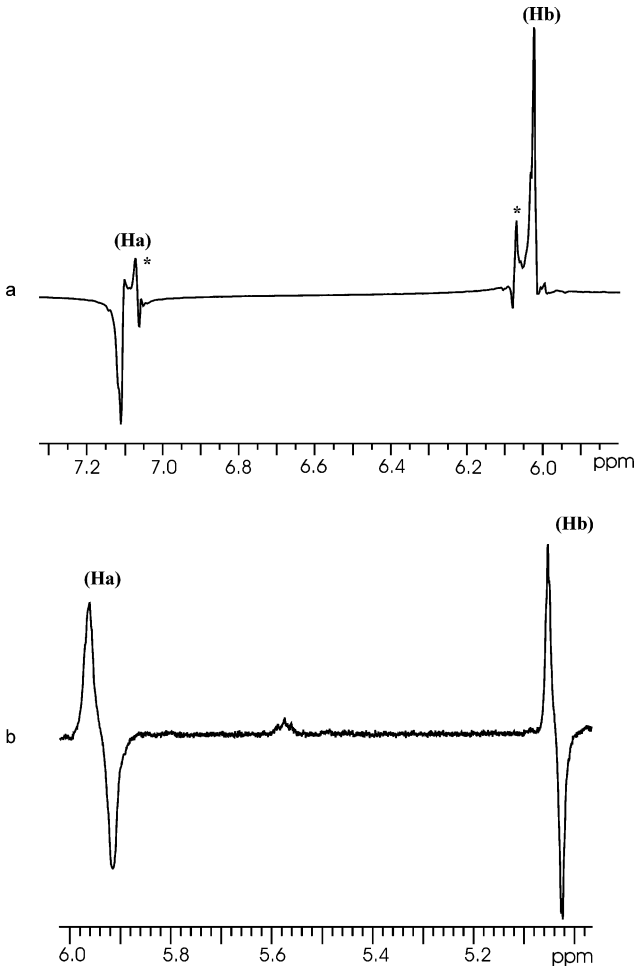


Fig. 1 ^{1}H NMR spectra of (a) a 0.06 mM solution of *para*-hydrogenated phenylpropriolate, and (b) a 0.06 mM solution of *para*-hydrogenated deuterated propargylamine, recorded immediately after shaking (3 atm *p*-H_2, CD_3OD, room temperature; * denotes the *cis*-isomer).

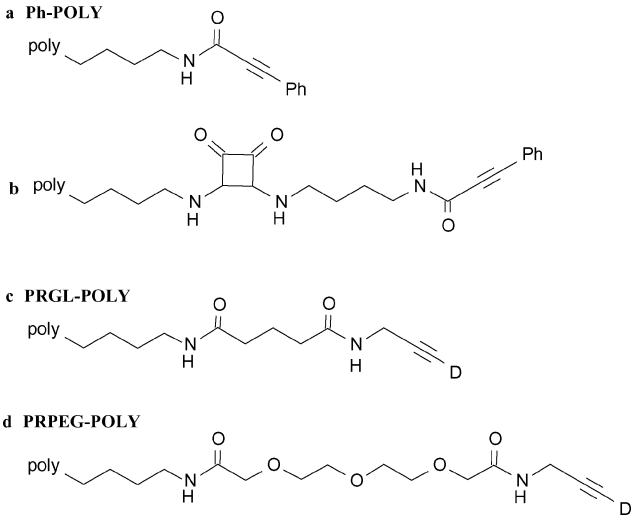
Table 1 Ha and Hb ^{1}H longitudinal relaxation times of *para*-hydrogenated substrates (CD_3OD, 400 MHz, room temperature)

Substrate	T_1 (Ha) [s]	T_1 (Hb) [s]
A	11.8	9.5
PhPOLY-40	1.5	1.8
B	19.5	21
PRGLPOLY-40	3.6	4
PRDGPOLY	10.0	10.0

For T_1 measurements, **A** and **B** have been synthesized from normal H_2, and purified in order to avoid the presence of any metal containing compound. Relaxation times were measured on outgassed CD_3OD solutions by means of the inversion recovery pulse sequence,[14] at 9.4 T and 298 K. The obtained values are the following: $T_1(H_a) = 11.8$ s and $T_1(H_b) = 9.5$ s for product **A**; $T_1(H_a) = 19.5$ s and $T_1(H_b) = 21.0$ s for product **B** (Table 1).

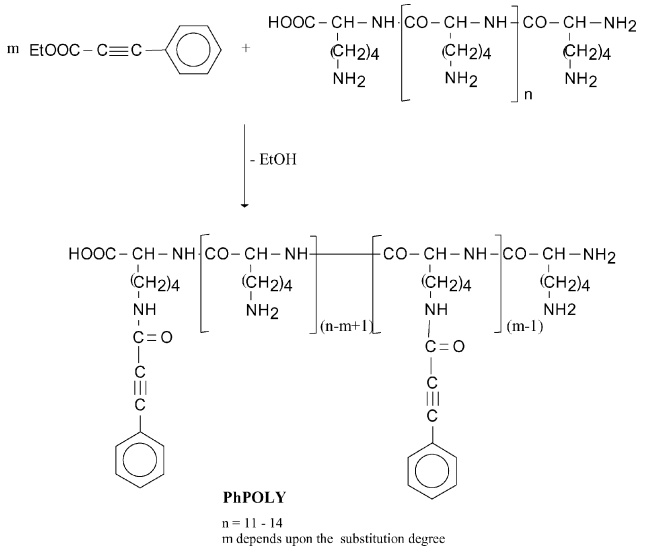
(2) *para*-Hydrogenation of phenylpropiolate moieties conjugated to poly(lysine) chains

The functionalization of a small poly(lysine) chain (11–14 lysine units per molecule) with phenylpropiolate moieties (Scheme 2a) has been carried out in methanol at room temperature by the direct reaction between poly(lysine) and ethylphenylpropiolate in appropriate molar ratio,[16] (see Experimental section for the procedure details), according to Scheme 3. The product has been fully characterized by ^{1}H and ^{13}C-NMR spectroscopy.



Scheme 2

According to the employed stoichiometric ratio, two degrees of functionalization have been achieved. In the first case (termed PhPOLY-100) all of the poly(lysine) arms have been conjugated to the alkynyl moieties (100% substitution). This product is soluble solely in dimethylsulfoxide, generally known as an unsuitable solvent for catalytic hydrogenation. In the second case (PhPOLY-40), where only about 40% of the poly(lysine) arms have been substituted, the product is partially soluble in methanol by the presence of free NH_2 groups, and hence suitable for catalytic hydrogenation.



Scheme 3

We then performed the *para*-hydrogenation of PhPOLY-40 dissolved in methanol solution directly in the NMR tube, under the same experimental conditions used for phenylpropiolate (see Experimental section for details). In this case, the reaction is so slow that no enhanced signals can be detected in the ^{1}H NMR spectrum when recording it after the same time (about 20 s after shaking) as for **A**. The measurement of the spin-lattice relaxation times of the olefinic protons of hydrogenated PhPOLY-40 afforded 1.5 and 1.8 s for H_a and H_b respectively (Table 1, see Scheme 1 for attributions), *i.e.* about 13–19% of those measured for hydrogenated phenylethylpropiolate.

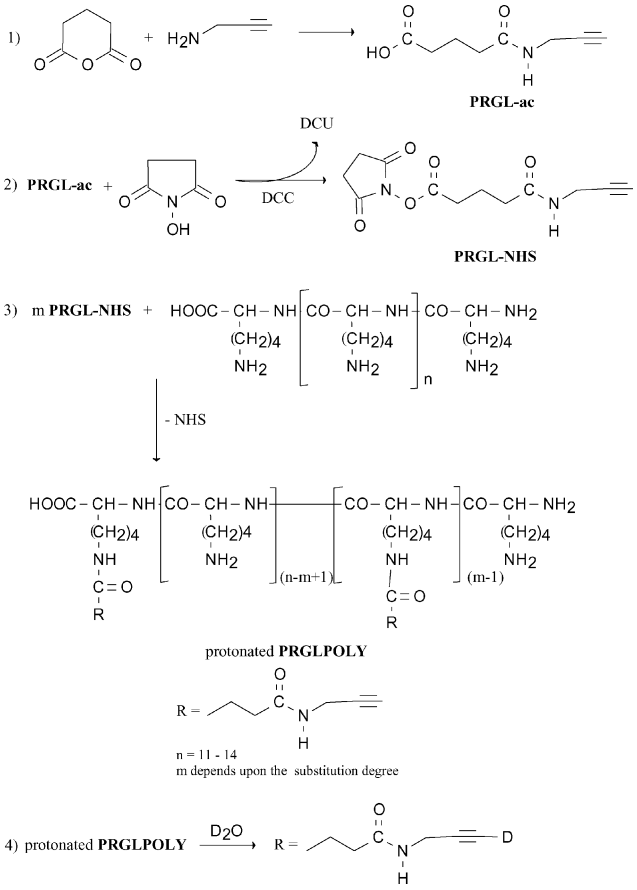
In order to limit both the steric hindrance of the unsaturated group and the decrease in the proton relaxation times in PhPOLY-40, we prepared other poly(lysine) derivatives, with the alkynyl fragment more spaced from the poly(amino acid) chain by means of a mobile alkyl chain. The obtained product, depicted in Scheme 2b, consists of a PhPOLY-like molecule, with 4-aminobutylamide of squaric acid as a spacer between the lysine NH group and the propiolate CO group. Unfortunately again this compound is insoluble in all solvents but DMSO, and therefore not suitable for *para*-hydrogenation experiments.

(3) *para*-Hydrogenation of deuterated propargylamine moieties conjugated to poly(lysine) chains

In order to prepare these kind of multimeric derivatives, it has been necessary first to synthesize a derivative of propargylamine suitable for conjugation to the ε-amino groups of the poly(lysine) chains. A good candidate was deemed to be the derivative with a glutaric acid unit (PRGL), which should also enable the unsaturated moiety to have a sufficiently high flexibility (Scheme 2c).

The synthetic route, depicted in Scheme 4, involves the reaction of glutaric anhydride with propargylamine, which affords the monopropargylamide derivative of glutaric acid (PRGL-ac). PRGL-ac is then reacted with *N*-hydroxysuccinimide (NHS) in the presence of dicyclohexylcarbodiimide (DCC) as the activating agent for the formation of the peptide bond, to yield the reactive ester. The latter is then reacted with poly(lysine) in the appropriate molar ratio to afford the final product PRGLPOLY.

As in the case of PhPOLY, two different degrees of functionalization have been achieved: PRGLPOLY-100 (100% substitution), insoluble in CH_3OH; and PRGLPOLY-40 (with a degree of substitution of about 40%), again fairly soluble in methanol due to the presence of free NH_2 groups. PRGLPOLY-40 is completely soluble in water, thus allowing complete deuteration of the triple bond, simply by dissolving the poly(lysine) derivative in



Scheme 4

D_2O. The proton–deuterium exchange is very fast, with complete deuteration occurring in a few minutes at room temperature. Its characterization has been carried out by ^{1}H and ^{13}C NMR spectroscopy.

Unlike in the case of PhPOLY-40, PRGLPOLY-40 undergoes hydrogenation fast enough to allow the detection of *para*-H_2 effects when *para*-H_2 is used. The ^{1}H NMR spectrum is reported in Fig. 2 (see Scheme 1 for attribution). Clearly, the enhancement of the olefinic signals is rather low if compared with the enhancements usually observed for small-sized molecules (see in Fig. 1 the spectrum relative to the *para*-hydrogenation of deuterated propargylamine under the same experimental conditions). Nevertheless, the observation of hyperpolarized absorption/emission signals is indicative that the introduction of a mobile spacer between the triple bond and the poly(lysine) chain is effective (1) in lowering the steric hindrance around the unsaturation, thus allowing faster hydrogenation, and (2) in slightly increasing the relaxation times of the olefinic protons in the hydrogenated product, allowing the polarization to be maintained for a longer time. In fact, the olefinic protons T_1 are in this case 3.6 s and 4.0 s for H_a and H_b, respectively (Table 1), *i.e.* about 18–19% of those measured for deuterated propargylamine.

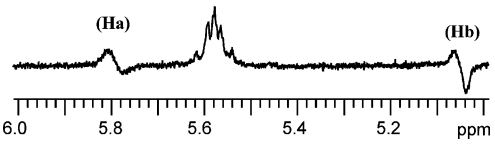


Fig. 2 ^{1}H NMR spectrum of a 0.15 mM solution of *para*-hydrogenated PRGLPOLY-40 recorded immediately after shaking (3 atm *p*-H_2, CD_3OD, room temperature). The signal at about 5.6 ppm is attributed to cyclooctene, which is formed during the first step of the catalytic cycle.

Insertion of a diethylene glycol (DG) chain between the poly(lysine) arms and the alkynyl moieties. A further improvement in the observed enhancement may be obtained by increasing the length of the mobile spacer between the alkynyl moiety and the poly(lysine) chain. In order to overcome the problem that lengthening the alkyl chain would lead to a decreased solubility of the compound, a diethylene glycol chain has been used as spacer (PRDGPOLY, Scheme 2d). The diethylene glycol chain increases the flexible carbon atoms number from 3 (glutaric acid) to 8.

The synthetic procedure used to prepare PRDGPOLY (reported in Scheme 5) is quite similar to that used in the PRGLPOLY case. The starting material is a COOH terminated DG, which has been converted to the chloride by using $SOCl_2$. The chloride has then been reacted with an equimolar quantity of propargylamine at low temperature, and successively with water to yield the monopropargylamide (PRAM-DG-COOH). This intermediate was purified before proceeding with the following steps. PRAM-DG-COOH has then been converted to the reactive NHS ester by using DCC as activating agent for the formation of the peptide bond, and this ester has been reacted with poly(lysine) at room temperature in the opportune stoichiometric ratio to yield PRDGPOLY (40% substitution). Deuteration has been achieved by dissolution in D_2O.

para-Hydrogenation of this substrate yields the ^1H spectrum shown in Fig. 3 (see Scheme 1 for the assignment): the pattern is the same as observed for the PRGLPOLY case, but the signal intensity is about twice that observed in the previous case. Furthermore, the polarization decay takes about 1 min to complete, while in the PRGLPOLY case no polarized signal could be observed already 20 s after introducing the NMR tube into the magnet. Also, the T_1 results are encouraging: hydrogenated PRDGPOLY olefinic protons show relaxation times of 10 s for both H_a and H_b (Table 1), *i.e.* just about the 50% of those measured for the parent deuterated propargylamine.

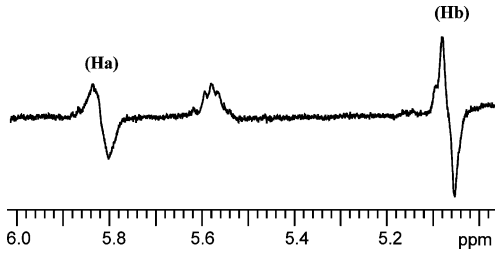
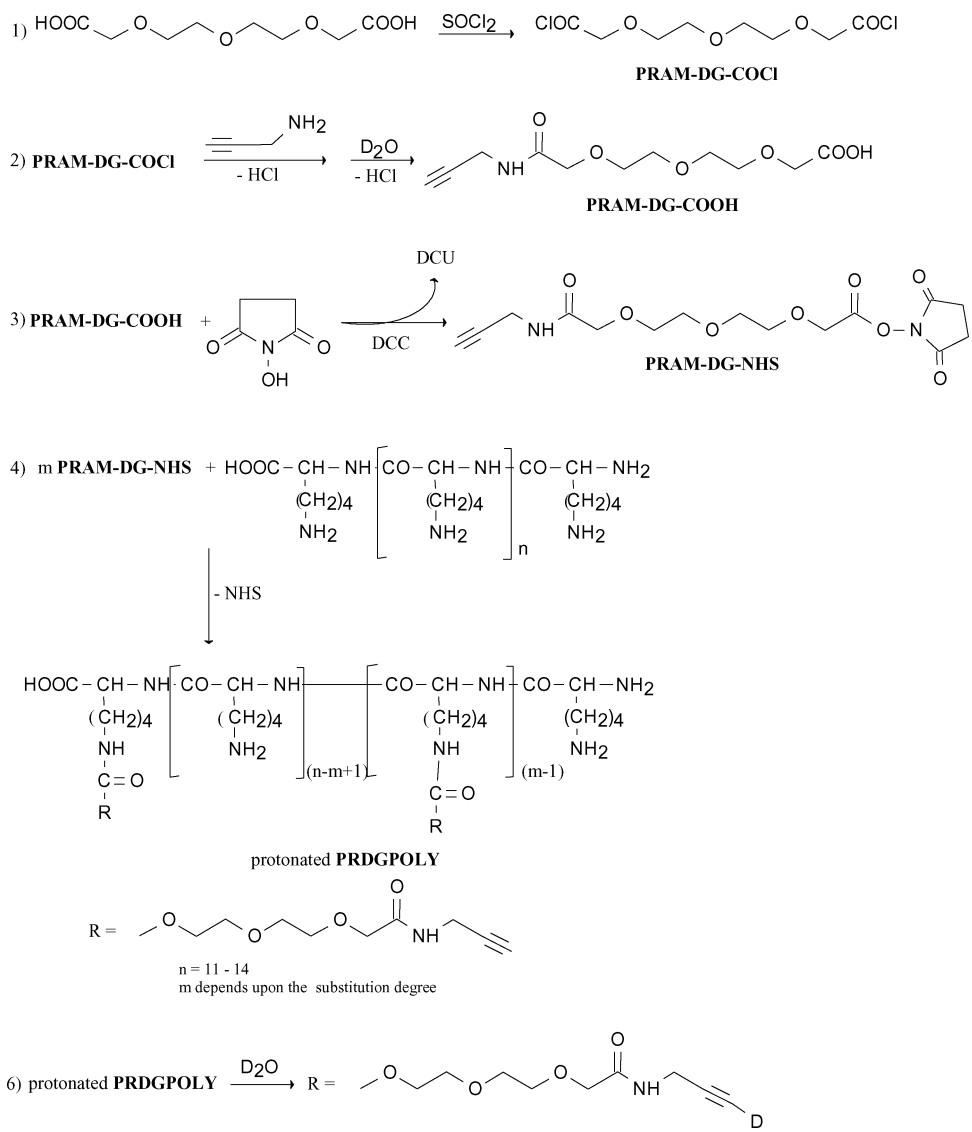


Fig. 3 ^1H NMR spectrum of a 0.15 mM solution of *para*-hydrogenated PRDGPOLY-40 recorded immediately after shaking (3 atm *p*-H_2, CD_3OD, room temperature). The signal at about 5.6 ppm is attributed to cyclooctene, which is formed during the first step of the catalytic cycle.



Scheme 5

Discussion

Poly(lysine) provides a suitable backbone for the synthesis of multimeric derivatives containing many alkynyl moieties ready for the reaction with *para*-hydrogen. It is available (or it can be synthesized) to any polymerization degree and the conjugation reaction can be controlled in order to prepare systems endowed with the required solubility. In this study it has been found that the complete saturation of all the ε-amino groups of the poly(lysine) leads to derivatives that are insoluble in the solvent (methanol) used for the hydrogenation reactions. Therefore systems still containing 60% of non-conjugated ε-amino groups have been synthesized as they show enough solubility for the hydrogenation step.

The hydrogenation process occurs first through the dissociation of the H_2 molecule at the Rh centre, followed by the transfer of the two hydrogen atoms to the unsaturated carbons of the substrate. This implies that the catalyst molecules must find enough space to coordinate simultaneously as many as possible unsaturated moieties of the multimeric substrate. The catalyst [Rh(COD)(dppb)][BF_4] has noticeable steric requirements and therefore the length of the spacer between the unsaturated moiety and the peptidic backbone becomes a primary requirement for the hydrogenation step. Upon comparing the four multimeric products (Scheme 2), clearly only PRDGPOLY appears to provide sufficient space for the action of the catalyst. In methanol we expect that the poly(lysine) backbone has no definite structure and the substituents point in different directions to minimize any possible interaction; thus, in this respect, poly(lysine) represents a good substrate for the preparation of these kinds of multimers.

For the envisaged MRI applications, the polarization of the hydrogen pair has to be transformed into enhanced ^{13}C magnetization of a neighbouring carbon nucleus.[12] For pursuing a good polarization/magnetization coupling it is necessary that the multimeric species maintain, as much as possible, the relaxation properties of the small molecules that have been used for conjugation. In fact, in order to pursue the optimization of the hyperpolarization process it is also necessary to deal with a sufficiently long T_1 for the olefinic protons arising from the addition of the *para*-H_2 molecule. The slow relaxation of the proton resonances is of major importance for the maintenance of the spin-order in the two-proton system prior the application of the field-cycling process, which is responsible for the magnetization transfer to the ^{13}C nuclei.[12]

Large macromolecules induce low relaxation times of their protons as a consequence of their longer reorientational times. Herein, we foresee that the introduction of long flexible spacers between the poly(lysine) backbone and the unsaturated moieties yields beneficial effects for the maintenance of long T_1 for the olefinic protons. Indeed, the T_1 of the olefinic protons increases as the length and flexibility of the spacer increases. For PRDGPOLY-40 (MW = 2840 Da) T_1 is 10 s, that is only about half of the value found for the monomeric product of the hydrogenation of the propargylamine.

Conclusions

The new functionalized poly(lysine) derivatives here reported represent potential candidates for the development of new contrast agents in MRI angiography based on the principle of hyperpolarization generated by *para*-hydrogen. We have shown that *para*-hydrogenated moieties in specifically designed macromolecular systems may maintain long relaxation times, which is the primary requisite for pursuing hyperpolarized ^{13}C molecules *via* transfer of the polarization order of the *para*-hydrogen system. Furthermore, the insertion of DG spacers between the macromolecule backbone and the unsaturated moieties allows the attainment of high hydrogenation rates by releasing steric hindrance and favouring the interaction with the hydrogenation catalyst.

Work is still needed in order to improve the solubility properties of these macromolecular systems. This may be attained by introducing hydrophilic substrates on the residual primary amino groups. We think that such derivatization could also be beneficial as far as long T_1 have to be maintained in blood serum. In fact optimal systems are those lacking any binding interaction with serum macromolecules or cell membrane surfaces.

Although more work has still to be done in order to identify suitable candidates for further development, the herein reported results support the view that the use of macromolecular systems containing several *para*-hydrogenated moieties suitable for MRI applications might be a possible target in the field of hyperpolarized contrast agents.

Experimental

NMR measurements

NMR spectra were recorded on a JEOL EX-400 instrument, operating at 399.75 MHz and 100.00 MHz for proton and ^{13}C respectively. 45° pulses were used for both nuclei; for ^{13}C spectra, experiments were carried out in the absence of NOE, with a relaxation delay of 12 s in order to obtain good integration ratios. T_1s were measured by the inversion recovery pulse sequence.[14]

Hydrogenation experiments were carried out as follows: 5 mg of catalyst and the correct quantity of substrate (7.0 μmol of triple bonds, *i.e.* 28.0 μmol of phenylethylpropiolate and propargylamine, 7.0 μmol of PhPOLY, PRGLPOLY and PRDGPOLY) were dissolved in 0.5 ml of CD_3OD, directly inside the 5 mm NMR tube, equipped with a vacuum valve. The tube was connected to a vacuum line, air was removed by a freeze-pumping process, and then H_2 (or *para*-H_2) was introduced on the frozen sample (the final pressure as the sample was heated to room temperature was about 3 atm). The sample was heated to room temperature near the magnet, shaken and introduced into the spectrometer. In *para*-H_2 experiments, 1 scan spectra were immediately recorded after loading the sample (about 20 s after shaking). In hydrogenation experiments ^1H spectra were recorded every ten minutes until no changes were detected in the spectra. T_1 measurements were made on samples after complete hydrogenation and/or relaxation of *para*-H_2 effects.

Materials and preparative methods

All preparations and manipulations were carried out under Ar atmosphere. Distilled and de-oxygenated solvents were used. Acetonitrile was distilled from P_2O_5, dichloromethane and methanol from CaH_2, diethyl ether from sodium benzophenone. [Rh(COD)(dppb)][BF_4] and all chemicals were purchased from Sigma-Aldrich. H_2 gas was obtained from a CLAIND generator, model HG300. *para*-Enriched hydrogen (about 50%) was prepared by storing H_2 over Fe_2O_3 at 77 K for 3–4 h.

Synthesis of PhPOLY-100. 100 mg of poly-L-lysine hydrobromide (*ca.* 0.04 mmol) were dissolved in 2 ml of water and 5.6 ml of NaOH 0.1 M were added in order to neutralize HBr (final pH: *ca.* 10). Water was then removed, and the residue was dissolved in about 3 ml of a mixture water–methanol 2 : 1, under Ar atmosphere. 76 μl of phenylethylpropiolate (0.5 mmol) were added dropwise to the solution. The mixture was then stirred at room temperature for about 40 h: a white precipitate was formed, that was filtered, washed two times with small quantities of methanol, and dried under vacuum. Yield 90%. δ_C(400 MHz, DMSO-d_6): 171.58 (1C), 152.25 (1C); 132.09 (2C), 130.17 (1C), 128.96 (2C), 120.01 (1C); 84.20 (1C), 82.92 (1C); 54.28 (1C, d), *ca.* 40 (1C), 31.67 (1C), 28.56 (1C), 22.88 (1C, t). The evaluation of the percentage of substituted arms (for both PhPOLY-100 and PhPOLY-40) was achieved by integration of the ^{13}C NMR signal attributed to the amidic groups formed on the arms (152.25 ppm) with respect to the signal attributed to the chain's peptidic

carbonyls (171.58 ppm; the integration ratio is about 1 : 1 in PhPOLY-100).

Synthesis of PhPOLY-40. The procedure is the same as for PhPOLY-100, but the quantity of ethylphenylpropiolate to be used is only 38 μl (0.25 mmol). In this case, the product is partially soluble in the reaction solvent; therefore, at the end of the reaction, it is necessary to evaporate the solvent, and then to wash repeatedly the residue with small quantities of water to eliminate NaBr. PhPOLY-40 is soluble also in water at pH = 6.7, and partially soluble at pH = 11. Yield = 85%. δ_C (400 MHz, CD_3OD): 178.3 (1C), 174 (1C), 155.9 (0.4C); 133.78 (0.8C), 130.45 (0.4C), 130.09 (0.8C), 121.84 (0.4C); 86.08 (0.4C), 84.25 (0.4C); 55.5 (1C), 49.3 (broad, 1C), 41.1 (broad,1C), 31.7 (broad,1C), 24.36 (broad,1C).

Synthesis of PRGL-ac. 2.33 g of glutaric anydride (21.56 mmol) were dissolved in 100 ml of dichloromethane, 1.4 ml of propargylamine (20.4 mmol) were added, and the mixture was refluxed for 1 h. The solvent was removed in vacuum. The residue was loaded on an XAD 1180 chromatographic column (height 40 cm), and washed with 100 ml of water. The pure product was collected by elution with 50 ml of methanol. Another fraction was collected immediately after that, containing just impurities. The chromatographic curve was obtained by measuring the UV absorbance of the fractions (215, 254 nm). Yield 81%. δ_C (400 MHz, D_2O): 177.23 (1C), 175.01 (1C); 79.63 (1C), 71.88 (1C); 34.54 1(1C), 32.90 (1C), 28.69 (1C), 20.54 (1C). δ_H (400 MHz, D_2O): 2.50 (1H, t, J = 2.4 Hz); 3.84 (2H, d, J = 2.4 Hz); 2.29 (2H, t), 2.20 (2H, t), 1.77 (2H, t).

Synthesis of PRGLPOLY-100. 100 mg of PRGL-ac (0.59 mmol), 110 mg of *N*-hydroxysuccinimide (NHS, 0.96 mmol) and 220 mg of dicyclohexylcarbodiimide (DCC, 1.07 mmol) were dissolved in about 25 ml of acetonitrile. The mixture was heated to 65 °C and stirred at this temperature for 6 h. After this period, the mixture was left to cool to room temperature and the solvent was evaporated in vacuum. The residue was then redissolved in a few ml of anhydrous dichloromethane and filtered upon celite. The solution was dried again, yielding a slightly yellow solid residue of reactive ester. This was dissolved again in 10 ml of acetonitrile, and added to about 20 ml of acetonitrile solution of poly(lysine) (obtained by neutralizing the commercial poly(lysine) hydrobromide with tetrabutylammonium hydroxide in water up to pH = 11). The mixture was stirred at room temperature for 48 h: during this period a white precipitate of PRGLPOLY-100 is formed. The solid was isolated, washed once with acetonitrile and once with a few ml of water, and then dried in vacuum. It is soluble in DMSO. Yield 60%. δ_C (400 MHz, DMSO-d_6): 171.77 (3C); 81.40 (1C), 72.83 (1C); 52.60 (broad, 1C); 38.52 (broad, 1C), 34.97 (1C), 34.60 (1C), 31.70 (broad,1C), 28.96 (broad,1C), 27.88 (1C), 23.01 (broad,1C), 21.49 (1C). The evaluation of the percentage of substituted arms (for both PRGLPOLY-100 and PRGLPOLY-40) was achieved by integration of the ^{13}C NMR signal attributed to the chain CH groups (52.60 ppm) with respect to the signal attributed to the triple bond carbon atom (81.40 ppm), in the absence of NOE (the integration ratio is about 1 : 1 in PRGLPOLY-100).

Synthesis of PRGLPOLY-40. The procedure is the same described for PRGLPOLY-100, but the following quantities were used: 37 mg of monopropargylamide of glutaric acid, 41 mg of NHS, 82 mg of DCC in about 12 ml of acetonitrile. In this case, the product is water-soluble, therefore for the purification a de-salting process was carried out, eluting the product on a Sephadex G10 column with water. Yield 55%.δ_C 400 MHz, D_2O): 175.53 (0.8C), 173.84 (1C, broad); 79.26 (0.4C), 71.69 (0.4C, t, J_{CD} = 70 Hz); 53.72 (1C, broad); 39.54 (1C, broad), 34.97(0.4C), 34.71 (0.4C), 30.69 (1C, broad), 28.80 (0.4C), 28.00 (1C, broad), 22.31 (1C, broad), 21.76 (0.4C). The H/D exchange of the acidic acetylene H atom occurs in a few min when PRGLPOLY-40 is dissolved in D_2O at room temperature, and is maintained in further manipulations (lyophilization and further dissolution for hydrogenation).

Synthesis of PRAM-DG-COOH. 2.53 g of DG-COOH (PM = 220, 11.5 mmol) were introduced into a three-necked recipient, connected to a gas inlet and a bubbler. Ar was introduced, then about 10 ml of $SOCl_2$ were dropped slowly, while mixing. The mixture was mixed at room temperature until no gas was detected in the bubbler, then it was connected to a vacuum pump, and the excess of $SOCl_2$ was removed at room temperature. The residue was dissolved in about 40 ml of dichloromethane under Ar atmosphere, 5.0 g of K_2CO_3 were added, and the suspension was cooled to 273 K. 40 ml of a dichloromethane solution of propargylamine (0.604 ml, 9.43 mmol) were then added in about 1 h, while vigorously stirring the suspension. Stirring was carried on for 1 h after the end of the addition. About 0.20 ml of water (11.1 mmol) were added at room temperature, and the mixture was stirred for 1 h. The suspension was filtered and PRAM-DG-COOH was extracted by addition of about 40 ml of water to the solution. The aqueous phase was washed twice with 40 ml of dichloromethane and brought to pH 4.5 by adding HCl. The product was purified by reverse phase column chromatography, gradient elution water → water–acetonitrile 5% (R_f = 033). Yield *ca.* 20%. δ_C (400 MHz, D_2O): 174.08 (1C, pH dependent); 172.20 (1C); 79.61 (1C), 70.55 (1C, J_{CH} = 250.6 Hz); 70.08 (1C), 69.98 (1C), 69.86 (1C), 69.69 (1C), 69.65 (1C), 69.53 (1C); 28.48 (1C). δ_H (400 MHz, D_2O): 4.13 (2H), 4.05 (2H), 3.98 (2H), *ca.* 3.7 (8H); 2.65.

Synthesis of PRDGPOLY-40. 67 mg of PRAM-DG-COOH (0.27 mmol), 57 mg of *N*-hydroxysuccinimide (NHS, 0.50 mmol) and 98 mg of dicyclohexylcarbodiimide (DCC, 0.47 mmol) were dissolved in about 20 ml of distilled acetonitrile. The mixture was heated to 70 °C and stirred at this temperature for 24 h. After this period, the mixture was left to cool to room temperature and filtered. The solution was then added to about 20 ml of an acetonitrile solution of poly(lysine) (obtained by neutralizing 100 mg of commercial poly(lysine) hydrobromide (0.04 mmol) with tetrabutylammonium hydroxide in water up to pH = 11). The mixture was stirred at room temperature for 3 d: during this period a white precipitate of PRDGPOLY-40 is formed. The solid was isolated and washed once with acetonitrile, and then dried in vacuum. The product was purified by means of a de-salting process, eluting it on a Sephadex G10 column with water. The H/D exchange of the acidic acetylene H atom occurs in a few min when PRDGPOLY-40 is dissolved in D_2O at room temperature, and is maintained in further manipulations (lyophilization and further dissolution for hydrogenation). Yield 50%. δ_C (400 MHz, D_2O): 173.68 (1C, broad), 172.62; 78.31 (0.4 C, t, J_{CD} = 10 Hz), 72.23 (0.4 C, d, J_{CD} = 70 Hz); 70.00 (0.4 C, t), 69.5 (2 C); 53.62 (1C, broad); 39.21 (1.4C, broad), 30.65 (1C, broad), 26.88 (1C, broad), 22.05 (1C, broad). The evaluation of the percentage of substituted arms was achieved by integration of the ^{13}C NMR signal attributed to the chain CH groups (53.62 ppm) with respect to the signal attributed to the triple bond carbon atom (78.31 ppm), in the absence of NOE.

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