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GMP polyamine hybrid hydrogels: enhanced gel strength probed by z-spectroscopy

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Abstract: The self-assembling tendencies of guanosine-5'-monophosphate (GMP) can be drastically increased using polyamines, with potential applications in the production of biocompatible smart materials, as well as for the design of anti-tumoral drugs based on G-quadruplex stabilization. Results from scanning electron microscopy (SEM), wide angle X-ray scattering (WAXS), rheology and nuclear magnetic resonance (NMR) z-spectroscopy studies are presented.

Introduction

Guanosine-containing compounds have the ability to self-organize into structures with interesting properties. For example, guanosine-5'-monophosphate (GMP) forms gels, in a process that starts with the self-organization of four GMP molecules and a cation into a tetrad (Figure 1). These tetrads further self-assemble into fibers that eventually form gels.[1] A similar phenomenon occurs in DNA and RNA sequences rich in guanine; these can form quadruplex structures that are similar to those formed by GMP, although with variations caused by the presence of bases other than guanine.[2] In the first case, GMP and GMP variants have been proposed as candidates for creating new materials;[3] in the second case, new families of drugs are being developed exploiting the fact that adoption of quadruplex conformations can influence various biological functions.[2e] One example of the latter is the development of anti-tumorals[2e, 4] whose mechanism of action relies on the fact that telomeres which adopt quadruplex conformations are poor substrates for telomerase, an enzyme needed to sustain cell multiplication.[5] Key in both cases is to increase the ability of guanine to self-organize. For example, GMP can form hydrogels, but the process is inefficient and high concentrations or low temperatures are needed.[3g] Similarly, the control of biological functions through DNA-quadruplex stabilization requires coercing guanine residues in DNA to self-assemble beyond their natural limit. Consequently, there is great interest in finding chemicals that encourage guanine to self-assemble. In the present paper we have focused our efforts on GMP, showing that its self-assembly ability can be significantly increased using polyamines; the lessons learned in the process are also of interest for the development of new anti-tumorals.

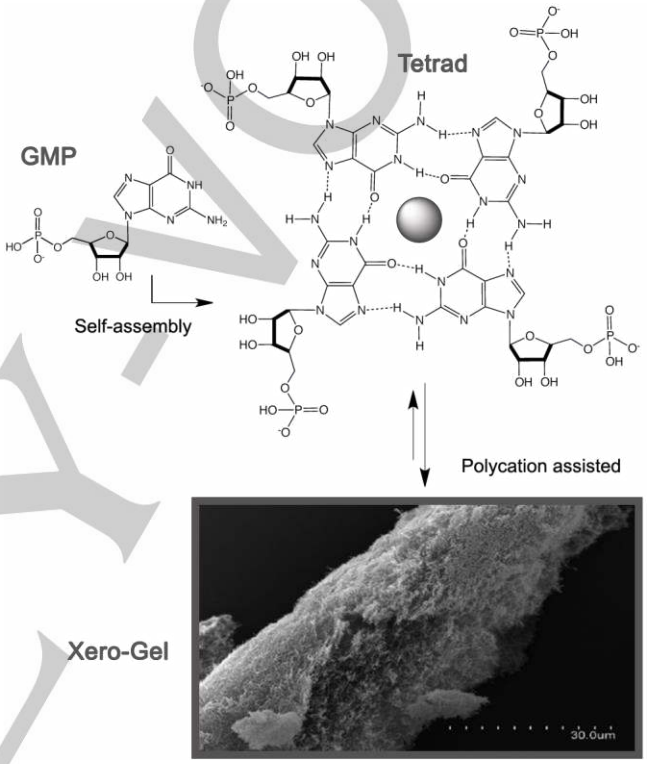

Figure 1. Tetrad assembly of GMP leading to fibre formation via tetrad stacking. GMP is an inefficient gelator, but can be converted into a very efficient one with the aid of polyamines. The figure shows a picture of a xerogel made of GMP and polyamine 343 (Figure 2).

The question is, then, how can free GMP be induced to self-assemble beyond its natural limit? Here we propose two mechanisms, both involving polycations. In the first case the equilibrium between the solution and the gel can be driven towards the latter by minimizing the repulsion between the negatively charged phosphates. In the second, the equilibrium can be further driven towards the gel by stabilizing the three-dimensional matrix using reversible cross-linkers, for example, polycations.

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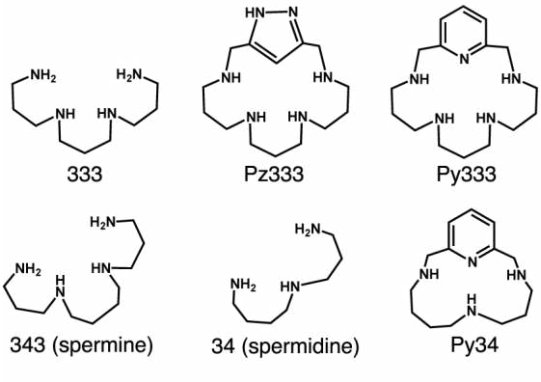

Figure 2. Polyamines used in this work.

Here we use polyamines for both purposes, but proteins, peptides, lipids, and even glycans could be used provided that they are polycationic. Several features make polyamines attractive, though. For example, in protonated form they have affinities for negatively charged phosphates that are orders of magnitude larger than those of simple cations.[6] This allows them to achieve feats such as the compaction of DNA into viral capsids.[7] In addition to the naturally occurring spermine and spermidine, four other synthetic polyamines[8] have been used in this work (Figure 2), but many others could be used; in fact, polyamines are easily engineered, and can even be made to respond to various external stimuli,[6b, 9] an advantage for the creation of smart materials.

Results and Discussion

To test whether the idea of promoting gel formation using polycations would work, polyamines were added to solutions of 30 mM GMP disodium salt. At this concentration GMP is unable to form a gel at room temperature. Vial inversion tests were used initially to assess gel formation. The gels were then characterized by rheology, wide-angle X-ray scattering, scanning electron microscopy, and NMR z-spectroscopy.

Table 1. Rheological properties of the gels obtained after mixing GMP and polyamines at their optimal ratios. The G'/G" confirms that the gels have a solid-like behavior. The larger the γ, the stronger the gel.

Polyamine	Polyamine:GMP molar ratio	G'	G"	Strength (γ/Pa)
34	1:3	356	47	63
Py34	1:3	4280	731	447
343	1:4	5403	773	562
333	1:4	13078	1363	1259
Pz333	1:8	30786	3171	1413
Py333	1:4	39195	4016	1778

Remarkably, all the polyamines are capable of turning this poor gelator into a very efficient one, with as little as 12.2 mg of GMP disodium salt being capable of gelating 1 g of water (< 2 % w/w). To produce a GMP gel with the same rheological properties as the gels produced using Py333, Pz333, or 333 but without the use of polyamines, requires at least one order of magnitude higher concentration of GMP (see the Supporting Information, Figure S1 and Table S1). However, different polyamines produce different results. Among the polyamines explored, the greater the number of amino groups in the polyamine, the stronger the gels produced (the higher yield stress), and for polyamines with the same number of amino groups, the cyclic derivatives formed the strongest gels (Table 1). Furthermore, the maximum gel strength is almost always achieved when all of the GMP charges are matched by the polyamine. A polyamine such as Py333 (Figure 2) forms the strongest GMP gels when its concentration is four times smaller than that of GMP. The one exception is polyamine Pz333, for which the optimum polyamine:GMP ratio is 1:8; the reason for this deviation is not known. Apart from this abnormality, all the data (Table 2) suggest that the solid matrix of the gel has to be electroneutral. The rheological studies carried out, including the results of the gel inversion tests, are reported in the Supporting Information (Tables S2 and S3, and Figures S2-S9).

Table 2. Relationship between polyamine Py333/GMP ratio and gel strength (γ). Maximum strength is obtained when the number of protonated nitrogen atoms matches the number of negative charges provided by the phosphates.

Py333/GMP ratio	Phase type	Strength (γ/Pa)
1:3	Gel	1122
1:4	Gel	1778
1:5	Gel	1122
1:8	Gel	704

Wide-Angle X-Ray Scattering (WAXS) and Scanning Electron Microscopy (SEM) studies

A simple way to determine whether polyamines induce gel formation through a mechanism other than quadruplex stacking is to use wide-angle X-ray scattering (WAXS). Using this technique it is possible to estimate the average quadruplex-quadruplex interplanar distance (or to infer the absence of regular spacing). The tetrads of a gel made using just GMP are known to be separated by a distance of 3.36 Å,[1b] and even guanine-rich DNA fragments that adopt G-quadruplex conformations show a similar distance.[10] In the present case, WAXS measurements revealed distances ranging from 3.30-3.43 Å, implying no major change in the interplanar distance, although the natural polyamines produced sharper WAXS peaks than the synthetic ones, with the Pz333 producing the broadest peaks (see the Supporting Information, Figure S10).

Scanning electron microscopy was subsequently used to study the morphology of the gel matrix. For this purpose the gels were dried using methods designed to preserve the structure of the matrix as far as possible (the drying process is described in the Experimental Section).

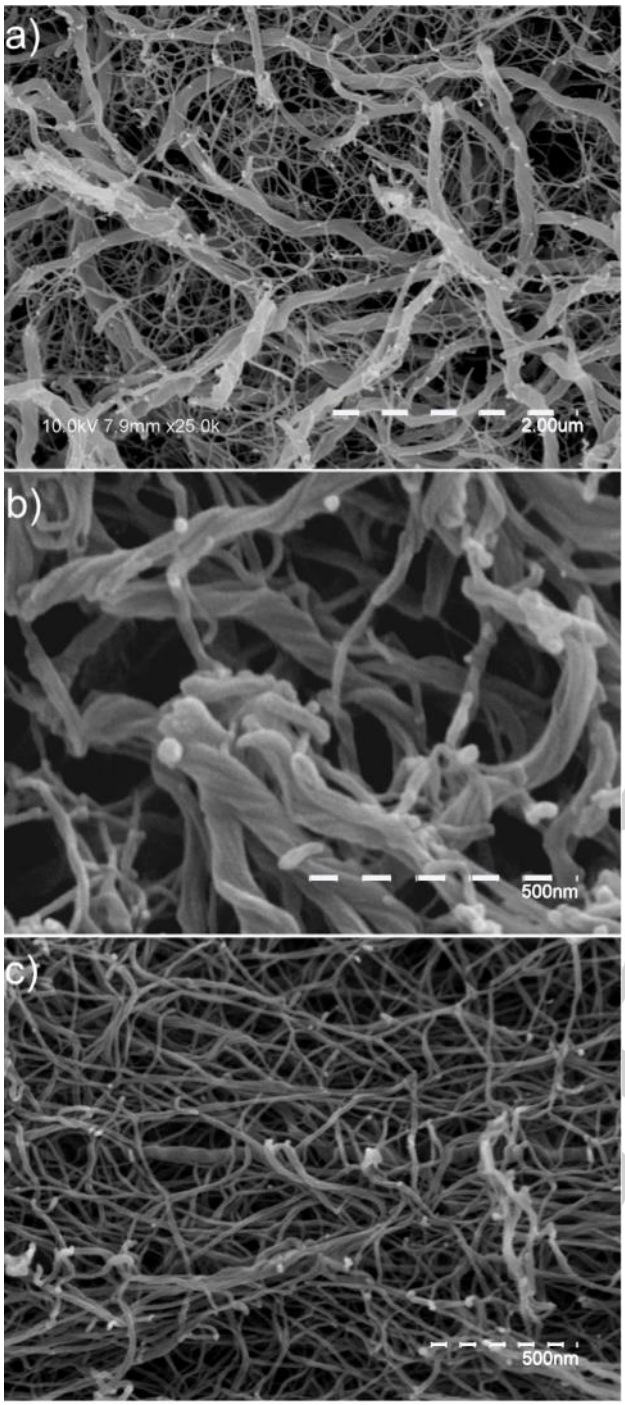

Figure 3. Scanning Electron Microscopy (SEM) pictures of gels dried while trying to preserve their structure. In a) and b) GMP and 343 (spermine) were used, and in c) GMP and Py333 were used. In all cases fibers bunch, forming super-fibers. In a) and b) the super-fibers are left-handed, but in c) there is no sign of left- or right-handedness.

SEM photographs revealed that the gels were always made of fibers (Figure 3 and Figures S11-S15), and that the polyamines Py34, 343, Pz333 formed left-handed helices. No handedness could be discerned in the rest of the cases, including those of gels made just of GMP. In addition, SEM images confirmed the presence of a phenomenon, fiber bunching, that is important for industrial applications because it can change the properties of the gels. For example, the more the fibers bunch, the smaller the surface of the solid matrix per unit of gelator. Fiber bunching can also change the ways gels react to particular stimuli, as inner parts of the bunched super-fiber are shielded from the environment. Examples of bunching can be seen in Figure 3b. The same bunching phenomenon has been observed in DNA-polyamine systems.[11]

Nuclear Magnetic Resonance (NMR) studies

All of the techniques described above probe static properties; however, what characterizes a supramolecular gel is its dynamic behavior. This stems from the fact that the gelator is in equilibrium between the free and the self-organized states. Liquid-state NMR was chosen to investigate this aspect of the gel. It would seem at first sight that liquid-state NMR spectroscopy is ill-suited for this purpose, and that the only signals that should be detected are uninformative leftovers. This is, however, not the case. It is true that the solid matrix is difficult to study directly by liquid state NMR techniques, because relatively immobile spins, such as those in the backbone of a gel have very short spin-spin relaxation times (T_2) and therefore their lines are too broad to be measured directly using a liquid-state spectrometer. However, where mobile and immobile species are in chemical exchange on the timescale of spin-lattice relaxation or faster, the resonances of immobile spins can be detected indirectly by the transfer of saturation between the immobile and mobile magnetization pools. Thus if the resonance of a mobile species is measured as a function of the frequency of a presaturating radiofrequency field, the decrease in its signal maps out the form of the much broader, “invisible”, spectrum of any immobile species with which it is exchanging magnetization, whether by direct chemical exchange, through an intermediary species, or through the nuclear Overhauser effect. This phenomenon can then be exploited to reveal the presence, of the “invisible” matrix, as well as to study the relationship between species in solution and the solid matrix. Figure 4 shows a pulse sequence that can be used for such purposes; it is an adaptation of a technique known as z-spectroscopy, which was first used in magnetic resonance imaging (MRI) to study related phenomena in living tissue.[12]

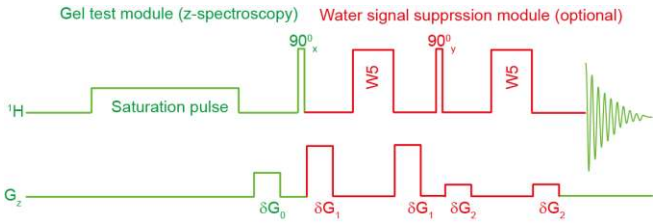

Figure 4. Z-spectroscopy pulse sequence used in the present work. The part of the pulse sequence that has been colored green is a simple presaturation pulse sequence that is followed by a read pulse. A pulsed field gradient is used to dephase any unwanted transverse magnetization created by the saturation pulse. The experiment is performed by moving the frequency of the saturation pulse over some 50 to 100 kHz either side of the liquid-state signals. Signals of interest are then plotted against the frequency of the saturation pulse. Typical results can be seen in Figure 5. The red part of the sequence is

a Robust5 module that serves to suppress solvent signals, as is necessary when samples are dissolved in H_2O rather than D_2O.[13]

The use of this technique confirmed that magnetization is exchanged between GMP in solution and the gel, and between polyamine in solution and the gel. This seems to imply chemical exchange from the liquid phase to the solid one and *vice versa*. However z-spectroscopy cannot conclusively prove the mechanism by which magnetization exchange proceeds, or the location of the exchanging species within the fiber. Whatever the exact mechanism of exchange, it must involve molecules in solution becoming associated with the gel fibers for a significant period of time in a state of low mobility. Nevertheless, WAXS measurements show that GMP quadruplex are formed, implying that phosphates are pointing outwards, while rheological ones revealed that polyamines are necessary to stabilize the fiber, thus suggesting that they must be part of the fiber, and probably close to the phosphates. Thus unless the stability of the ensemble is extremely high, the self-assembled structure must exchange GMP and polyamines with the liquid phase, as suggested by z-spectroscopy. Taking that as a working hypothesis, it is worth noting the differences found between the different systems. For example, the effects of the pre-saturation pulse seem to be weaker on GMP signals than on polyamine ones in the case of gels made using the linear polyamines 333, (Figures 5b and c), spermine or spermidine. In the case of the cyclic polyamines (Py34, Py333 and Pz333) the effects of the pre-saturation pulse on polyamine and GMP signals are almost the same, indicating either a faster rate of exchange of GMP between the gel matrix and solution, or more efficient exchange of magnetization between the gel matrix and GMP molecules within the gel, with the latter implying a different mode of binding for the cyclic polyamines (see the Supporting Information, S16-S20) a possibility also suggested by WAXS results.

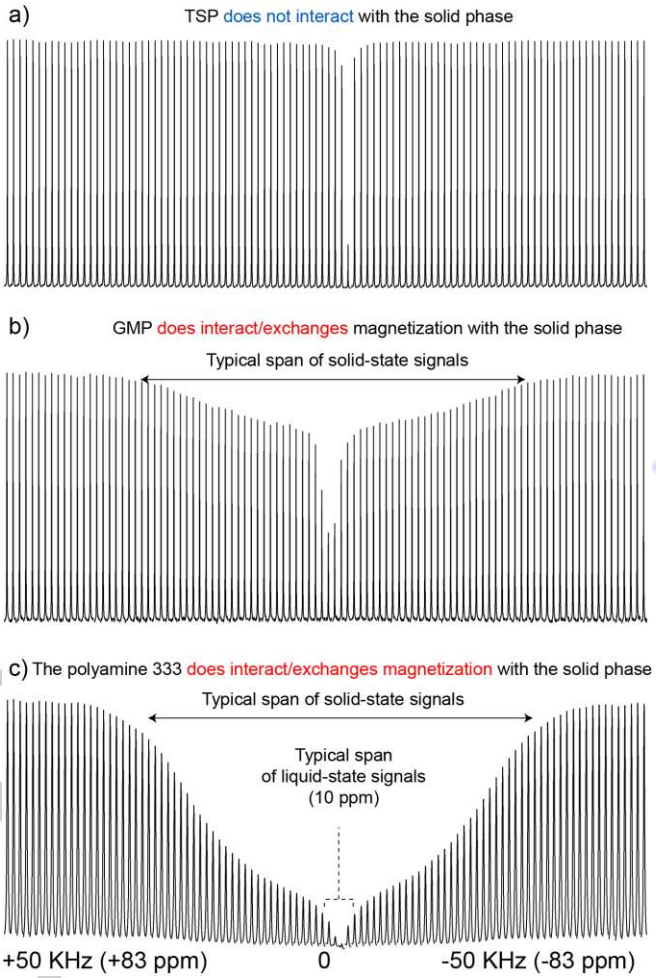

Figure 5. Z-spectra of a 333–GMP gel in D_2O with a coaxial capillary containing the reference material TSP-d_4 (3-(trimethylsilyl)-2,2′,3,3′-tetraadeuteriopropionic acid) in D_2O. Selected liquid-state signals of TSP (0 ppm signal, (a)), GMP (8.1 ppm signal, (b)), and the polyamine 333 (3.02 ppm signal, (c)), are plotted as a function of the frequency of the presaturation pulse, which was varied to cover the typical range of a solid-state signal (in this case the gel matrix). Signals of species in solution exchanging or in contact with the matrix are saturated when the latter is saturated. The TSP does not interact with the solid matrix and, as a result, its signal is only saturated when the saturation frequency is close to it, resulting in a dip only a few tens of Hz wide. In contrast, signals from GMP in solution (8.1 ppm) are attenuated even when the pulse is applied far from any liquid-state GMP signal (b). This reveals that GMP is exchanging magnetization with the gel matrix, as expected from the equilibrium between free and self-associated GMP. The polyamine (3.02 ppm signal) shows even stronger effects, (c), probably reflecting a greater rate of exchange.

Conclusions

We have presented a simple efficient way of promoting GMP to self-assemble. The results presented are of interest for the production of smart biocompatible materials as well as for the production of stabilized G-quadruplexes in telomeric DNA, which have potential applications as anti-tumorals.

Experimental Section

Synthesis of the ligands: All chemicals were purchased from commercial sources and used as received. The hydrochloride salts of spermine, spermidine, and *N,N'*-Bis(3-aminopropyl)-1,3-propanediaminepolyamine were obtained by dissolving the polyamines in anhydrous chloroform and adding 4 M hydrogen chloride in dioxane stepwise until a precipitate was formed. The white solid was filtered off and dried in vacuo.

The cyclic polyamines were synthesized using a modified Richman-Atkins procedure.[14] The full characterisation of ligands Py333 and Pz333 is reported in reference [8a] and the full characterisation of polyamine Py34[8b] is reported below.

1,5-tri-(*p*-tolylsulfonyl)-1,5,10-triazadecane

Yield: 70 %; m.p. it is an oil; ^{1}H NMR (300 MHz, $CDCl_3$, 25ºC) δ= 7.73 (d, 4H, *J* = 8Hz), 7.72 (d, 4H, *J* = 8 Hz), 7.62 (d, 2H, *J* = 8 Hz), 7.29 (d, 6H, *J* = 8 Hz), 5.38 (t, 1H, *J* = 6 Hz), 4.98 (t, 1H, *J* = 7 Hz), 3.11 (t, 2H, *J* = 7 Hz), 3.05-2.85 (m, 6H), 2.42 (s, 9H), 1.74 (m, 2H), 1.50 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$, 25ºC) δ=143.7, 143.5, 143.5, 137.1, 137.0, 136.0, 129.9, 129.9, 127.2, 127.2, 49.0, 46.23, 42.7, 40.2, 29.4, 26.6, 26.0, 21.6.

3,7,12 -Triaza-3,7,12- *p*-tolylsulfonyl-1-(2,6)-pyridinacyclotridecaphane.

Yield: 89 %. m.p; 151.6-152.9ºC; ^{1}H NMR (300 MHz, $CDCl_3$, 25ºC) *δ*= 7.76-7.69 (m, 5H), 7.59-7.53 (m, 3H), 7.48 (d, 1H, *J* = 8 Hz), 7.33 (d, 2H, *J* = 8 Hz), 7.32 (d, 2H, *J* = 8 Hz), 7.27 (d, 2H, *J* = 8 Hz), 4.33 (s, 2H), 4.29 (s, 2H), 3.27-3.19 (m, 2H), 3.11 (t, 2H, *J* = 7 Hz), 2.80 (t, 2H, *J* = 7 Hz), 2.63 (t, 2H, *J* = 7 Hz), 2.44 (s, 3H), 2.43 (s, 3H), 2.41 (s, 3H), 1.59-1-54 (m, 2H), 1.32-1.30 (m, 2H), 1.17-1.14 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, 25ºC) *δ*= 157.0, 143.6, 137.9, 136.1, 130.0, 129.9, 128.8, 127.4, 127.3, 123.2, 55.5, 55.3, 50.5, 49.8, 48.6, 48.5, 29.6, 26.9, 26.5, 21.7, 21.6.

3,7,12-triaza-1-(2,6)-pyridinacyclotridecaphane trihydrobromide.

Yield: 98 %; m.p. 279 ºC; ^{1}H NMR (300 MHz, D_2O, 25ºC) δ= 8.03 (t, 1H, *J* = 7 Hz), 7.60 (d, 2H, *J* = 7 Hz), 4.56 (s, 2H), 4.53 (s, 2H), 3.36 (t, 2H, *J* = 7 Hz), 3.29 (t, 2H, *J* = 7 Hz), 3.28 (t, 2H, *J* = 7 Hz), 3.18 (t, 2H, *J* = 7 Hz), 2.28 (quin, 2H, *J* = 7 Hz), 2.28 (quin, 2H, *J* = 7 Hz), 1.94 (quin, 2H, *J* = 7 Hz), 1.92 (quin, 2H, *J* = 7 Hz); ^{13}C NMR (75 MHz, D_2O, 25ºC) δ= 150.8, 150.7, 140.2, 124.9, 50.2, 50.1, 45.8, 44.2, 44.1, 42.3, 21.9, 21.4, 21.3; ESI-MS observed 248.5 *m/z*; calcd for $[M]^+$ 248.2; elemental analysis calcd (%) for $C_{14}H_{24}N_4(HBr)_3$: C, 34.4; H, 5.6; N, 11.5; found: C, 34.0; H, 5.2; N, 10.6.

Gel sample preparation and rheological measurements: The gels were prepared by weighing the appropriated amount of hydrobromide/chloride polyamine salt in a vial and adding 1 mL of a 30 mM guanosine 5'-monophosphate disodium salt solution. The pH was adjusted to 5 by adding drops of concentrated HCl or NaOH solution. The result was sonicated and heated until a solution was obtained. The sample was cooled at room temperature for a period of 30 min. Subsequently, a gelation "inversion test" was carried out. Several polyamine:GMP molar ratios were tried; the results are shown in Tables S2, S3, and Figures S2-S9.

Oscillatory stress sweep measurements were used to compare the strengths of the gels obtained using different conditions. Rheology experiments were performed using a *TA Instrument Advanced Rheometer 2000*. A parallel-rough-plate geometry (25 mm) was used with a gap of 1 mm and 1 mL sample in each case. Samples were prepared by placing the melted gels in a 25 mm cylindrical block and leaving them to set for 15 min to allow gel formation at 10 ºC. Oscillatory stress sweep measurements were performed over a range of 0.1-10000 Pa with a constant frequency of 1 Hz. The rheometer was controlled by the *Rheology Advantage Instrument control* (v5.8.2) programme and the analysis of the data was carried out using the *Rheology Advantage Data Analysis* (v5.7.0) programme.

The estimated values of G', G", and γ obtained for each GMP gel at different GMP concentrations without polyamines are summarized in Table S1. The raw data are shown in Figure S1.

Wide angle X-ray scattering measurements (WAXS): WAXS measurements were performed on a Bruker GADDS D8 WAXS machine with cross-coupled Göbel mirrors and pin-hole collimation for point focus geometry, using a sealed tube X-ray source operated at 30 kV and 10 mA to produce Cu Kα radiation of wavelength 1.54 Å. The WAXS camera was fitted with a Hi-star 2D detector (effective pixel size 100 µm). The spectra were smoothed by the procedure of Savitzky and Golay.

It was necessary to increase the concentration of GMP six-fold (180 mM) to prepare all of the samples for the WAXS measurements. The ratio polyamine:GMP was consistent across experimental methods.

The spectra of the samples (Figure S10) show broad peaks between 26 and 27 degrees, which corresponds to a distance of 3.30-3.43 Å.

Scanning electron microscopy analysis: The morphologies of the gels were investigated using an S-4800 (HITACHI) scanning electronic microscope with a spotlight of field emission (FEG) with a resolution of 1.4 nm at 1 kV. The pictures were taken at 8000 µm work distance using an emission current of 10100 nA. Images were acquired with the QUANTAX 400 programme.

Samples for SEM were prepared using the critical point drying method. This allows wet samples to be dried without collapsing or deforming the structure. In order to carry out the procedure, water was replaced by ethanol, and then ethanol was replaced with liquid CO_2 operating at the CO_2 critical point (32 ºC and 72 atm approximately). Finally, samples were sputter-coated with Au/Pd.

Nuclear Magnetic Resonance (NMR) studies: Z-spectra were acquired using the pulse sequence shown in Figure 4 but leaving out the solvent suppression module. A 600 MHz Varian spectrometer equipped with an Agilent OneNMR probe able to deliver a maximum pulsed field gradient of 62 G cm^{-1} was used to acquire 100 experiments varying the frequency of the saturation pulse to cover 50 kHz either side of the water signal. One scan per saturation frequency was collected, comprising 65536 complex data points, with a spectral width of 12.6 kHz. The repetition time was 5.6 s, of which 2.6 s comprised the acquisition time and 3.0 s the saturation pulse. The saturation pulse strength ($\gamma B_1/2\pi$) was 156 Hz. A pulsed field gradient of 6 G cm^{-1} (0.5 ms) was used to eliminate any signal produced by the saturation pulse. 16 dummy scans were used. Reference ^{1}H spectra are shown in Figures S21-S26.

Acknowledgements

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Keywords: GMP-gels • polyamines • self-assembly

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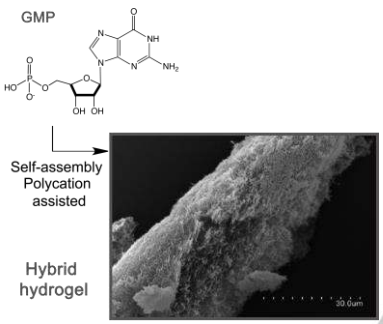
FULL PAPER

The self-assembling tendencies of guanosine-5'-monophosphate (GMP) can be drastically increased using polyamines, with potential applications in the production of biocompatible smart materials, as well as for the design of anti-tumoral drugs based on G-quadruplex stabilization.



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GMP polyamine hybrid hydrogels: enhanced gel strength probed by z-spectroscopy