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RESEARCH ARTICLE

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A short oxazolidine-2-one containing peptide forms supramolecular hydrogels under controlled conditions

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Low-molecular-weight hydrogels are made of a small percentage of small organic molecules dispersed in an aqueous medium, which may aggregate in several manners using different methods. However, often the organic gelator in water has poor solubility, so the addition of a solubilising agent is required. In the case of acidic gelators, this mainly consists of the addition of a strong base, that is sodium hydroxide, that deprotonates the acidic moiety, so the gelator molecules become more soluble and tend to assemble into micelles, forming a dispersion. Some gelators, however, are sensitive to the harsh pH and get hydrolysed. This is the case of some molecules presenting carbamates in their features, like Fmoc-protected or oxazolidinone-containing peptides. In this paper, we present a valid alternative to sodium hydroxide, by dissolving a tripeptide containing an oxazolidinone moiety in a phosphate buffer (PB) medium at pH 7.4. The results obtained with the NaOH dissolution are compared with the ones with PB, as both methods present advantages and drawbacks. The use of NaOH produces transparent but weak hydrogels, as it exposes the gelator to harsh conditions that end up in its partial hydrolysis, which is more pronounced at high concentrations (≥10 mM). Using PB to dissolve the gelator, this problem is completely avoided as no hydrolysis product has been detected in the hydrogels, which are very stiff although more opaque. By tuning the preparation conditions, we can obtain a wide variety of hydrogels, with the properties required by the final application.

KEYWORDS
hydrogels, oxazolidin-2-one, self-aggregation, supramolecular material, tripeptide

1 | INTRODUCTION

Low-molecular-weight (LMW) gelators are small molecules able to form supramolecular gels.[1–4] These are solid-like materials consisting of a bundle of fibres formed through weak interactions that entrap the solvent and that can support their own weight when subjected to gravity. Particular attention has been paid to the functional groups present in these gelators, such as aromatic rings, proton donors and acceptors, and hydrophobic moieties, that are particularly able to form these interactions.[5–9] Amide bonds are involved in H-bonds, as well as polar side chains.[10–12] Aromatic moieties of protecting group or of side chains offer stacking interactions,[11,13,14] whereas apolar side chains can contribute with weak hydrophobic interactions.[15–17] Because of their usual biocompatibility[12,18,19] and the ease of rationalizing a good gelator by varying either protecting groups or amino acids, peptides have been thoroughly explored for their gelation abilities.

However, predicting if a certain molecule will form a gel or not is not straightforward. Most of the gelators were discovered

serendipitously or starting from small changes in the chemical structure of a known gelator.[20] Nonetheless, gelators with similar structures often behave differently, some forming gels, some others forming crystals or precipitates.[15,21,22] In these last years, a large number of compounds and gelation conditions were tested, and the research in this field led to a remarkable improvement in understanding of general principles behind LMW gel formation and properties.[23,24] It should also be noticed that in this field, it is of extreme importance to work in a reproducible way, carefully controlling all the parameters and steps involved in the gelation process (solvent, concentration, time, temperature, etc.).[25]

The use of triggers has been widely studied by several researchers,[26–31] as a careful choice of the trigger strongly impacts the gel's final properties. For instance, the replacement of HCl with a hydrolysing reagent such as glucono-δ-lactone (GdL)[32,33] or 1,3-propanesultone[34,35] induces gelation by a slow pH change allowing the formation of strong homogeneous hydrogels. The presence of a reagent that slowly triggers the gelation is useful not only to achieve homogeneity through the gelation process, but also to create transient systems, that is, that evolve over time.[36–38] Moreover, several studies have been devoted to the use of salts to form supramolecular polymers by electrostatic interactions.[39–42] In particular, divalent cations proved to be a valid method to obtain gels over a wide range of pH, crosslinking the carboxylate groups derived from the dissolution of dipeptides at high pH.[43–48]

In contrast, the dissolution step is often disregarded, although it may lead to the hydrolysis of the gelator itself. For example, carbamates-containing peptides and in particular the Fmoc-protected ones undergo deprotection at a pH higher than 10.5.[49,50] This problem can be overcome by reducing the amount of base, then filtering out the undissolved gelator or by reducing the time of dissolution during which the gelator is at a basic pH.[51] Although the deprotection is limited when ordered structures such as micelles are formed,[52,53] care should always be taken when using sensitive gelators.

In this paper, we show our recent studies on the formation of hydrogels from the self-aggregation of the protected tripeptide Boc-L-Phe-D-Oxd-L-Phe-OH **1**. The gelator contains the Oxd moiety that is an unnatural amino acid, readily obtained from threonine. The heterocyclic ring imparts a local constraint to the molecule, so that it can readily adopt stable secondary structures even with a reduced number of amino acid in the chain.[54–56] Some dipeptides and tripeptides containing the Oxd moiety act as gelator, both for water and for organic solvents.[13,57] The dissolution of the gelator in a milder environment given by a phosphate buffer at pH 7.4 avoids the hydrolysis, allowing us to obtain materials stiffer than the ones obtained with dissolution in NaOH. However, the materials obtained from dissolution in harsher conditions showed higher transparency compared with the other ones, so the two methodologies can be used alternatively depending on the final application of the material.

2 | MATERIALS AND METHODS

2.1 | Synthesis of the gelator

Boc-L-Phe-D-Oxd-L-Phe-OH **1** was prepared with liquid phase synthesis, following a procedure that was previously reported by our group.[58] All the characterisation data matched the literature values.

2.2 | Gel preparation

2.2.1 | Method A

Hydrogels A–I were prepared dissolving **1** at the required concentration (see Table 1) in distilled water and NaOH (1 equiv.), by alternating ultrasound sonication and vigorous shaking over a short period of 2 min. After complete dissolution, the trigger was added. Gels with GdL were formed by adding pure GdL (1.2 equiv.) in the solution, swirling the resulting solution for a few seconds until complete dissolution of GdL and leaving the gel to form overnight. Gels with $CaCl_2$ were formed by adding 100 mM $CaCl_2$ aqueous solution (either 0.5 or 1.0 equiv.) to the gelator solution, then leaving the gel to form overnight.

TABLE 1 pH values and hydrolysis percentage of gels A–I.

Gel	Gelator concentration (mM)	pH_0	Trigger (mM)	pH_f	Hydrolysis (%)
A	3.71	8.5	GdL (4.45)	4.1	3
B	9.26	7.6	GdL (11.1)	4.1	5
C	18.5	7.0	GdL (22.2)	3.8	14
D	3.71	8.6	$CaCl_2$ (1.85)	6.0	8
E	9.26	7.6	$CaCl_2$ (4.63)	6.0	13
F	18.5	7.2	$CaCl_2$ (9.26)	5.6	20
G	3.71	8.8	$CaCl_2$ (3.71)	7.1	3
H	9.26	7.4	$CaCl_2$ (9.26)	5.9	19
I	18.5	7.2	$CaCl_2$ (18.5)	5.3	22

Note: pH_0 = starting pH (before trigger addition); pH_f = final pH.

TABLE 2 pH values and hydrolysis percentage of gels J–O.

Gel	Gelator concentration (mM)	PB concentration (mM)	pH_0	Trigger (mM)	pH_f	Hydrolysis (%)
J	3.71	9.6	6.9	GdL (7.41)	4.8	N.D.
K	9.26	24.0	6.9	GdL (18.5)	4.8	N.D.
L	18.5	48.0	6.8	GdL (37.1)	4.7	N.D.
M	3.71	9.6	6.9	$CaCl_2$ (3.71)	6.4	N.D.
N	9.26	24.0	6.9	$CaCl_2$ (9.26)	6.6	N.D.
O	18.5	48.0	6.8	$CaCl_2$ (18.5)	6.3	N.D.

Note: pH_0 = starting pH (before trigger addition); pH_f = final pH; N.D. = not detected.

2.2.2 | Method B

Hydrogels J–O were prepared dissolving **1** at the required concentration (see Table 2) in PB at different concentrations by alternating ultrasound sonication and vigorous shaking over a short period of 2 min. For the gels at 0.2% w/V of gelator concentration, the final concentration of PB was 9.6 mM; for the ones at 0.5% w/V, it was 24 mM, and for the ones at 1.0% w/V, it was 48 mM, in order to adjust the PB concentration with the gelator concentration. After complete dissolution, the trigger was added. Gels with GdL were formed by adding pure GdL (2.0 equiv.) in the solution, swirling the resulting solution for a few seconds until complete dissolution of GdL and leaving the gel to form overnight. Gels with $CaCl_2$ were formed by adding 100 mM $CaCl_2$ aqueous solution (1.0 equiv.) to the gelator solution, then leaving the gel to form overnight.

Gels used for the study of the critical gelation concentration (CGC) were prepared by diluting a 0.2% w/V solution of gelator in either water containing NaOH (1.0 equiv.) or PBS (9.6 mM, at pH 7.4) with MilliQ water, then the trigger was added.

Gels used for photographs and rheology were prepared on a total volume of 2 mL in a Sterilin Cup®.

Gels used for spectrophotometric analysis were prepared on a total volume of 1 mL into disposable cuvettes with 10 mm optical path.

Xerogels used for microscopy were prepared by transferring a small amount of the gel prepared in Sterilin cups onto a microscope glass slide. The samples were left to dry over a period of 16 h at room temperature in a box to avoid the deposition of dust.

Gels used for HPLC-MS analysis were prepared on a total volume of 1 mL in glass vials for HPLC. The gels were then transferred in a larger vial and dissolved with 3 mL of fresh CH_3CN, then 0.1 mL of the resulting solution was diluted with 0.9 mL of fresh CH_3CN. These samples were injected.

2.3 | Rheology

The rheological measurements were performed using an Anton Paar (Graz, Austria) MCR102 rheometer with a vane and cup measuring system, setting a gap of 2.1 mm. The gels were prepared as described and tested directly in the Sterilin Cup®, which fits in the rheometer. Oscillatory amplitude sweep experiments (γ: 0.01%–100%) were performed at 23°C using a constant angular frequency of 10 rad/s.

2.4 | Spectrophotometric analysis

The spectrophotometric analyses were performed using a Cary 300 UV-vis double beam spectrophotometer, using a cuvette with water as reference.

2.5 | HPLC-MS

HPLC-MS analysis was carried out with an Agilent 1260 Infinity II liquid chromatography coupled to an electrospray ionization mass spectrometer (LC-ESI-MS), using a Phenomenex Gemini C18-3 μ - 110 Å column, H_2O/CH_3CN with 0.2% formic acid as acid solvent at 40°C (positive ion mode, m/z = 50–2000, fragmentor 70 V).

2.6 | Optical microscopy

The images of the xerogels deposited on glass slides were recorded using a Nikon (Minato, Japan) 13 ECLIPSE Ti2 Inverted Research Microscope with a 40× magnifier.

3 | RESULTS AND DISCUSSION

The protected tripeptide Boc-L-Phe-D-Oxd-L-Phe-OH **1** is a cheap molecule that may be readily obtained by the liquid-phase synthesis on the multi-gram scale. The synthesis and characterisation of this product were previously reported by our group, as well as some preliminary studies of its gelling ability using the solvent switch method, mixing water with alcohols.[58]

Now we report here our recent results for the preparation of hydrogels, avoiding the use of organic solvents and using two different techniques as trigger: the pH variation method and the addition of cations, able to form electrostatic interactions with the gelator. To prepare the hydrogels, we used two methods (method **A** and method **B**) that differ for the crucial step of the gelator dissolution in water.

Following method **A**, the gelator was dissolved in basic water, adding the NaOH that is required to promote the gelator dissolution, as at neutral pH, the molecule is not water-soluble. After the dissolution, we tested the gelation ability of 1, adding the trigger to increase concentrations of the gelator (0.2, 0.5 and 1.0% w/V) and with different triggers (Table 1), obtaining a gel in any tested condition, as shown in Figure 1.

We probed the critical gelation concentration (CGC) of this gelator in the presence of GdL and calcium and found that a concentration of gelator lower than 0.2% w/V produces only clear solutions; therefore, the CGC is 0.2% w/V (Table S1 and Figure S1).

As in previous work, we observed that a similar scaffold containing the Oxd moiety coupled with 3,4-diflurophenylalanine underwent hydrolysis when dissolved in the presence of NaOH,[59,60] we checked the stability of the gelator under these conditions by means of HPLC-MS (Table 1). Even though the major component of the final material was **1**, we could detect a significative percentage of the Boc-L-Phe-OH, ranging between 3% and 22%, which increases with the gelator concentration, in line with the kinetics parameters of the reaction. When the gelator is dissolved in NaOH, the imide bond is hydrolysed, producing two acids, Boc-Phe-OH and H-Oxd-Phe-OH (Figures 2 and S2). As the hydrolysis rate increases with the reagent concentration, the more concentrated gels contain a higher amount of acids (Boc-Phe-OH and H-Oxd-Phe-OH), thus reducing the final pH of the gel.

For this reason, we studied the possibility to obtain stable gels, avoiding the initial harsh basic pH that favours the gelator hydrolysis.[59] Following method **B**, we dissolved the gelator in a phosphate buffer (PB) solution with a concentration adjusted with the gelator concentration to obtain a final pH ranging between 6.8 and 6.9 (see Section 2 for details), then we added the same triggers previously reported in Table 1. Unfortunately, the interference of phosphate ions with either the pH variation or the Ca^{2+} activity required higher amounts of both triggers (Table 2).

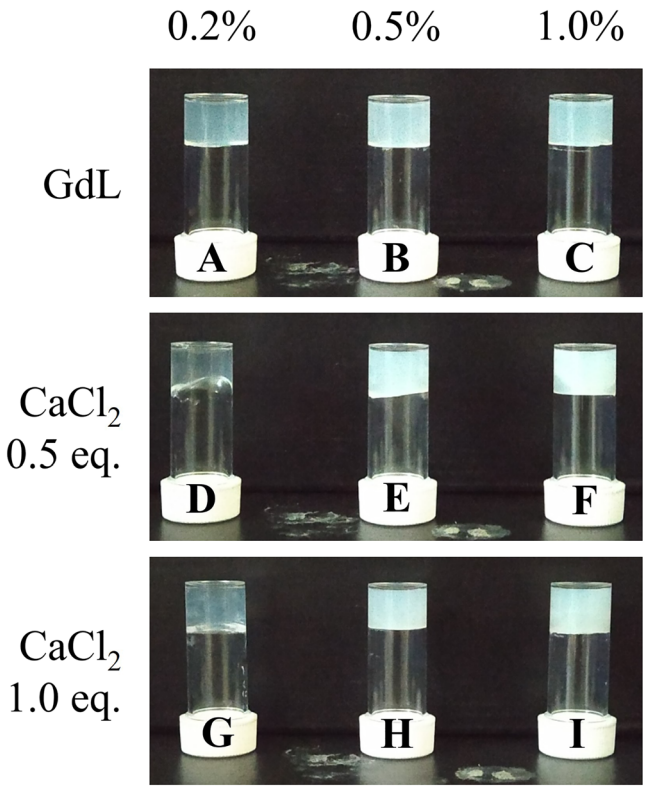

FIGURE 1 Photographs of the gels A–I obtained with method **A**, varying both the gelator concentration and the trigger, as reported in Table 1.

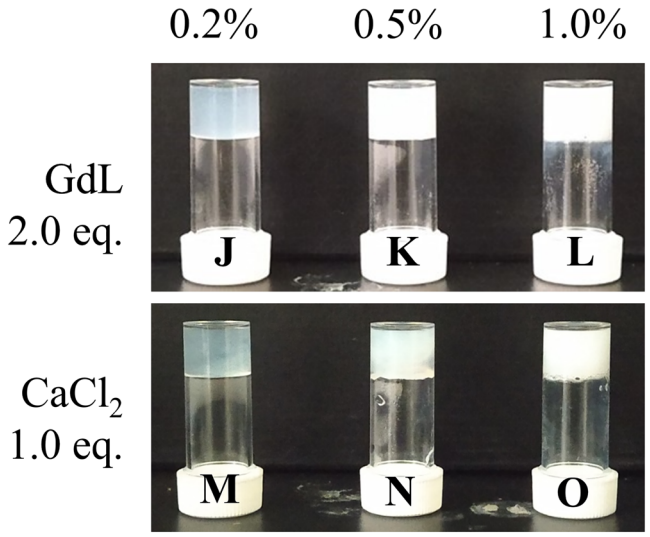

FIGURE 3 Photographs of the gels J–O obtained with method B, varying both the gelator concentration and the trigger, as reported in Table 2.

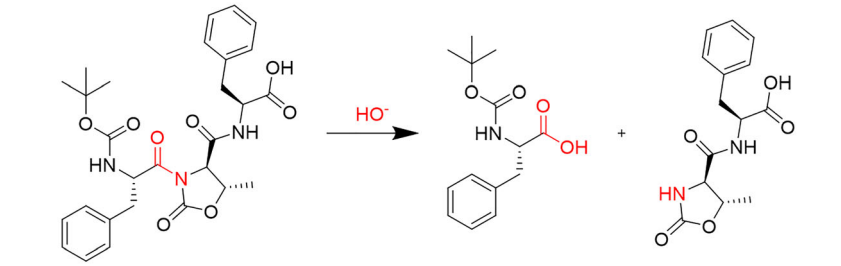

FIGURE 2 Schematic representation of the hydrolysis of the gelator Boc-L-Phe-D-Oxd-L-Phe-OH **1**.

Thus, hydrogels J–L were prepared by adding 2.0 equiv. of GdL, which led to the formation of stable gels with a final pH around 4.8 (Figure 3, up), as 1.2 equiv. led to the formation of weak hydrogels, with final pH ranging between 5.8 and 6.0 (Figure S3). Likewise, we formed hydrogels M–O with 1.0 equiv. of calcium ions (Figure 3, down), as with the addition of 0.5 equiv. of calcium ions, only partial gels (0.2% and 0.5% w/V concentration) or weak gels (1.0% w/V concentration) are obtained (Figure S4).

With this methodology, the CGC is reduced, as gels are formed with concentrations lower than 0.2% w/V. Indeed, the gel triggered with GdL has a CGC of 0.05% w/V, whereas the one triggered with $CaCl_2$ has a CGC of 0.1% w/V (Table S2 and Figures S5 and S6).

The HPLC-MS analysis demonstrates that the PB methodology (method **B**) allowed us to avoid the problem of the hydrolysis (Figure S7 and Table 3). This methodology may be adopted for gelators with pK_a lower than 7.4 for the formation of hydrogels, as PB is a solution widely used in biological and biomedical applications, representing a valid alternative to the harsh basic environment (about pH 10) obtained with NaOH. Indeed the dissolution at a high pH can cause the hydrolysis of many other gelators, for example, those possessing a carbamate group or interfering with basic sensitive protecting groups, as reported, for instance, in the case of the widespread Fmoc-protected gelators.[49]

The mechanical and optical properties of hydrogels A–O were characterised, to outline the stiffness and transparency of these media, compare the properties of the hydrogels, and understand if the partial hydrolysis of the gelator has an impact on them (Table 3).

The hydrogels obtained with method **A** (A–I) have a G' modulus higher than the G'' and a significant elasticity, confirmed by the long linear viscoelastic range, LVER (Table 3 and Figure S8). The hydrogels D, E, G, and H, prepared with Ca^{2+} ions as trigger, are even more elastic since they do not present a crossover point (breaking point of the gel network) in the range of shear strain studied, having the G' always higher than the G''. In addition, hydrogels A, D, and G show high transparency, taken as the value of transmittance at 630 nm,[60] ranging between 58.8% and 77.8%, in contrast with their modest strength, which ranges between 0.26 and 1.64 kPa. Reasonably, as the gelator concentration increases, the stiffness of the gel

TABLE 3 Storage modulus (G'), loss modulus (G''), linear viscoelastic region (LVER), crossover point and transparency of hydrogels A–O.

Gel	G' (kPa)	G'' (kPa)	LVER (γ %)	Crossover point (γ %)	Transparency at 630 nm (%)
A	1.64 ± 0.76	0.12 ± 0.05	1.0	100	70.2
B	5.48 ± 1.14	0.62 ± 0.27	1.5	21.0	46.3
C	24.8 ± 7.97	3.42 ± 0.98	1.5	55.1	26.4
D	0.26 ± 0.12	0.04 ± 0.01	0.5	N.D.	77.8
E	4.80 ± 0.90	0.62 ± 0.11	1.0	N.D.	18.2
F	16.9 ± 10.3	2.33 ± 1.80	1.0	85.1	5.3
G	0.35 ± 0.08	0.04 ± 0.02	2.2	N.D.	58.8
H	6.09 ± 3.82	0.66 ± 0.16	0.5	N.D.	14.1
I	21.8 ± 1.25	3.36 ± 0.26	2.2	60.2	1.9
J	4.55 ± 2.87	0.41 ± 0.25	1.5	37.7	46.0
K	28.2 ± 11.6	3.47 ± 1.26	2.2	19.1	0.7
L	90.2 ± 34.1	6.87 ± 0.28	0.7	N.D.	0.6
M	1.03 ± 0.29	0.20 ± 0.09	2.2	N.D.	30.7
N	31.2 ± 13.9	6.93 ± 2.98	0.7	33.9	2.5
O	76.4 ± 40.3	9.94 ± 4.09	0.5	14.7	1.2

Note: G' and G'' are taken at γ = 0.046% as at that strain none of the gel has inflections in the trend of their moduli; N.D. = not detected.

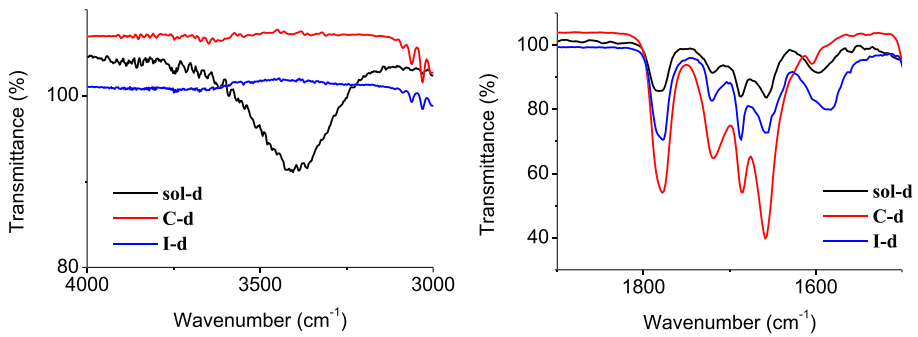

FIGURE 4 FT-IR spectra of 1% solution (sol-d) and gels C-d and I-d, replacing water with D_2O and NaOH with NaOD.

increases accordingly, while the transparency drops (Figure S9). Indeed, hydrogels at the concentration of 0.2% w/V are the least stiff and the most transparent, whereas the ones at the concentration of 1.0% w/V are the stiffest and the least transparent.

Coming to hydrogels J–O, obtained with method **B**, we notice an increase in mechanical properties (Table 3 and Figure S10) coupled with reduced transparency (Table 3 and Figure S11). In particular, hydrogels L and O, which contain the gelator in 1.0% w/V concentration, are extremely strong ($G' = 90.2$ kPa and 76.4 kPa, respectively) but not transparent (transparency = 0.6% and 1.2%, respectively).

To have a better understanding of the formation of entangled fibres that form the final gel, we analysed the gels by IR spectroscopy. By comparing the differences in the FT-IR spectra of solution and gel and analysing what bonds have appeared and disappeared, it becomes possible to infer the driving forces of hydrogelation.[61–63] First, we recorded the FT-IR spectra of the two solutions (in NaOH and in PB) and of the corresponding gels C, I, L, and O, all at 1% w/v concentration. As all the samples are in water solution, we could not get any information on the NH region. In the region between 1800 and 1500 cm^{-1}, we noticed that the peak positions show very small variations between the solution and the gels (Figure S12). This effect is probably due to the presence of water molecules that efficiently form hydrogen bonds with the carbonyls of **1**, mimicking what happens in fibre formation. Indeed the carbonyl peaks have all wavenumber lower than the previously reported peaks of Boc-L-Phe-D-Oxd-L-Phe-OBn, recorded in 3 mM concentration in dichloromethane solution, where hydrogen bonds are not possible (Table S3).[58] The FT-IR spectra of the corresponding xerogels show a similar behaviour (Figure S13). Here, the NH bonds are clearly visible and range between 3351 and 3322 cm^{-1},

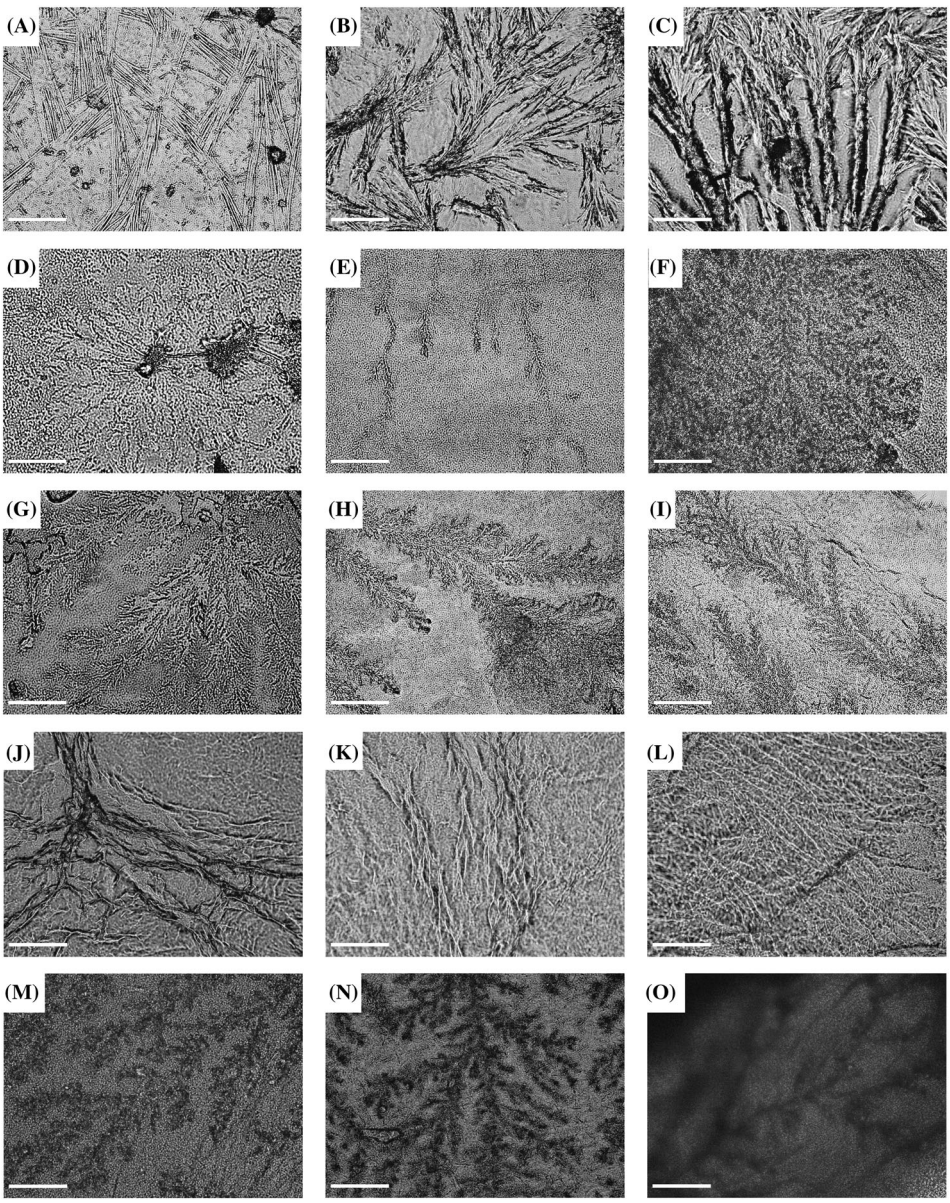

FIGURE 5 Images of xerogels A–O of **1** obtained with the different conditions tested. The scale bar is 50 μm.

thus showing that NH groups are involved in hydrogen bonds, typical of solids. In Table S3, we summarize these results, and we show the comparison of the main NH (when visible) and CO peaks under different conditions.

To check the behaviour of the NH groups in solution, we prepared a 1% solution and gels C and I, replacing water with D_2O and NaOH with NaOD (for this reason, named sol-d, C-d, and I-d).[64,65] Comparing the spectra in the NH region, the signal of sol-d is centred at 3403 cm^{-1}, which is typical of non-bonded NH groups,[66,67] whereas the formation of supramolecular interactions may be confirmed by its disappearance in gels (Figure 4). In the CO region, no peak seems to have undergone a pronounced shift from solution to gel. In contrast, a shift variation among the peaks at 1584 ($CaCl_2$), 1597 (solution) or 1604 (GdL) cm^{-1} was recorded, and it is attributed to the C–O stretching of carboxylic acid, where the shift variation may be due to the different ions coordinating the carboxylate group.

To complete the analysis of these materials, we prepared the xerogels of all the samples of hydrogels A–O and analysed them with an optical microscope (Figure 5). The analysis revealed that they generally have a fibrous structure. Morphologically, the gels sharing the same trigger obtained both from NaOH and PB are similar. Differences arise varying the trigger, where bundles of long fibres are found in GdL triggered gels and branched fibres are present in calcium-triggered gels.

4 | CONCLUSIONS

In this work, we studied the ability of the tripeptide Boc-L-Phe-D-Oxd-L-Phe-OH to form hydrogels under controlled conditions. We prepared 15 hydrogels, having different properties, including gelator concentration, stiffness, pH and transparency.

A particular attention has been devoted both to the technique needed for the gelator dissolution and for the hydrogel formation. So, we compared two methods for solubilisation, employing either NaOH or PB, which both present advantages and drawbacks. The use of NaOH exposes the gelator to harsh conditions during the dissolution that ends up in its partial hydrolysis, which is more pronounced with high concentrations (≥10 mM). Using PB to dissolve the gelator, this problem is completely avoided, as no hydrolysis product has been detected in the hydrogels. Unfortunately, the use of PB salts requires a higher concentration of both triggers and produces more opaque hydrogels. Thus, if the preparation of transparent gels is required, NaOH aqueous solutions may be employed to dissolve gelators in low concentrations such as 1–5 mM, as the reduced concentration cuts down the hydrolysis rate.

This study could help researchers avoiding the preparation of unstable, irreproducible, and not reliable materials. We think that a careful study of the dissolution conditions of the gelator coupled with the analysis of the gelator stability in gels should become a general approach for the preparation of supramolecular gel materials.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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