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SYNTHESIS AND STUDY OF A NEW MOLECULAR HYDROGELATOR DERIVED FROM L-VALINE

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ABBREVIATIONS

ATP	Adenosine triphosphate
Boc	Tert-butoxycarbonyl
Bpoc	2-p-bi-phenylylisopropyloxycarbonyl
Cbz/Z	Carbobenzyloxy
COSY	Correlated Spectroscopy
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	Dicyclohexylurea
DIPEA	N,N-Diisopropylethylamine
DMF	N,N-Dimethylmethanamide
DMSO-d_6	Deuterated dimethyl sulfoxide
eq.	Equivalents
Et_3N	Triethylamine
Fmoc	Fluorenylmethyloxycarbonyl
HMBC	Heteronuclear Multiple Bond Correlation
HOSu	Hydroxysuccinimido
HOXt	N-alkoxycarbonylamino acid
HR ESMS	High resolution Electro-spray Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
$(HVal)_2Oct$	(S)-2-amino-N-(8-((R)-2-amino-3-methylbutanamido)octyl)-3-methylbutanamide
LMWG	Low Molecular Weight Gelator
MeOH	Methanol
mgc	Minimum Gelation Concentration
m/z	Mass/charge
NHS	N-hydroxysuccinimide
NMR	Nuclear Magnetic Resonance
Nps	2-nitrophenylsulfenyl
Oct	Octamethylene alkyl chain
Pyr	Pyridine

(PyrSucVal)$_2$Oct	N^1,$N^{1'}$-((2S,2'S)-(octane-1,8-diylbis(azanediyl))bis(3-methyl-1-oxobutane-1,2-diyl))bis(N^4-(pyridin-4-ylmethyl)succinamide)
Qui	Quinoline
rt	Room temperature
Suc	Succinic acid
(SucVal)$_2$Oct	(6R,19S)-6,19-diisopropyl-4,7,18,21-tetraoxo-5,8,17,20-tetraazatetracosanedioic acid
TBTU	2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorphosphate
TEM	Transmission Electron Microscopy
TFA	Trifluoroacetic acid
T_{gel}	Gel melting temperature
THF	Tetrahydrofuran
TMS-Cl	Trimethylsilyl chloride phenol
Val	Valine
wt	Weight
(ZVal)$_2$Oct	Benzyl ((5R,18S)-5-isopropyl-19-methyl-3,6,17-trioxo-1-phenyl-2-oxa-4,7,16-triazaicosan-18-yl)carbamate
ZValOH	Carbobencyloxy-*L*-valine
ZValOSu	2,5-dioxopyrrolidin-1-yl ((benzyloxy)carbonyl)-*L*-valinate

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INTRODUCTION

1. INTRODUCTION

1.1 Supramolecular gels

1.1.1 Formation and applications of supramolecular gels

Supramolecular gels are large structures or networks formed by the self-assembly of small organic molecules, called low molecular weight gelators (LMWGs), by non-covalent interactions, like π-π stacking, hydrogen bonding, van der Waals interactions, ion pairing, solvophobic forces and coordination. Due to these interactions, the organic molecules are organized into fibers that immobilize the solvent in the interstices of a solid matrix. As a result, a viscoelastic semi-solid is obtained.[1]

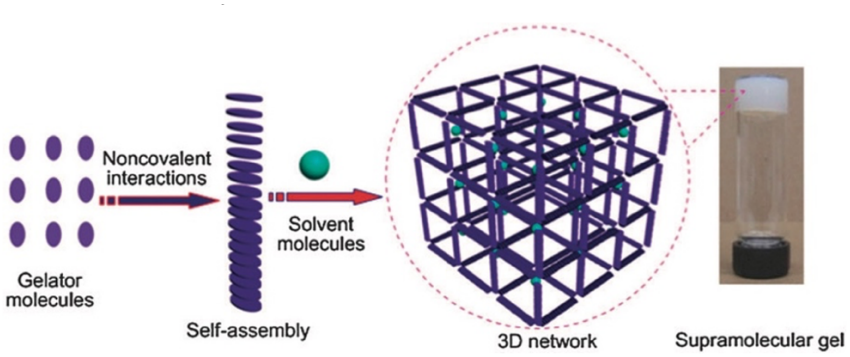


Figure 1. *Representation of the formation of a supramolecular gel.*[2]

In many cases, the gels' properties do not change over time. Still, as the non-covalent interactions are weak and reversible, it is possible to reconfigure the gel matrix by gel-to-gel transitions when exposed to external stimuli, such as temperature, pH, light, enzyme, redox and chemical products. In this way, it is possible to change their properties to give them the desired application.[1]

Although scientists started to study supramolecular gels only thirty years ago approximately, a lot of fields where they can be applied have been discovered, for instance:

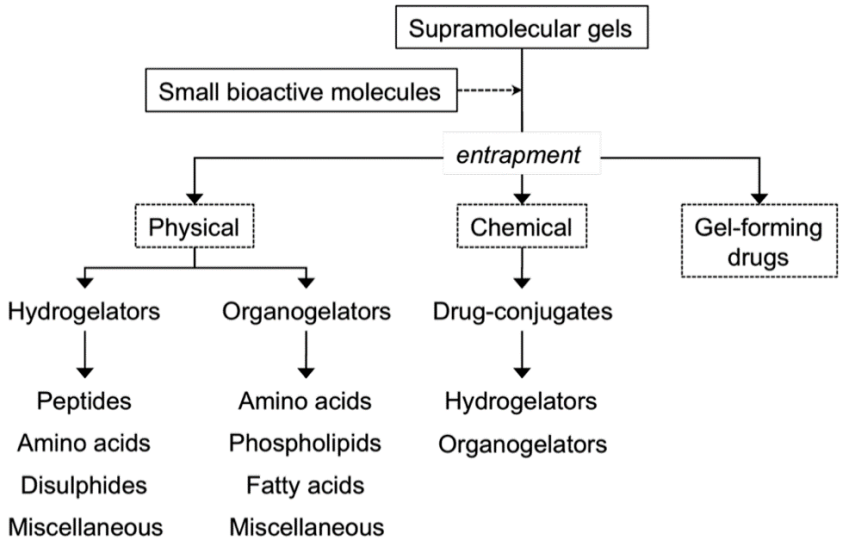
- Catalysis.
- Cell culturing.
- Biomaterials.
- Electronic materials.
- Sensors.

- Tissue engineering.
- Environmental remediation.
- Biomedicine.

Supramolecular gels have become particularly popular in the last application named above, biomedicine. In this field, especially in drug delivery, they are being studied to solve drug solubility problems in water and/or provide controlled release. Therefore, using supramolecular gels could improve drug bioavailability and therapeutic efficiency.[2]

1.1.2 Entrapment of actives in supramolecular gels

Supramolecular gels can be classified according to how they trap the bioactive molecules.



Scheme 1. *Classification of supramolecular gels.*[3]

In physical entrapment, the gel is prepared using a stock solution of the corresponding bioactive molecules, or a solution of these molecules is allowed to diffuse in an already prepared gel.[4] Depending on the solvent, there are two types of gels in physical entrapment:

- Hydrogels, where the solvent is water.[5] We can distinguish between:
  - Peptide-based hydrogels.
  - Amino acid derivatives-based hydrogels.

- Disulfide-containing hydrogels which are formed by linking amino acids through S-S bonds, often based on the oxidized dimer of the amino acid cysteine.
- Miscellaneous. This group is constituted by LMW hydrogelators that cannot be included in the above categories, like carbohydrates derivates or amphiphilic molecules with a long hydrophobic chain.[3]

- Organogels, where the solvent is a non-polar biocompatible organic solvent, such as oils.[5] We can distinguish between:
  - Amino acid derivatives-based organogels.
  - Phospholipid-based organogels, made up of amphiphilic phospholipids in oils, being lecithin the most used.
  - Fatty acid-based organogels, formed by simple fatty acids derivates.
  - Miscellaneous. This group is constituted by LMW organogelators that cannot be included in the above categories, like compounds that contain amides or esters.[3]

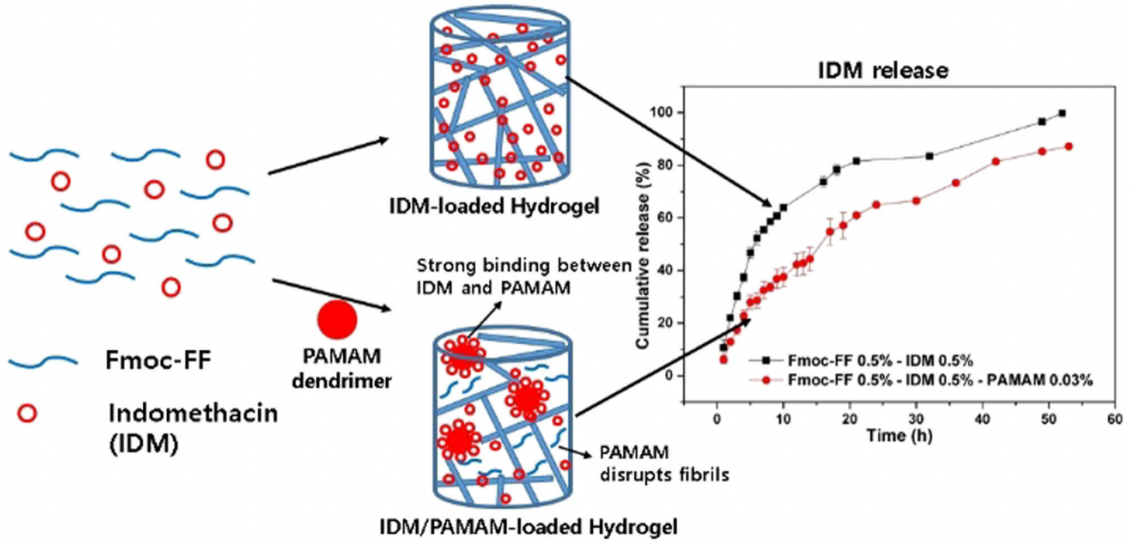


Figure 2. *Comparison of the drug indomethacin (IDM) release when it is incorporated, during the self-assembly process, in two physical entrapped hydrogels: a) Fmoc-diphenylalanine (Fmoc-FF) and b) Fmoc-FF with small amount of polyamidoamine dendrimer (PAMAM).*[6]

In chemical entrapment, the bioactive molecules are conjugated to the gel by covalent bonds. In this case, the resulting gel must be non-cytotoxic, present good activity and resist proteolytic degradation.[4] Depending on the solvent, there are two types of gels in chemical entrapment:

- Drug-conjugates as hydrogels, where the solvent is water.
- Drug-conjugates as organogels, where the solvent is a non-polar biocompatible organic solvent.

In both cases, most common gels are constituted by drugs conjugated to peptides, usually prepared from L-amino acids.[3]

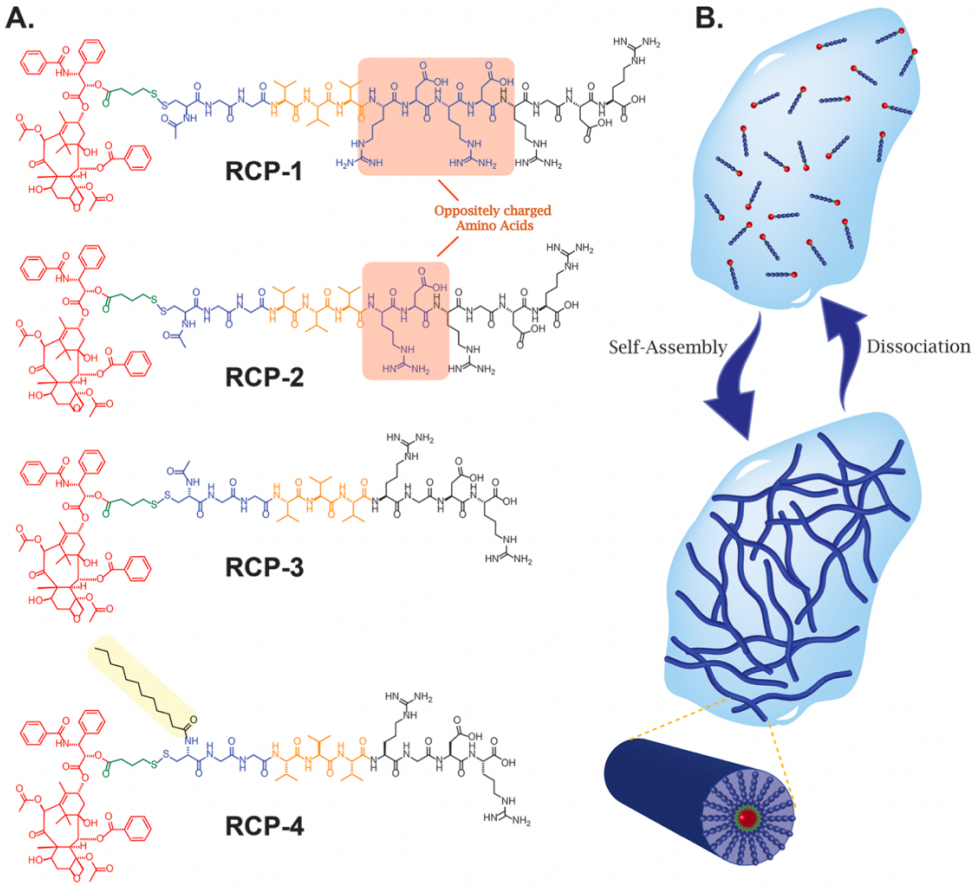


Figure 3. *Representation of a) molecular design of four drug amphiphiles (RCP-X) containing: the paclitaxel drug (red), a reducible disulfylbutyrate (buSS) linker (green), a two glycine spacer (blue), three valines β-sheet-forming segment (orange) and a tumor-penetrating sequence (black), and b) amphiphiles self-assembly in supramolecular filaments by chemical entrapment after dissolution in water, which can reversibly dissociate.*[7]

Gel-forming drugs are those that are able to form gels by themselves or by the formation of a salt. They are the group in which there is less literature available until this moment.[3]

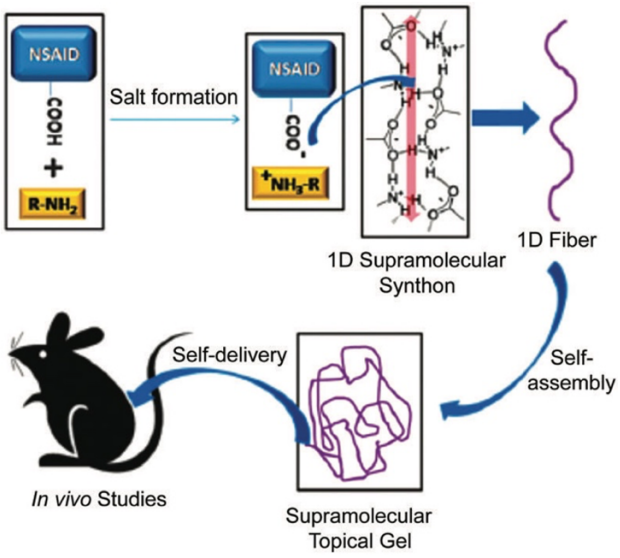


***Figure 4**. Schematic representation of formation and self-delivery of supramolecular gels based on gel-forming drugs.[3]*

1.1.3 Gel to gel transitions

Although this project does not study so-called "gel to gel transitions", considering the possibility of future work in the group, a brief revision of transformations of supramolecular gels into polymorphic or new gels is included in this point of the introduction section.

Typically, a ca. 1 wt% concentration gelator is dissolved or colloidal dispersed in the solvent to form a supramolecular gel. Then, the gelation process is activated by a trigger, yielding 1D or 2D anisotropic structures. Finally, a 3D network is formed, where the solvent is trapped inside by surface tension and capillary forces. The way these gels form depends on the environmental conditions, such as the solvent, temperature, ionic strength etc.

If gels are exposed to external stimuli, the network structure can change. When conditions are modified to promote self-assembly, the network changes its structure to arrive at a thermodynamically lower energy state. In this process, the errors in the original network can be corrected.[1]

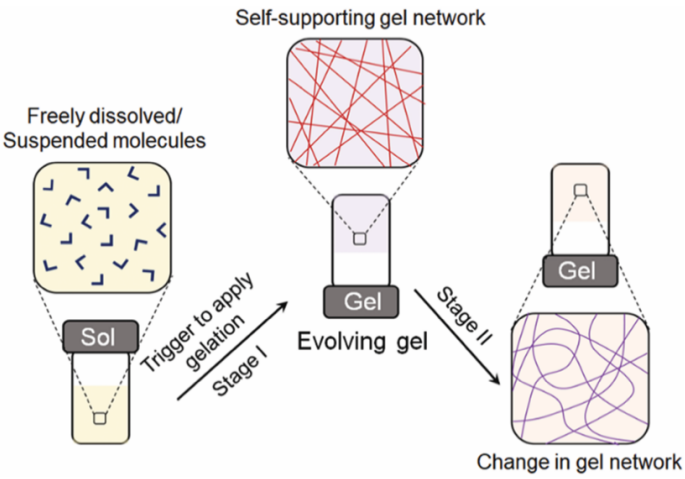


Figure 5. *Schematic representation of gel formation followed by a change in the gel network.*[1]

Sometimes it is possible to find some problems when the non-covalent interactions are strong, so these errors cannot be corrected during self-assembly. This means that the structure does not reach the minimum energy state and is in a kinetically trapped state, where it is stable. In this case, reconfiguring the gel matrix is possible when external stimuli provide enough energy to go from one thermodynamically favorable state to another. This process is called post-assembly fabrication.[1]

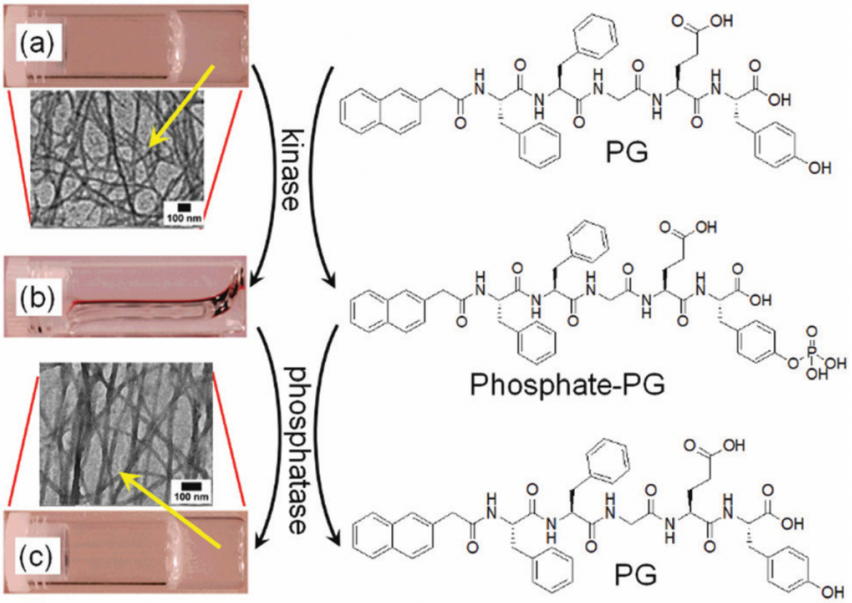


Figure 6. *Structure, phase and fibers arrangement (TEM) changes of a pentapeptide gelator (PG) when: a) original gel is formed, b) treated with kinase and ATP to form the phosphorylated compound, and c) treated with phosphatase to restore the gel.*[8]

It can also be the case that the gel exchanges matter with the medium, causing a slow transformation in the structure from the kinetically trapped state to a thermodynamically favorable, known as a metastable gel. In this state, it is possible to program autonomous gel-to-gel transitions without the presence of external stimuli.[1]

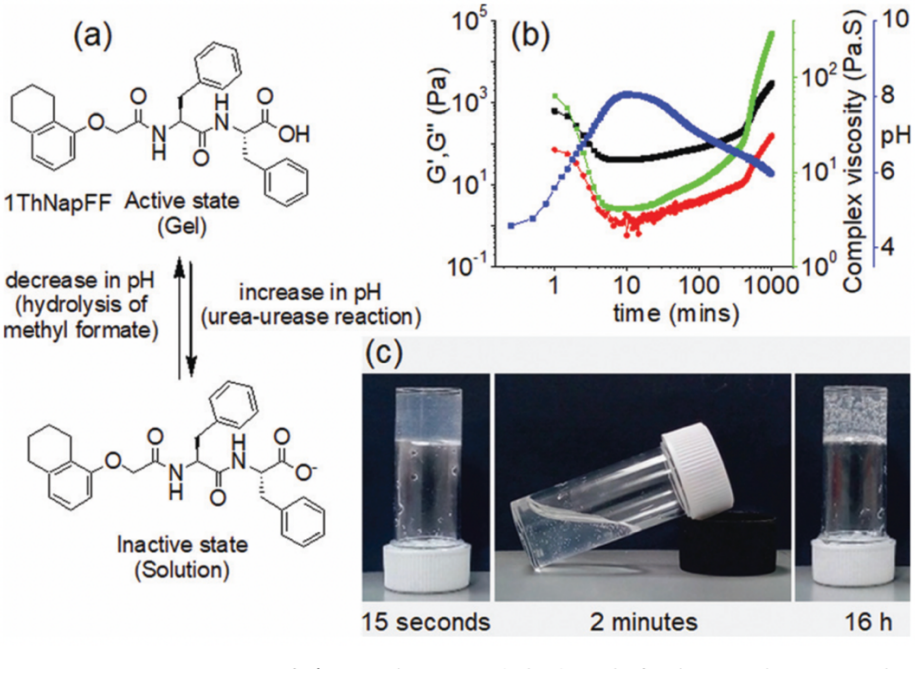


Figure 7. *Representation of a) annealing peptide hydrogel of 1ThNapFF by a pH cycle, constructed by coupling the urease-urea enzymatic reaction with methyl formate, (b) variation of rheological moduli with pH over time: G' (black), G'' (red), viscosity (green) and pH (blue), and (c) a gel-sol-gel transition seen during the annealing process.*[9]

One way to cause the gel to transition from a kinetically trapped state of a gel is to functionalize it once assembled. To do that, there are different possibilities:

- Thermal annealing: Consists of heating the supramolecular gel to obtain a solution of the gelator dissolved in the solvent, called gel-to-sol transformation, due to the weakening of the interactions between the gelator molecules. After cooling, it re-gels in a thermodynamic minimum state. This is not always possible because there are gelators with functional groups that can participate in secondary reactions, bringing on undesired permanent chemical changes, such as Diels-Alder reactions. In addition, some gelators can change to another gel

state without forming a sol state by reorganizing the hydrogen bonds in the network.

- Enzymatic reaction: When the gelator skeleton has enzyme responsive centers, they react specifically in the presence of an enzyme, forming a covalent bond between them. It means that the chemical structure of the gel has changed, so the non-covalent interactions change too, provoking self-assembly. This method is beneficial in biomedical research.
- UV light-induced reaction: If the gelator is functionalized with photoresponsive chromophores or non-covalently bonded to photoresponsive fragments, these groups absorb this light of a specific wavelength when it is irradiated, leading to photoreactions that change the physical and chemical properties. Therefore, supramolecular interactions change, causing transformations in the gel matrix. Some important photoreactions are trans-cis photoisomerization, photodimerization/polymerization or electrocyclic reactions. This method is used in cell culture and optoelectronics.
- pH changes: This approach works when the gelator has ionizable groups, such as amines or carboxylic acids or active cleavable bonds incorporated in its structure. Consequently, depending on the pH of the medium, it is possible to produce reversible sol-gel transitions because the gelator's solubility changes with the degree of de/protonation of these groups. The transition to a solution occurs when the pK_a of the gelator is crossed. The pH of the medium also modifies the nature of the non-covalent interactions, resulting in changes in morphology and molecular conformation. On the other hand, to give stability against pH changes and avoid gel-to-gel transitions, it is common to increase the hydrophobicity of the gelator, introducing, for example, an alkyl chain in its structure so at elevated pH it acts as a surfactant, forming micellar aggregates. This method is used in biomedical research.
- Dynamic covalent chemistry: Consists in creating or breaking reversible dynamic covalent bonds in the gelator structure under mild reaction conditions, changing the gel matrix and its properties. In thermodynamically stable products, this process is slower than in kinetically trapped products, and correcting errors and imperfections in the last ones is possible. Some used reactions are the formation

of imines, hydrazones, boronic esters, Diels-Alder reaction and disulfides exchange reaction.

- Redox reactions: A redox-responsive gel has redox-active groups in its skeleton, such as ferrocenes, disulfides, or redox-active metals trapped inside its fibers. In the first case, redox changes in the structure also modify the interactions between molecules. In the second case, changes in the oxidation state of these metals, which act like metal ligands, modify the coordination sphere and the electrostatic interactions. Redox-responsive gels can be prepared chemically, where chemical reagents activate the molecule, or electrochemically, where an electrical field changes the gel's properties.
- Post-assembly fabrication of gels in chemical analytes: In this case, chemical analytes, like metal ions, amino acids or carboxylic acids, are joined to specific positions in the gelator structure, which cause modifications in the properties and aggregation of gels. Gelators interact electrostatically with ionic analytes and through hydrogen bonding and stacking interactions with neutral ones. They may be used in the future as antibacterial agents, water purifying agents or conductive materials.[1]

The structure of metastable gels can be changed by time programmable self-regulating gel to gel transitions, which usually need to have access to both states limited externally. To do that, there are different methods:

- Aging: Gels formed under kinetic control can suffer a gel-to-gel transition over time, reorganizing into a more stable assembly, which provokes changes in the gel microstructure and properties. This phenomenon is common in multicomponent gel systems. Many factors influence the way the gel ages and how long it takes to do it. It does not always work well, because the gel network can sometimes be weakened and lead to crystallization.
- pH regulated autonomous programming gel to gel transitions: They take place when the gel has a specific chemical reagent in its structure, which reacts slowly in the first stages of the reaction, achieving a specific gel network, and then this reaction is accelerated, leading to a spontaneous change in pH. Consequently, a new gel network is achieved.

- Redox responsive self-regulating gel to gel transitions: One way to prepare gels that show this type of transition is to synthesize a system formed by an organic gel and a metal, followed by a change in the oxidation state of this metal during the self-assembly process. As so many biological processes are carried out in response to a change in pH, this type of gels can be used to obtain biomimetic hydrogels or tissue engineering.
- Chemically fuelled systems: consist of gels modified in the first steps of the gelation process by a chemical reaction (addition of a fuel). The modified molecules form a high-energy, metastable gel. In the absence of fuel, the molecules can react with the medium (solvent, for example) to generate a new gel.[1]

1.1.4 Antecedents of supramolecular hydrogels in the group

Previously, the research group studied supramolecular hydrogels based on amino acid derivatives. Therefore, this project is based on the following results obtained by the group.

Firstly, in 2012 gelators were prepared from L-valine and L-isoleucine with pyridine terminal units to study their thermodynamic parameters for the aggregation in water, enthalpy and entropy, and how they changed with temperature.[18]

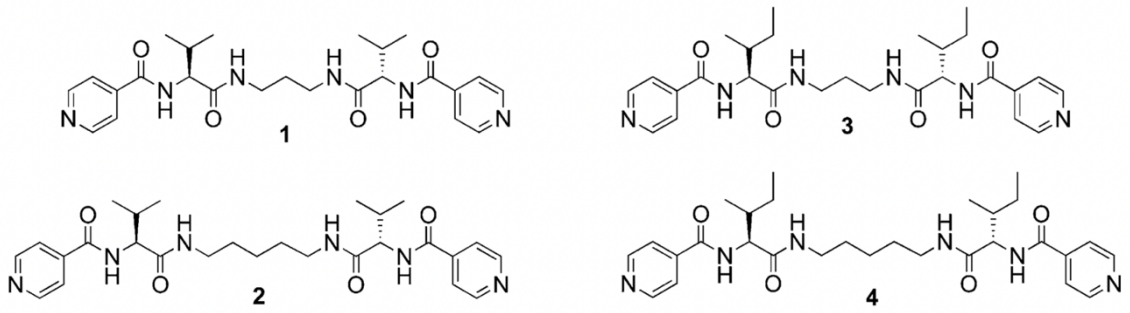


Figure 8. *Chemical structure of pyridine-terminated gelators.*[18]

A few years later, in 2016 a unit of succinic acid was employed as a building block to prepare pH-responsive hydrogels. The formation of the fibrillar networks of these hydrogels was biologically mediated by the production of CO_2 from baker yeast.[19]

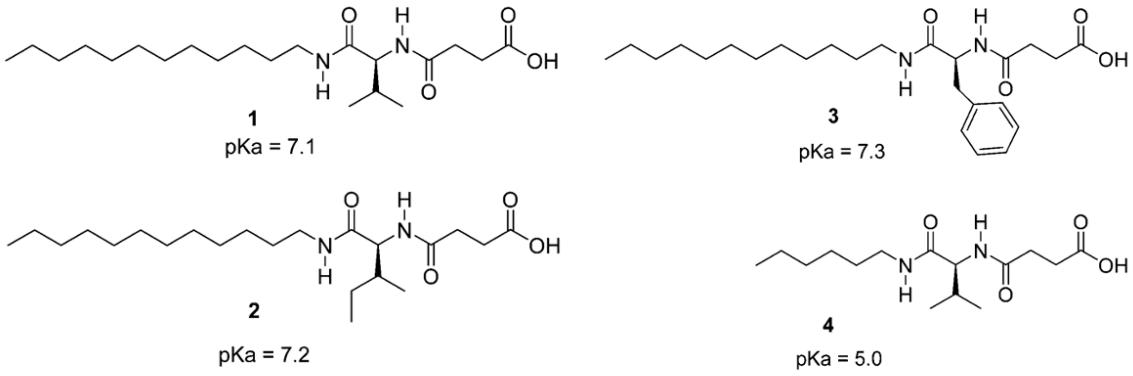


Figure 9. *Chemical structure of hydrogelators with succinic acid-type terminal units.*[19]

In 2018, the group also explored pyridine analogues such as quinoline and how they changed the hydrogels' properties, like rheological ones, biocompatibility, gelation capability and minimum gelation concentration (mgc).[20]

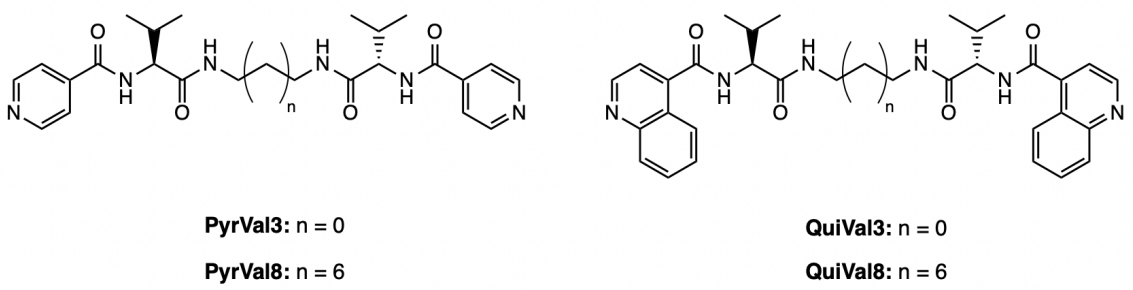


Figure 10. *Chemical structure of pyridine and quinoline-derived hydrogelators.*[20]

In this project, as described in the objectives section, introducing a succinic acid block and a terminal pyridine in an L-valine derivative are explored to modify or regulate the properties of the hydrogel.

1.2 General methods used in peptide synthesis

This part briefly reviews the principal methods used in N-protection of amino acids and peptide bond formation. These reactions have been used in the synthetic procedures described in the "3. RESULTS AND DISCUSSION" section.

1.2.1 N-Protecting groups

In peptide synthesis is important to protect the α-amino to avoid polymerization of the amino acid once it has been activated, because this peptide coupling usually become in the C to N direction. Due to this, α-amino-protecting groups are temporary, so they must be removed in mild conditions not to remove the rest of protecting groups, while these are permanent (removed at the last step of the synthesis) or semipermanent (removed if the peptide coupling takes place through the terminal C).

The α-amino-protecting groups must be easy to add and remove, and must resist the reaction conditions. They also are expected to increase the solubility in solvents, prevent the epimerization during the coupling, be stable and facilitate manipulation.

The most common α-amino-protecting groups in solid-phase synthesis are Fmoc and Boc, while in solution synthesis the most used are Cbz/Z, Nps and Bpoc.[10]

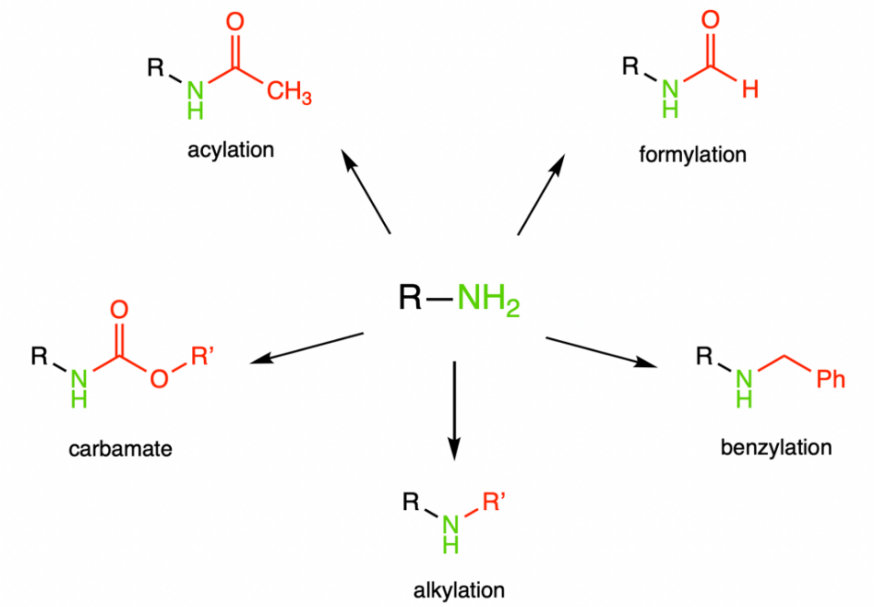


Figure 11. *Types of protection for amine functionality.*[11]

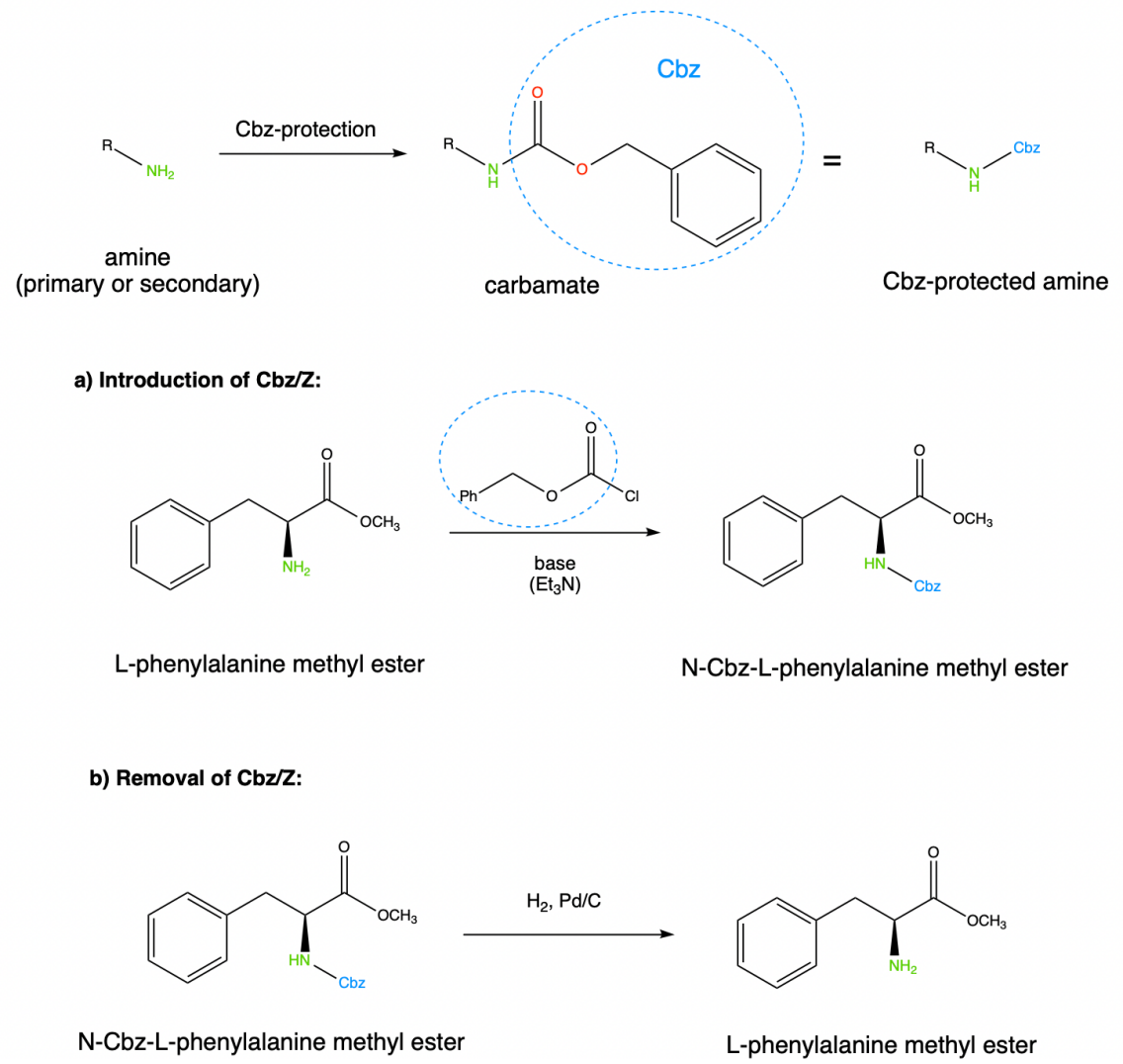
As there are many types of α-amino-protecting groups, the methodologies used can differ. Most of them are based on the reaction of the free amino acid (or with the side-chain protected) with an haloformate or dicarbonate of the protecting group under Schotten Bauman conditions: organic solvent - basic aqueous solution, or with the corresponding halide in an organic solvent. But in some cases, the free α-carboxylic acid can trigger the formation of dipeptides.

To avoid this, there are two types of methodologies: the ones that protect the α-carboxylic acid and deprotect it when the α-amino protection takes place, like the use of trimethylsilyl esters of the amino acid, and those that make use of less-reactive electrophiles in the protecting group introduction reagent, such as the use of N-hydroxysuccinimido (HOSu) derivative.

The protecting groups' removal can be carried out in acidic or basic conditions, depending on their properties. For example, the group Boc used in solid-phase synthesis is stable to bases, nucleophiles and catalytic hydrogenation so that it can be removed by trifluoroacetic acid (TFA) in DCM or by trimethylsilyl chloride phenol (TMS-Cl) in DCM or dioxane, etc.[10]

In this project, the protecting group used in the synthesis is carbobenzyloxy (Cbz/Z), which is one of the most popular for peptide synthesis in solution due to:

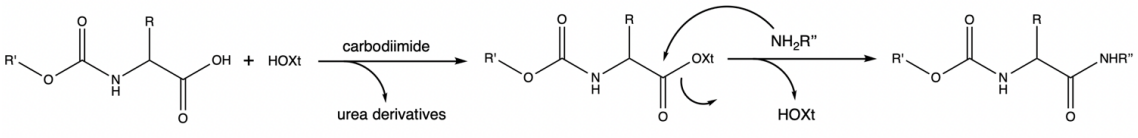
1. The easy preparation of Z-protected amino acids.
2. The high stability of the protected amino acid trough basic and mild acidic conditions, including the removal conditions of Boc.
3. The wide range of removal conditions that can be applied: catalytic hydrogenolysis or strong acids, such as HBr in acetic acid, trifluoroacetic acid (TFA) at high temperature, liquid HF, etc.
4. The suppression of racemization during the peptide-bond formation.[10]



Scheme 2. *Structure, general scheme of Cbz/Z protection and specific example of a) introduction of Cbz/Z and b) removal of Cbz/Z.*

1.2.2 Amino acid activation

In peptide synthesis it is common to activate the amino acid through the preparation of N-alkoxycarbonylamino acid (HOXt) derivatives, which are easy to store and reactive enough with amines because they are susceptible to the nucleophilic attack thanks to their electron-withdrawing properties. These active esters are prepared by the reaction between the carboxylic acid and a substituted phenol or hydroxylamine in the presence of a carbodiimide. In fact, the active esters are mixed anhydrides made from a carboxylic and a phenolic or hydroxamic acid.[12]

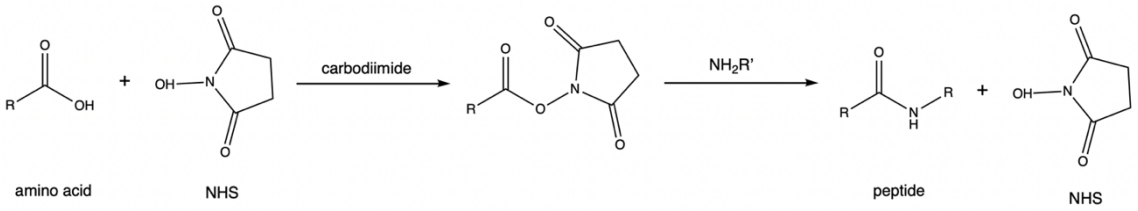


Scheme 3. *General scheme to prepare active esters and their reactivity with amines.*[12]

Esters can be activated from different pathways, as shown in *Table 1*. One of the most common involves using N-hydroxysuccinimide (NHS).

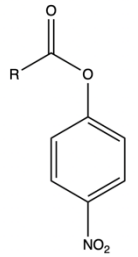
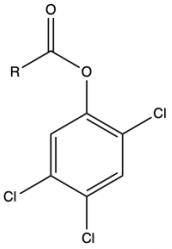
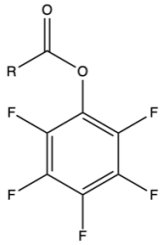
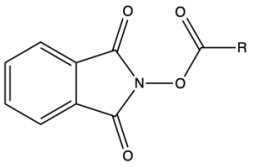
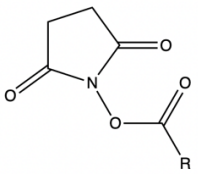
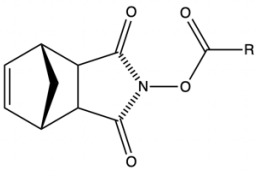
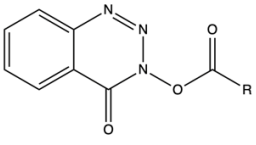
Furthermore, esters derived from hydroxamic acids act as active esters and as intermediates implicated in the coupling reactions in which N-hydroxy compounds are added to improve the carbodiimide-based coupling.

The aminolysis of these active esters can be carried out in polar solvents and catalyzed by mild acid conditions.[12]

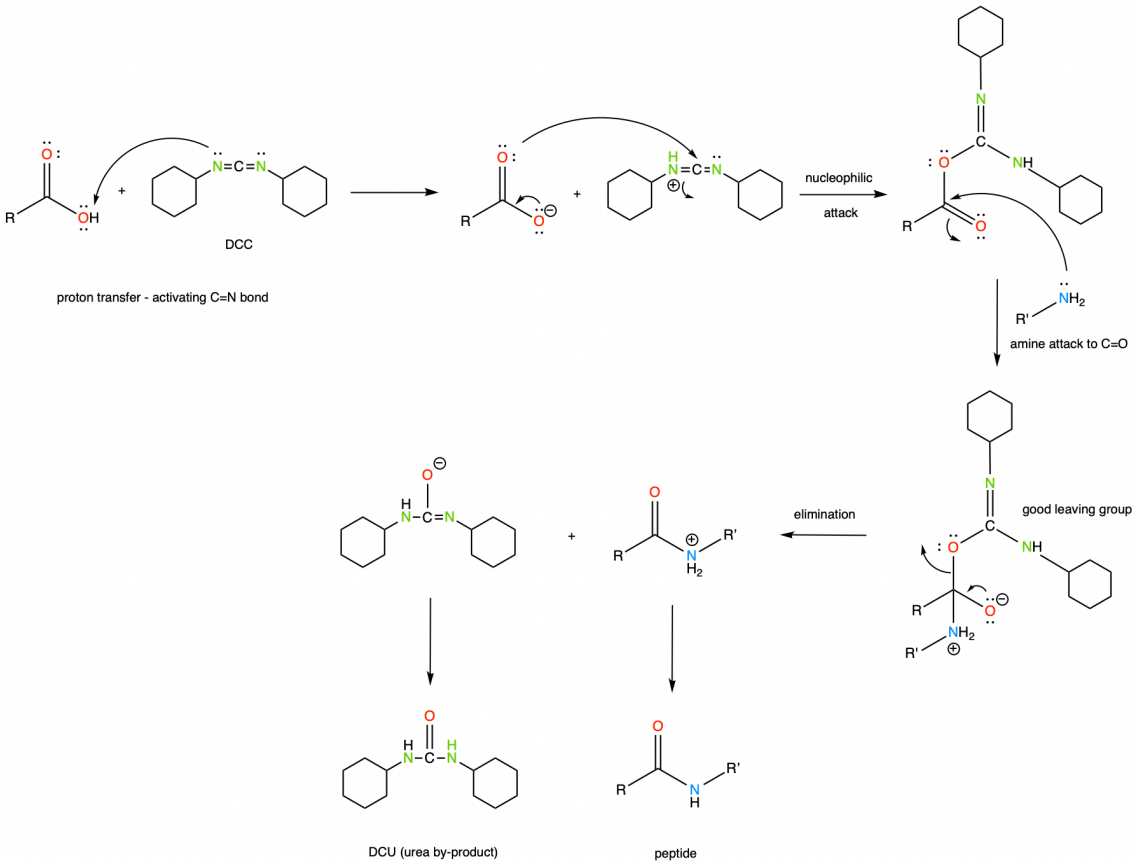


Scheme 4. *Specific scheme of preparation and reaction with amines of active esters made from NHS.*

Table 1. *Active esters used in peptide synthesis.*[12]

Name	Structure
p-nitrophenyl active ester	
2,4,5-trichlorophenyl active ester	
pentafluoro active ester	
o-phthalimido active ester	
N-succinimide active ester	
N-hydroxy-5-norbornene-endo-2,3-dicarboxyimide	
4-oxo-3,4-dihydrobenzotriazinyl esters	

To form a peptide, the carboxylic acid moiety of the amino acid must be activated with an appropriate coupling reagent. One of the most popular coupling reagents are carbodiimides, specifically the N,N'-Dicyclohexylcarbodiimide (DCC). These reagents were designed to avoid the undesired formation of N-acylurea and to facilitate the separation from the by-products, because it is very insoluble in most solvents. If separation of the by-product dicyclohexylurea (DCU) is not performed correctly, problems in the synthesis can appear. In addition, they are reasonably cheap and present a moderate activity.[13]



Scheme 5. *Mechanism of coupling reaction between two amino acids using DCC as coupling reagent.*

Another coupling reagent used in peptide synthesis is 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorphosphate (TBTU), which presents some advantages: it produces relatively lower racemization, and the coupling reactions where it is involved are fast. Due to these properties, TBTU is one of the most used in research laboratories and industry.[14]

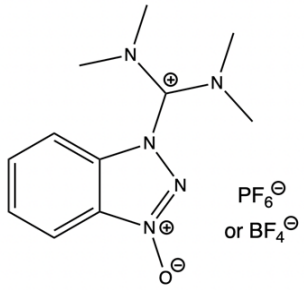
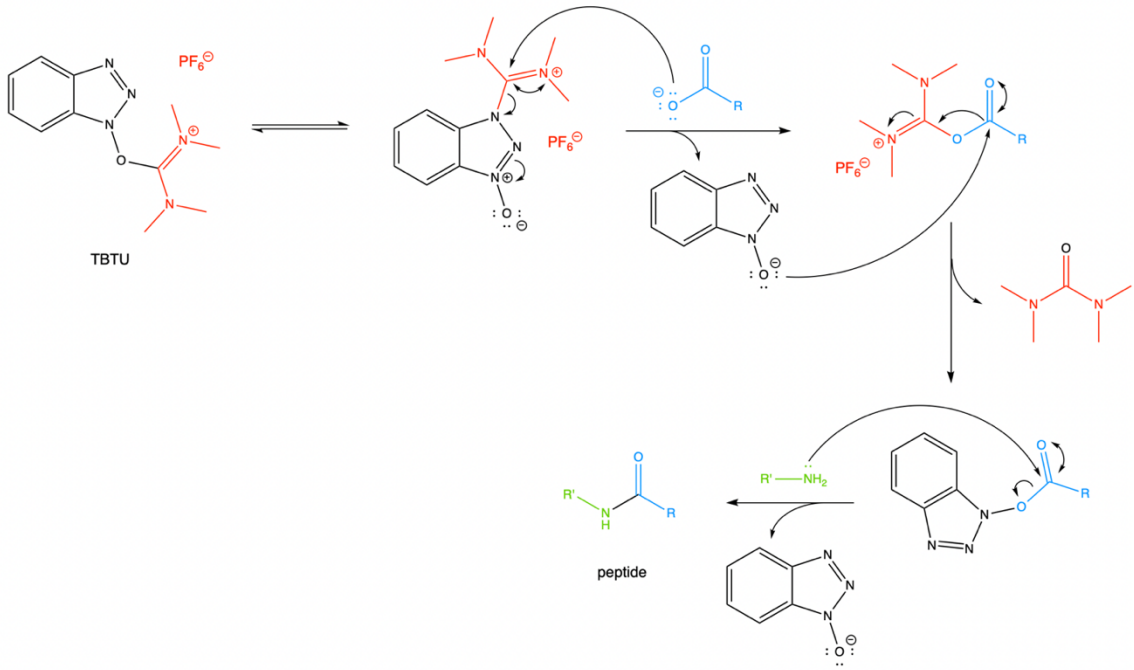


Figure 12. *Chemical structure of TBTU.*[15]



Scheme 6. *Mechanism of coupling reaction between two amino acids using TBTU as coupling reagent.*[15]

1.3 Rheology

In this project, some rheological measurements of gels are reported. Rheology is used here to study the mechanical and viscoelastic properties of the hydrogels. These properties are studied by applying a force that causes a deformation of the hydrogel and increasing the magnitude of this force until it breaks down.

In these measurements, shear storage modulus (G'), loss modulus (G'') and loss factor (G''/G') can be monitored against time, frequency and strain:

- Shear storage modulus (G'): represents the deformation energy stored during the shear process, that is, the stiffness of the hydrogel.
- Loss modulus (G''): represents the dissipated energy during the shear process, that is, the liquid-like response of the hydrogel.

Monitoring the evolution of G' and G'' against time is possible to observe the process of gelation, and monitoring them against strain is possible to observe the hardness of the hydrogel: when G' > G'', it is still behaving like an elastic solid, and when G'' > G', it has broken down and behaves like a liquid.[16]

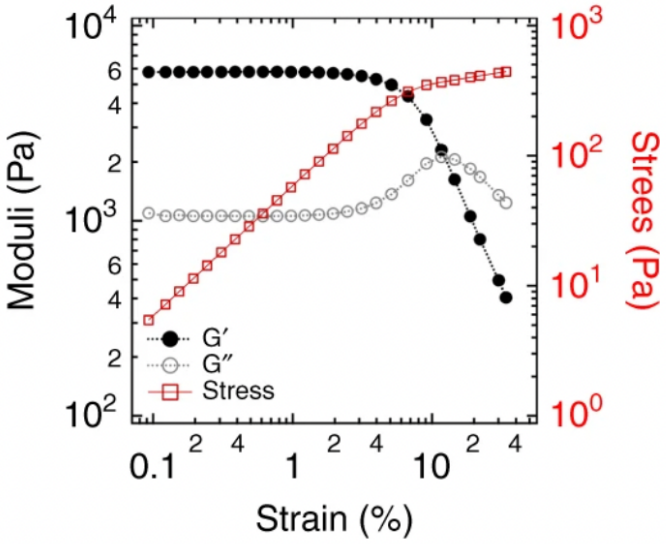


Figure 13. *Typical graphical representation of rheological measurements.*[17]

OBJECTIVES

2. OBJECTIVES

The general objective of this project is to prepare and study the hydrogels formed by the compound abbreviated as $(PyrSucVal)_2Oct$ (*Figure 14*). This compound represents a continuation of the work carried out in the group on molecular hydrogelators. The target molecule merges building blocks used in previous studies (see the introduction section) such as pyridine, succinic acid and L-valine units. This work aims to contribute to understanding the structure-properties relationship of this type of hydrogelators.

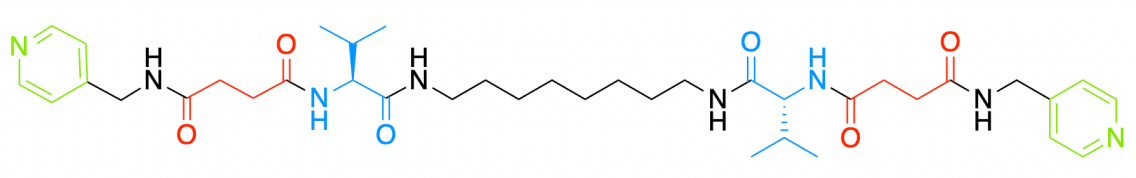


Figure 14. *Chemical structure of $(PyrSucVal)_2Oct$ containing pyridine, succinic acid and L-valine as building blocks.*

Therefore, the aims of this project are:

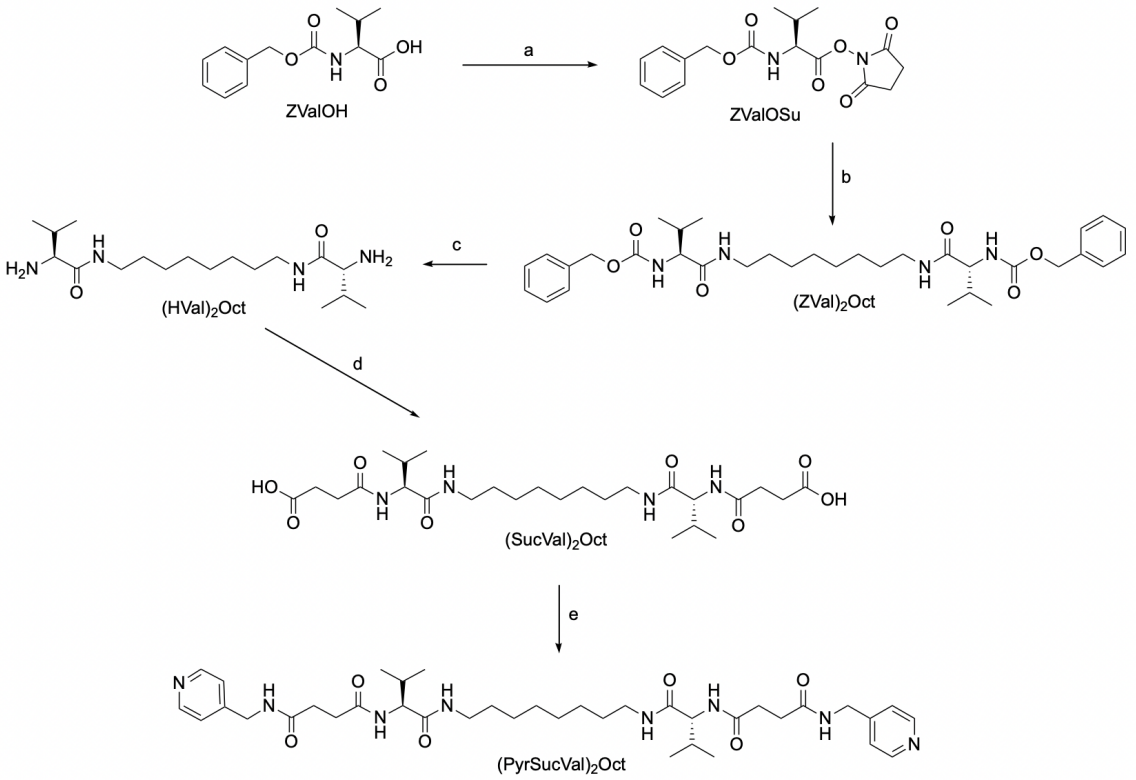
- Synthesize the compound $(PyrSucVal)_2Oct$.
- Characterize the compound $(PyrSucVal)_2Oct$ by NMR and mass spectrometry techniques.
- Determine the acid-base constants of $(PyrSucVal)_2Oct$.
- Determine the minimum concentration of gelation (mgc) and study the properties of $(PyrSucVal)_2Oct$ hydrogels prepared by three different procedures:
  - Heating-cooling.
  - Abrupt pH change.
  - Smooth pH change.
- Study the structure of $(PyrSucVal)_2Oct$ hydrogels by transmission electron microscopy (TEM).
- Study the strength of $(PyrSucVal)_2Oct$ hydrogels by rheology technique.

RESULTS AND DISCUSSION

3. RESULTS AND DISCUSSION

3.1 Synthesis of (PyrSucVal)$_2$Oct

The synthetic procedure for synthesizing **(PyrSucVal)$_2$Oct** is shown in the *Scheme 7*. (The abbreviation (PyrSucVal)$_2$Oct comes from the building blocks used: 4-aminomethyl**pyr**idine, **suc**cinic acid, L-**val**ine and **oct**amethylene alkyl chain).



Scheme 7. *Reagents and conditions: a) DCC, NHS in THF. 0ºC, 1h. b) octane-1,8-diamine in THF. 50ºC, overnight. c) H_2, 10% Pd/C in MeOH. rt, 4h. d) Succinic anhydride, Na_2CO_3 in THF. rt, overnight. e) 4-aminometil pyridine, TBTU, DIPEA in DMF. rt, overnight.*

The first step was activating the N-protected amino acid ZValOH by coupling, in the presence of DCC, the succinimide group to the carboxylic acid, producing the activated ester ZValOSu. Then, this activated ester and the diamine were coupled to form (ZVal)$_2$Oct, After that, the Z group was removed by hydrogenolysis, using Pd/C as catalyst, and obtaining (HVal)$_2$Oct. Next, this compound was acylated with succinic anhydride in the presence of the base Na_2CO_3, yielding the carboxylic acid (SucVal)$_2$Oct. Finally, 4-aminomethyl pyridine reacted with this carboxylic acid in the presence of TBTU and DIPEA, leading to the final compound (PyrSucVal)$_2$Oct.

The reactions of the four first steps had a yield between 67% and 81%, whereas the last step had a yield of 45%.

3.2 Characterization of $(PyrSucVal)_2Oct$

3.2.1 Nuclear Magnetic Resonance (NMR)

All NMR spectra can be found in section "7. ANNEX", with all the peaks assigned. All of them confirmed the presence of the pure target compound in each step.

Initially the ^{1}H-NMR spectrum ZValOSu, which is shown in *Figure 15*, showed two unwanted peaks corresponding to the by-product dicyclohexylurea (DCU, *Figure 16*). For this reason, it was necessary to recrystallize the compound until these mentioned peaks did not appear.

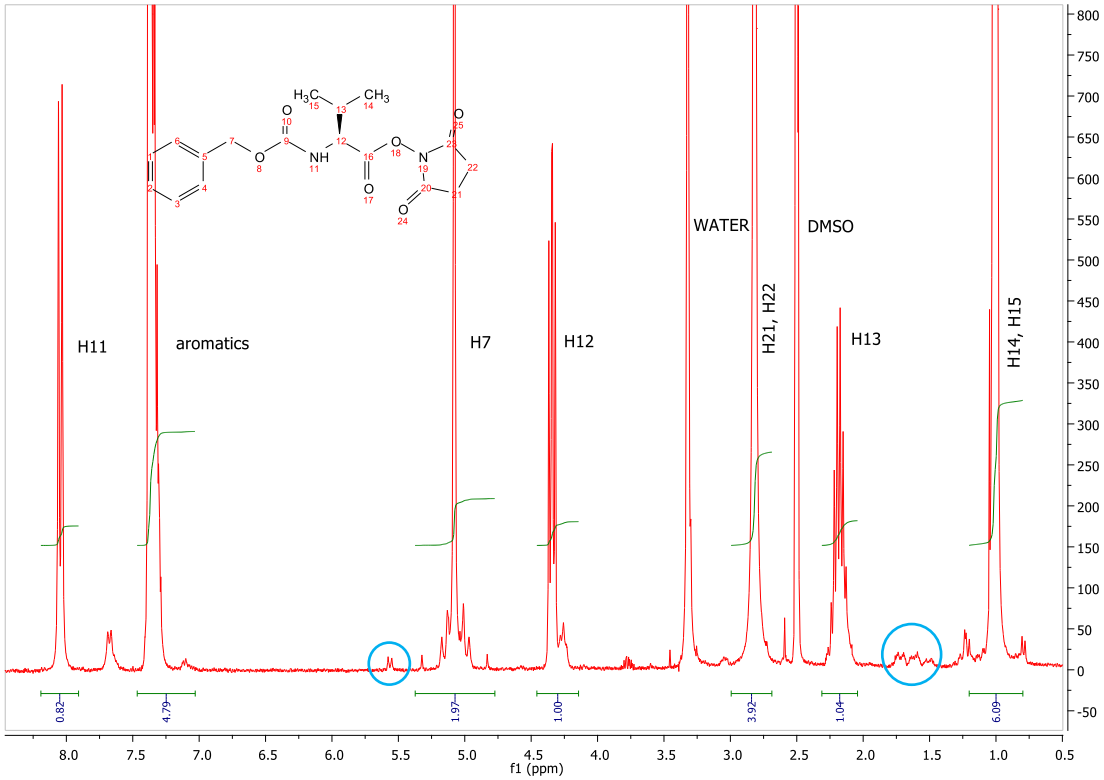


Figure 15. *^{1}H-NMR spectra of ZValOSu containing residual DCU.*

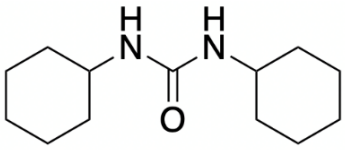


Figure 16. *Structure of dicyclohexylurea (DCU).*

3.2.2 Mass spectrometry

The exact mass calculated for $(PyrSucVal)_2Oct$, whose chemical formula is $C_{38}H_{58}N_8O_6$, is 722.447 and the mass found by high resolution mass spectrometry was 723.4550, corresponding to the protonated cation $[M+H]^+$ (*Figure 17)*.

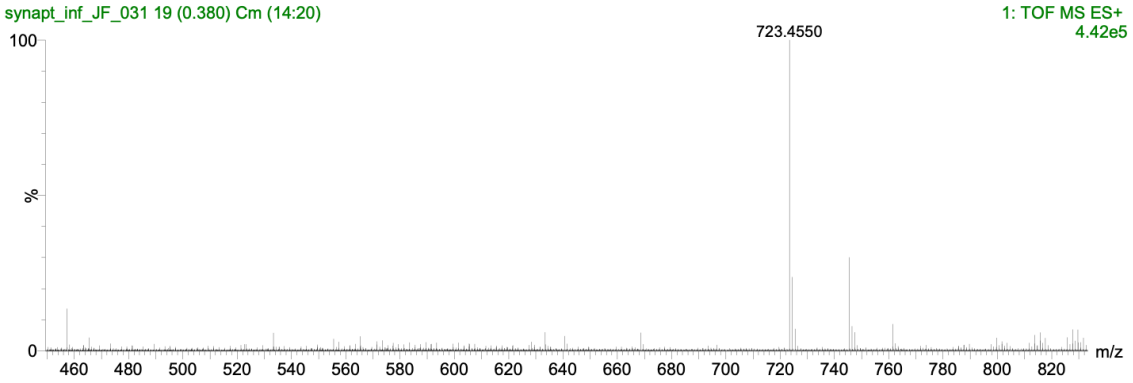
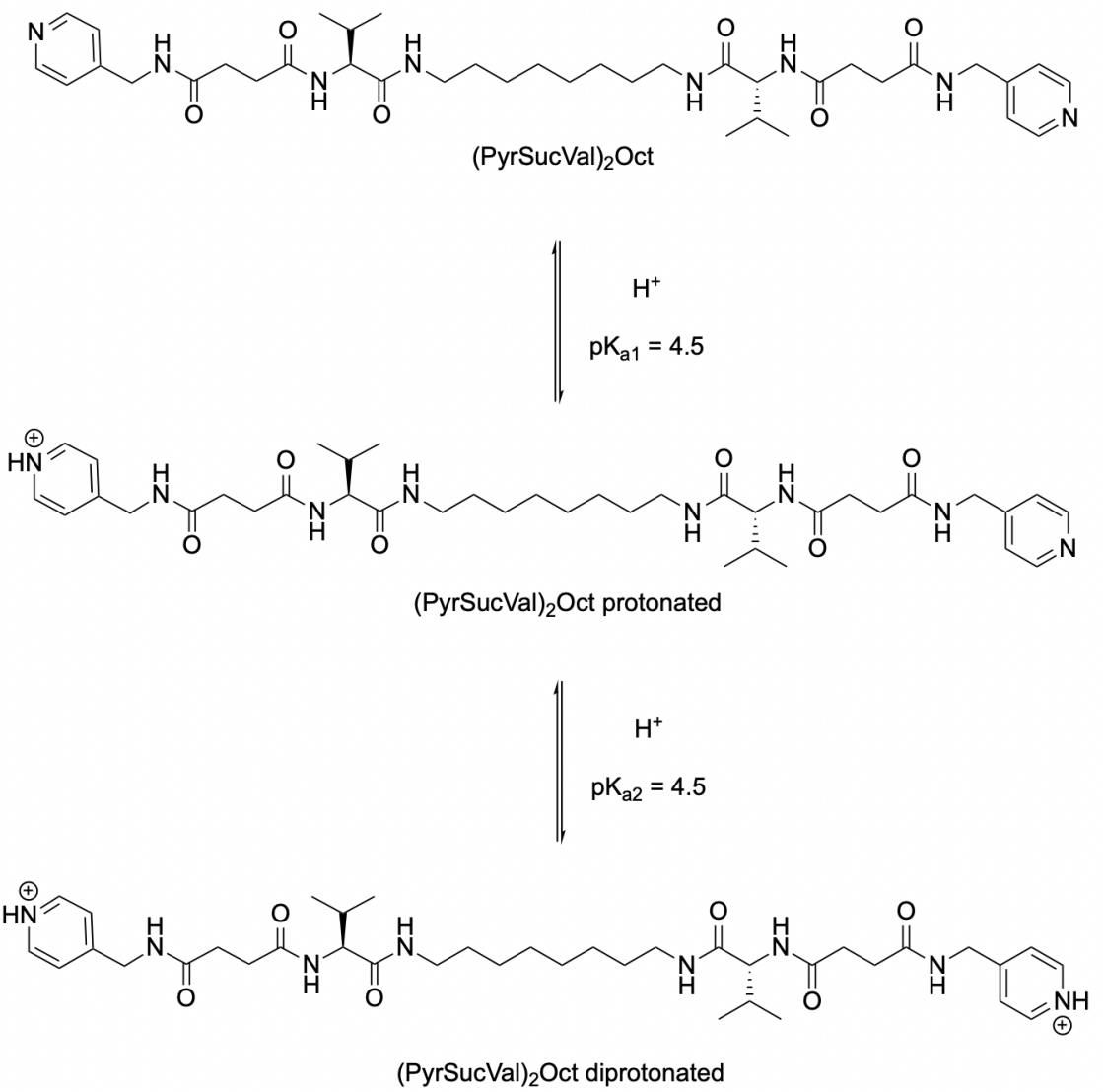


Figure 17. *Mass spectra of $(PyrSucVal)_2Oct$.*

3.2.3 pK_a determination

The determination of the acid-base constants of the two pyridine units of $(PyrSucVal)_2Oct$ was determined by potentiometric titration. The sample was acidified with 0.05 M aqueous HCl and titrated with 0.1M aqueous NaOH. In this way the values of pK_a for the pyridinium species could be calculated. The experiment was carried out in duplicated and the results were fitted iteratively with the HYPERQUAD program to obtain the desired constants. The pK_a values obtained are shown in *Scheme 8*.



Scheme 8. *(PyrSucVal)$_2$Oct protonation equilibrium.*

3.3 Hydrogelation studies

3.3.1 Gelation by heating-cooling

The procedure to determine the minimum gelation concentration (mgc) of (PyrSucVal)$_2$Oct in water starts by weighing the corresponding mass of (PyrSucVal)$_2$Oct, indicated in *Table 2*. Then, the compound was dissolved in 1 mL of water by harsh heating in a closed, pressure-resistant vial. Finally, the system was left to rest at room temperature for 10 minutes to promote hydrogel formation.

Table 2. *Concentration and mass of $(PyrSucVal)_2Oct$ weighed to determine the mgc in water by heating-cooling.*

VIAL	$m_{(PyrSucVal)2Oct}$ (mg)	Concentration (mg/mL)
1	1.1	1.1
2	2.1	2.1
3	3.1	3.1
4	4.2	4.1
5	5.2	5.2

It can be observed that gels (self-supported upon vial inversion) were formed for the samples except that of vial 1 (*Figure 18).* Therefore a value of 1.5 mg mL^{-1} was estimated for the mgc value.

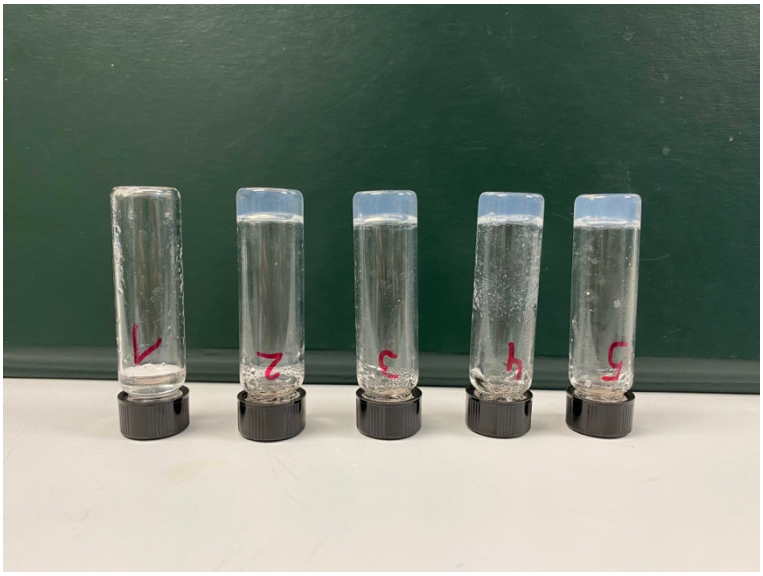


Figure 18. *Determination of mgc of $(PyrSucVal)_2Oct$ in water by the heating-cooling procedure.*

Four assays were performed to evaluate the reproducibility of forming hydrogels at 1.5 mg mL^{-1}, as shown in *Figure 19* in all the cases a consistent gel was obtained.

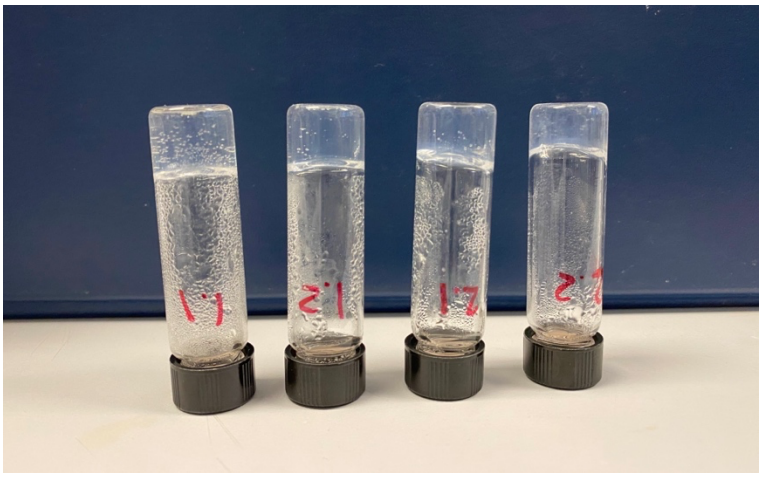


Figure 19. *Reproducibility of hydrogelation using $(PyrSucVal)_2Oct$ at 1.5 mg mL^{-1}. Four assays were carried out.*

To evaluate the reversibility of gel formation, the hydrogel was mechanically disrupted and monitored after one day. No gel was formed after this time, indicating that, in this time scale, the system is not thixotropic. However, upon an additional heating-cooling cycle, the hydrogel was restored.

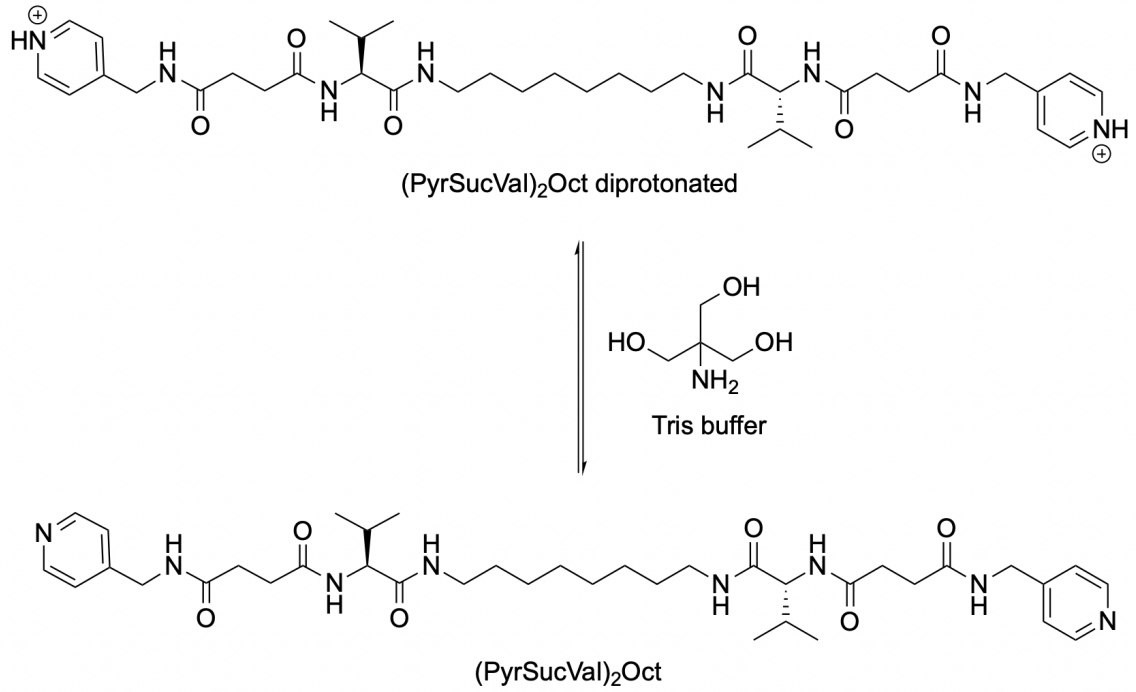
Finally, seven samples were prepared, shown in *Table 3,* to determine the influence of concentration on the gel melting temperature (T_{gel}, the temperature at which the system is no longer self-sustained). All the hydrogels were found to be remarkably stable at 100 ºC, the maximum achievable temperature at atmospheric pressure for hydrogels.

Table 3. *Concentration of vials used to determine the T_{gel} of $(PyrSucVal)_2Oct$ in water by heating-cooling.*

VIAL	Concentration (mg/mL)
1	1.65
2	2.1
3	3.1
4	3
5	4.2
6	5.2
7	7.5

3.3.2 Gelation by abrupt pH change

The mgc of the hydrogels formed by $(PyrSucVal)_2Oct$ in water by abrupt pH change was determined by dissolving the compound in HCl 0.1M and then neutralizing the medium by adding 800 µL of Tris buffer 0.5M, pH = 7. This way, the soluble pyridinium species are transformed into neutral, gel-forming species, as shown in *Scheme 9.*



Scheme 9. *$(PyrSucVal)_2Oct$ neutralization equilibrium with Tris buffer.*

All the samples with a concentration of 1 mg mL^{-1} or higher formed gels (*Table 4, Figure 20*).

Table 4. *Concentration and mass of $(PyrSucVal)_2Oct$ weighed to determine the mgc value in water by abrupt pH change.*

VIAL	$m_{(PyrSucVal)2Oct}$ (mg)	Concentration (mg/mL)
1	1	1
2	1.5	1.5
3	2	2
4	2.5	2.5
5	3	3
6	3.5	3.5
7	4	4
8	5	5

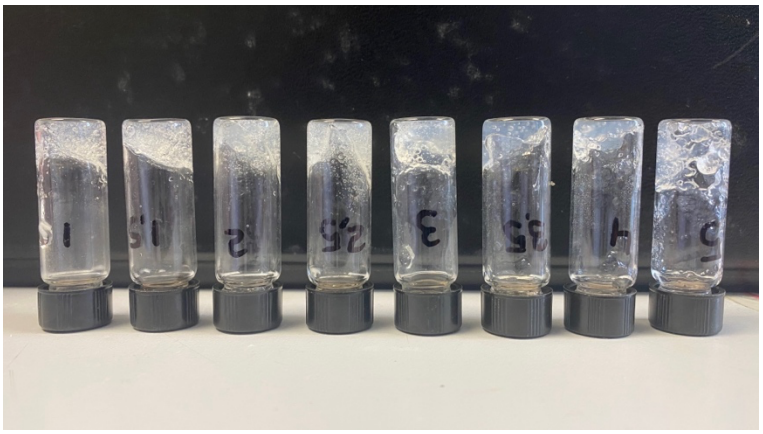


Figure 20. *Determination of mgc of $(PyrSucVal)_2Oct$ in water by abrupt pH change.*

As for thermal stability, reversibility and thixotropic behaviour, the results were similar to those for the hydrogels formed by the heating-cooling procedure.

3.3.3 Gelation by smooth pH change

Samples of 2 mg of $(PyrSucVal)_2Oct$ dissolved in 1 mL HCl 0.1M were exposed to ammonia vapors until basic pH was reached, as shown in *Figure 21*. Gels were formed, as shown in *Figure 22.*

Figure 21. *Procedure to determine if $(PyrSucVal)_2Oct$ forms a gel in water by smooth pH change. The vial with 2 mg of $(PyrSucVal)_2Oct$ dissolved in 1 mL HCl 0.1M is introduced in a closed system containing concentrated aqueous NH_3 25%.*

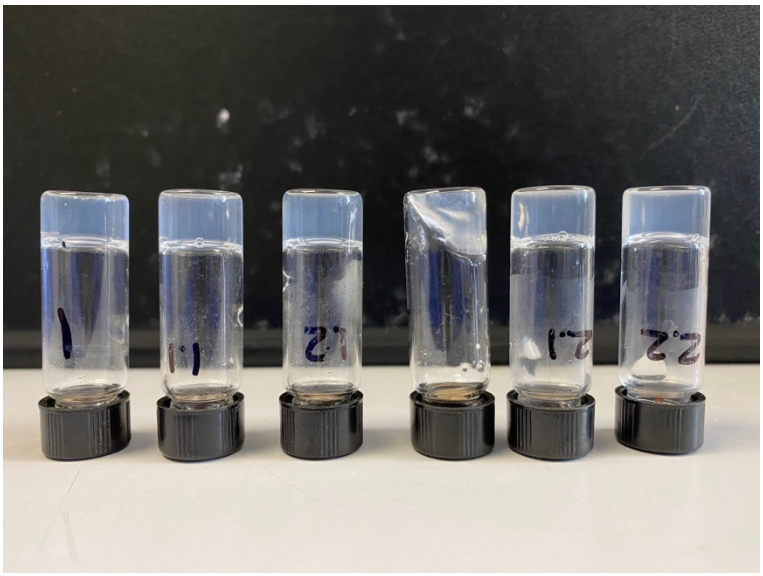


Figure 22. *Assays for the determination of gel formation by (PyrSucVal)$_2$Oct driven by smooth pH change.*

As in the previous case, thermal stability, reversibility and thixotropic behaviour, the results were similar to those for the hydrogels formed by the heating-cooling procedure.

3.4 Transmission Electron Microscopy (TEM)

The microstructure of the xerogels was evaluated by transmission electron microscopy. (*Figure 23*). The samples from the heating-cooling procedure showed agglomerated objects which show fibrillar structure upon magnification. On the other hand, the samples obtained by pH change showed fibrils that are seemingly more interconnected in the sample prepared by smooth pH change than in the one prepared by abrupt pH change.

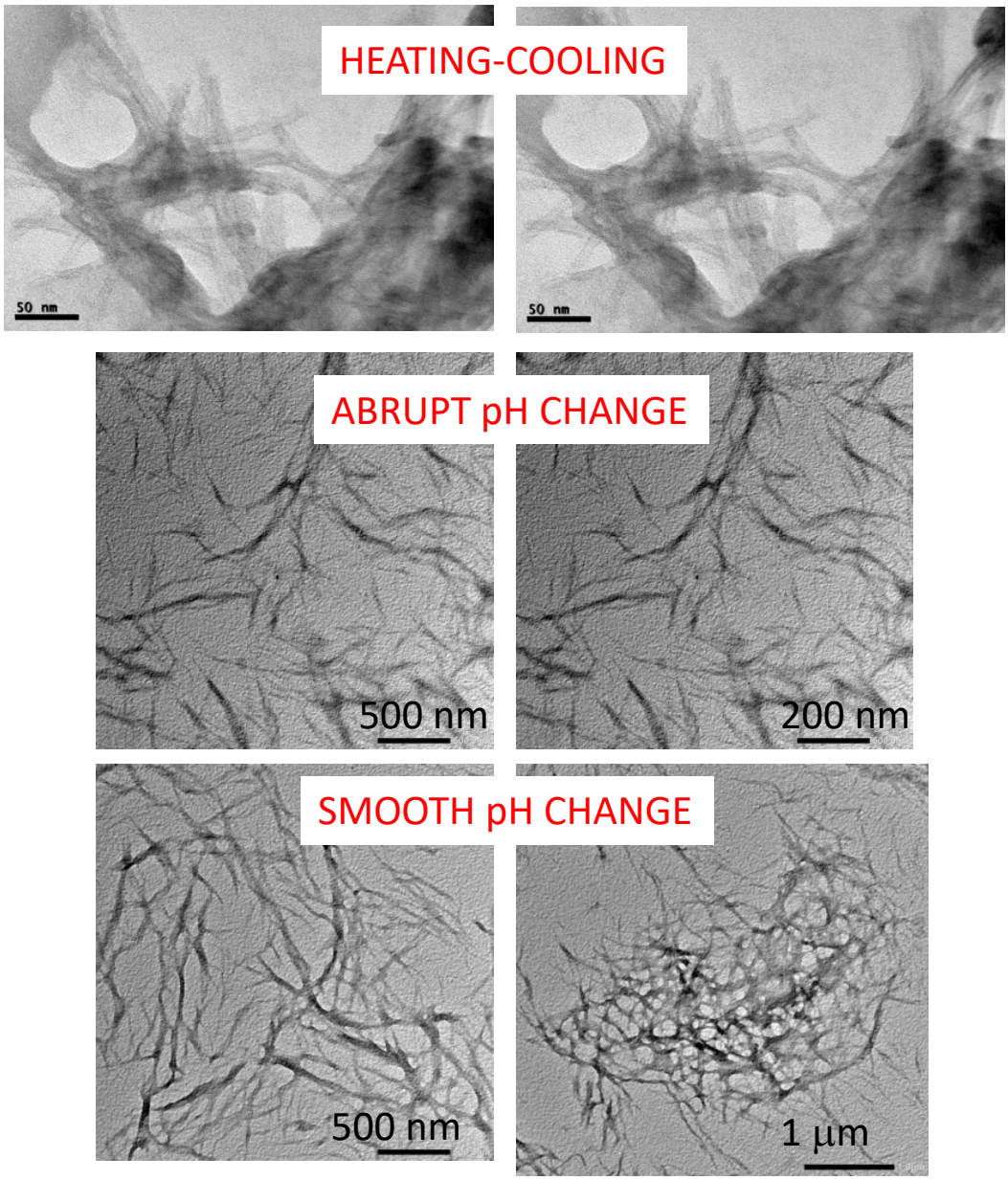


Figure 23. *Transmission electron microscopy (TEM) images obtained from hydrogels prepared by heating-cooling, abrupt pH change and smooth pH change.*

3.5 Rheology

Rheology studies were performed to obtain the elastic modulus (G') and viscous modulus (G") of the gels.

In *Figure 24*, a representation of the moduli obtained for a hydrogel prepared by the heating-cooling procedure. It can be observed that the G' value is above that of G",

indicative of a gel's presence. The loss of linearity at high oscillatory stress values indicates that the gel is starting to become a viscous liquid.

Figure 25 shows the comparison of the elastic modulus G' determined for hydrogels prepared using the three different methodologies described previously. It can be observed that while the gels prepared from pH change show similar strength, the hydrogel from thermal treatment is notably weaker, with a lower G' value.

Finally, the effect of the concentration of the strength of the hydrogel (related to the G' modulus) was studied. For example, as shown in *Figure 26*, for the hydrogels prepared by smooth pH change, there is a clear tendency to higher G' values as the concentration of gelator increases. This behavior is reasonable considering that an increase in concentration results is a denser fibrillar network. Similar results were obtained for the other gelation methods.

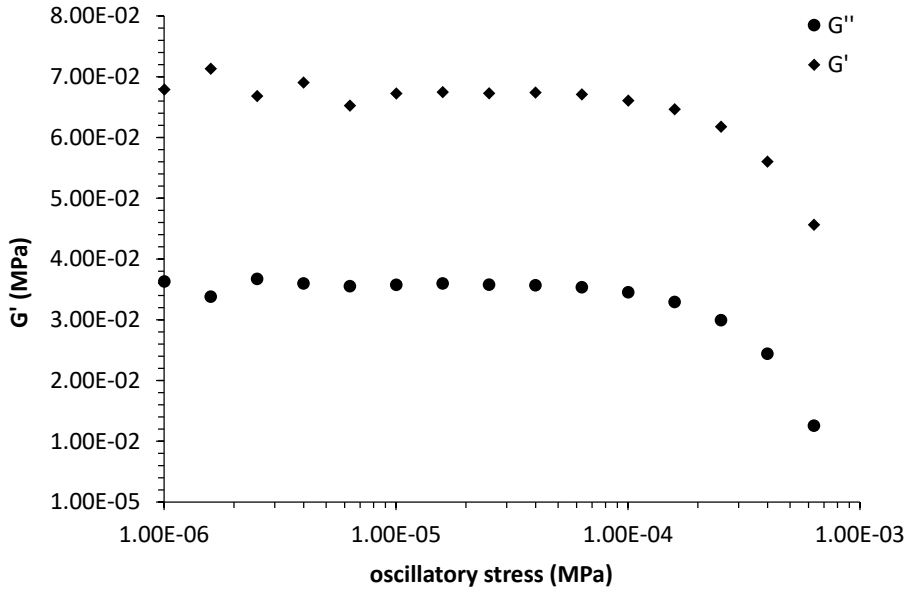


Figure 24. *Rheological study for the hydrogel obtained by the heating-cooling procedure (10 mg/mL).*

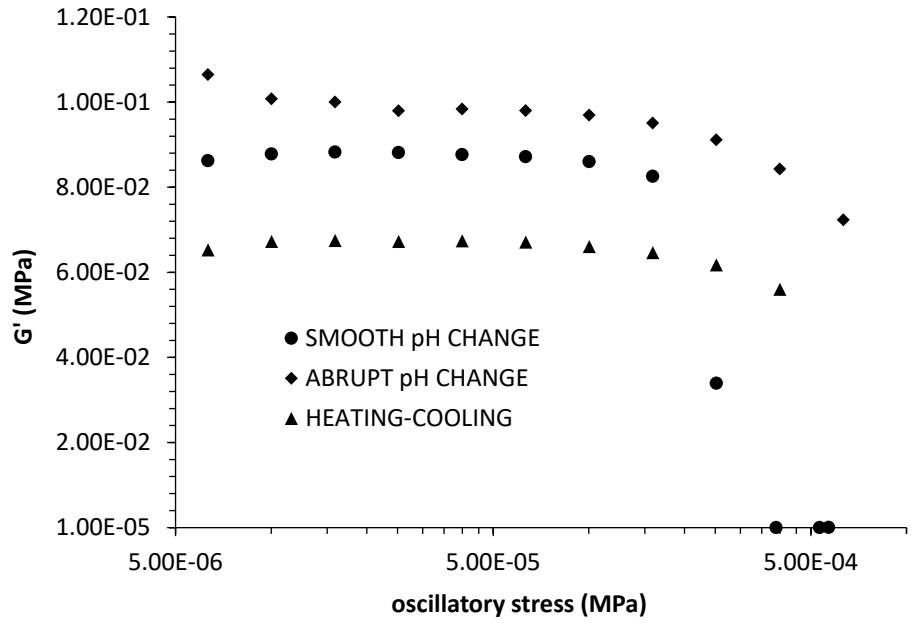


Figure 25. *Rheological study for the hydrogels obtained by three different procedures (10 mg/mL).*

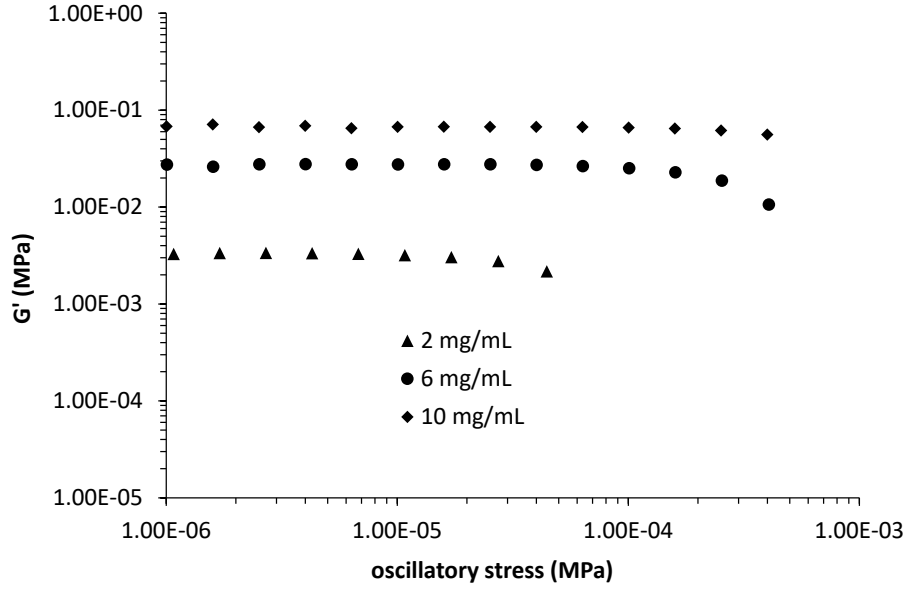


Figure 26. *Rheological study of the effect of gelator concentration for the hydrogels obtained by smooth pH change.*

CONCLUSIONS

4. CONCLUSIONS

Upon completion of this project about $(PyrSucVal)_2Oct$ hydrogels, it is concluded that:

- The synthesis of the compound $(PyrSucVal)_2Oct$ was made successfully, with relatively good yields.
- The NMR spectra and mass spectrometry study of the final compound agreed with the proposed structure.
- The pK_a value of $(PyrSucVal)_2Oct$ is 4.5 for both protonations, a value similar to common pyridine derivatives.
- The following methods yielded hydrogels of $(PyrSucVal)_2Oct$:
  - Heating-cooling
  - Abrupt pH change (addition of buffer Tris (pH = 7.4) to an acidic solution)
  - Smooth pH change (addition of NH_3 (g) to an acidic solution)
- Transmission electron microscopy confirms the presence of fibrillar networks
- Rheological measurements of the G' and G'' moduli confirm that rheologically speaking, the materials formed are gels.
- The strength of the gels (G') depends on the concentration of the gelator and the method used for their preparation.

EXPERIMENTAL PROCEDURE

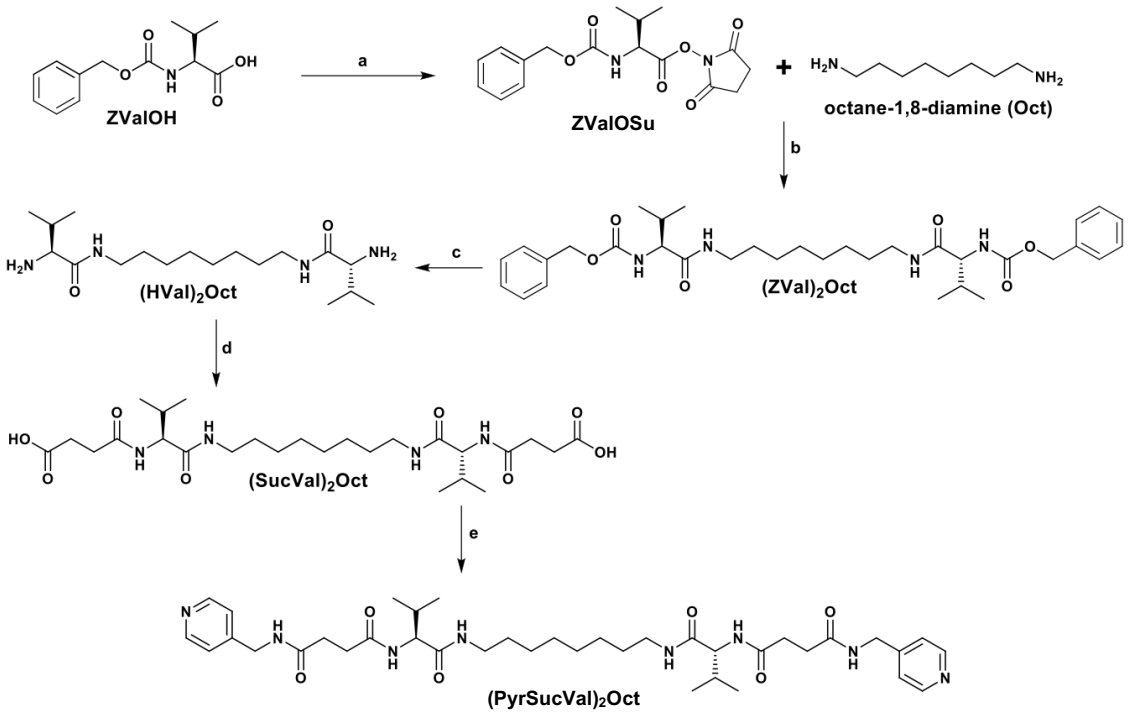
5. EXPERIMENTAL PROCEDURE

5.1 General methods

NMR $^{1}H/^{13}C$ spectra were recorded at 300/75 MHz in the indicated solvent at 30ºC. Signals of the deuterated solvent (DMSO-d_6 for ZValOSu, $(ZVal)_2Oct$, $(HVal)_2Oct$ and $(SucVal)_2Oct$, D_2O for $(PyrSucVal)_2Oct$) were taken as the reference in DMSO-d_6, the singlet at δ 2.50 ppm and in D_2O, the singlet at δ 4.79 ppm for ^{1}H-NMR. ^{1}H y ^{13}C signals were assigned with the aid of 2D methods (COSY, HSQC and HMBC).

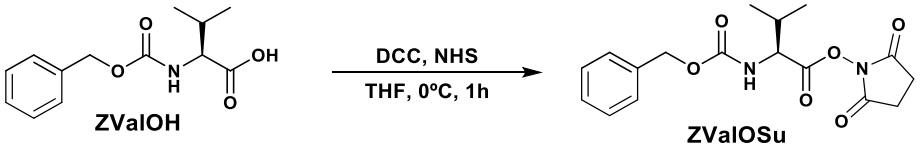
Reactions that required an inert atmosphere were carried out under N_2. Commercially available reagents were used as received.

5.2 Synthesis of $(PyrSucVal)_2Oct$



Scheme 10. *Reagents and conditions: a) DCC, NHS in THF. 0ºC, 1h. b) THF. 50ºC, overnight. c) H_2, 10% Pd/C in MeOH. rt, 4h. d) Succinic anhydride, Na_2CO_3 in THF. rt, overnight. e) 4-aminomethylpyridine, TBTU, DIPEA in DMF. rt, overnight.*

5.2.1 Activation of the amino acid



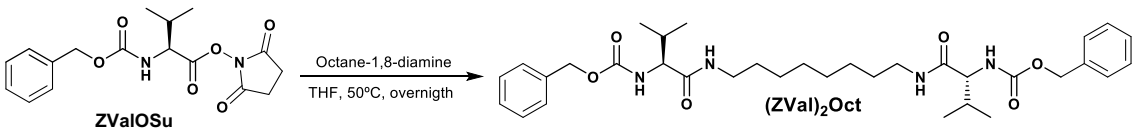
Scheme 11. *Amino acid activation reaction.*

A solution of *N,N'*-dicyclohexylcarbodiimide (DCC, 20.09 mmol, 1.01 eq.) in THF (20 mL) was added dropwise, under N_2 at 0ºC, to a solution of the commercial available carbobenzyloxy-*L*-valine (**ZValOH**, 19.89 mmol) and N-hydroxysuccinimide (NHS, 20.09 mmol, 1.01 eq.) in THF (50 mL). The mixture was stirred for 1h at 0ºC. Then the solution was placed in the refrigerator overnight to cause the precipitation of N,N'-dicyclohexylurea (DCU). After this time, the mixture was filtered under vacuum. The filtrate was concentrated in the rotary evaporator and left in vacuum overnight to remove the remaining THF. After this time, hot isopropanol was added and was allowed to rest and crystallize to obtain the pure compound. Once crystals were formed, the mixture was filtered under vacuum and the precipitate was dried in the stove at 54ºC.

*2,5-dioxopyrrolidin-1-yl ((benzyloxy)carbonyl)-L-valinate (**ZValOSu**):* A previously recrystallized white solid was obtained (yield 72%).

^{1}H-NMR (300 MHz, DMSO-d_6): 8.05 (d, *J* = 8.3 Hz, 1H), 7.34 (dt, *J* = 8.3, 3.8 Hz, 5H), 5.08 (s, 2H), 4.32 (dt, *J* = 24.9, 12.5 Hz, 1H), 2.82 (s, 4H), 2.32 – 2.01 (m, 1H), 1.02 (d, *J* = 8.3 Hz, 6H).

5.2.2 Coupling between activated ester and amine



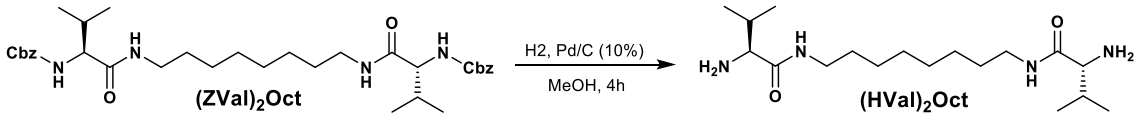
Scheme 12. *Ester-amine coupling reaction.*

A solution of the activated ester **ZValOSu** (26.22 mmol, 2 eq.) in THF (60 mL) was added dropwise, under N_2 at 50ºC, to a solution of octane-1,8-diamine (13.11 mmol) in THF (15 mL). The mixture was stirred for 24h at 50ºC. After this time, the obtained reaction mixture was concentrated in the rotary evaporator and left in vacuum for 24h. After this time, the mixture was washed 3 times: first with base (NaOH 0.5M) to remove the remaining reagent ZValOSu, then with acid (HCl 0.5M) to remove the remaining amine and finally with neutral medium to remove the excess of acid. Then the precipitate was dried in the stove at 54ºC.

Benzyl ((5R,18S)-5-isopropyl-19-methyl-3,6,17-trioxo-1-phenyl-2-oxa-4,7,16-triazaicosan-18-yl)carbamate (**$(ZVal)_2Oct$**): A white solid was obtained (yield 81%).

^{1}H-NMR (300 MHz, DMSO-d_6): 7.86 (t, *J* = 5.1 Hz, 2H), 7.32 (dt, *J* = 10.8, 4.2 Hz, 10H), 7.19 (d, *J* = 8.9 Hz, 2H), 5.02 (s, 4H), 3.89 – 3.69 (m, 2H), 3.02 (dtd, *J* = 25.5, 12.8, 6.5 Hz, 4H), 1.91 (dq, *J* = 13.4, 6.6 Hz, 2H), 1.37 (s, 4H), 1.23 (s, 8H), 0.84 (d, *J* = 6.7 Hz, 12H).

5.2.3 Deprotection of carbobenzyloxy group



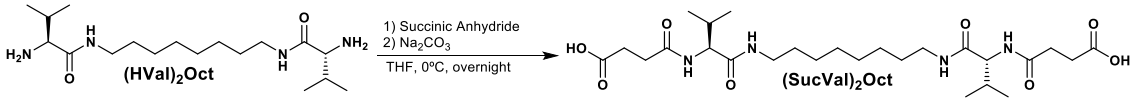
Scheme 13. *Deprotection of Z group reaction.*

Palladium on carbon catalyst (10% w/w) was suspended in a solution of **$(ZVal)_2Oct$** (10.54 mmol) in MeOH (100 mL) and stirred, under H_2 at room temperature, for 4h until the solution turned black. The reaction mixture was filtered under vacuum through Celite®, and the filtrate was concentrated in the rotary evaporator and left in vacuum for 24h to remove the remaining solvent.

(S)-2-amino-N-(8-((R)-2-amino-3-methylbutanamido)octyl)-3-methylbutanamide (**$(HVal)_2Oct$**): A white solid was obtained (yield 78%).

^{1}H-NMR (300 MHz, DMSO-d_6): 7.77 (t, *J* = 5.3 Hz, 2H), 3.14 – 2.96 (m, 4H), 2.88 (d, *J* = 5.1 Hz, 2H), 1.83 (dh, *J* = 13.4, 6.7 Hz, 2H), 1.55 (s, 4H), 1.45 – 1.32 (m, 4H), 1.24 (s, 8H), 0.93 – 0.71 (m, 12H).

5.2.4 Obtention of the carboxylic acid



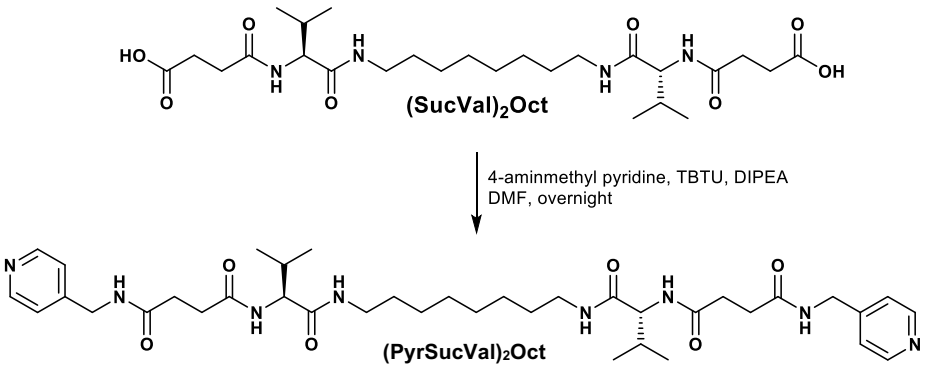
Scheme 14. *Obtention of the carboxylic acid reaction.*

Na_2CO_3 solid (62.74 mmol, 7.6 eq.) was added to a solution of **(HVal)$_2$Oct** (8.20 mmol) in THF (170 mL), and then a solution of succinic anhydride (32.82 mmol, 4 eq.) in THF (120 mL) was added dropwise, at 0ºC. The mixture was stirred for 24h at 0ºC. After this time, the reaction mixture was concentrated in the rotary evaporator and left in vacuum for 24h to remove the remaining solvent. Once the product is dry, distilled water (200 mL) and a few drops of HCl conc. were added until the solution turned white and pH was similar to 1. Then, chloroform (300 mL) was added and, as precipitate appeared, the solution was filtered under vacuum. The precipitate was dried in the stove at 54ºC.

(6R,19S)-6,19-diisopropyl-4,7,18,21-tetraoxo-5,8,17,20-tetraazatetracosanedioic acid (**(SucVal)$_2$Oct**): A white/yellow solid was obtained (yield 67%).

^{1}H-NMR (300 MHz, DMSO-d_6): 12.10 (s, 2H), 7.89 (d, *J* = 8.2 Hz, 4H), 4.17 – 3.99 (m, 2H), 3.20 – 2.87 (m, 4H), 2.47 – 2.29 (m, 8H), 1.94 (dq, *J* = 13.4, 6.6 Hz, 2H), 1.38 (s, 4H), 1.23 (s, 8H), 0.82 (d, *J* = 6.7 Hz, 12H).

5.2.5 Preparation of the final compound



Scheme 15. *Obtention of the final compound reaction.*

TBTU (11.19 mmol, 2.1 eq.), 4-aminometil pyridine (10.74 mmol, 2 eq.) and DIPEA (11.25 mmol, 2.1 eq.) were added sequentially to a solution of **(SucVal)$_2$Oct** (5.36 mmol) in DMF (25 mL), under H_2 at room temperature. The mixture was stirred for 24h. After this time, water was added to the solution until precipitate appeared, then it was filtered by vacuum. The precipitate was washed 3 more times with distilled water and a few drops of NaOH 1M to remove the remaining reagents. The precipitate was dried in the stove at 54ºC.

N^1,$N^{1'}$-((2S,2'S)-(octane-1,8-diylbis(azanediyl))bis(3-methyl-1-oxobutane-1,2-diyl))bis(N^4-(pyridin-4-ylmethyl)succinamide) (**(PyrSucVal)$_2$Oct**): A grey solid was obtained (yield 45%).

^{1}H-NMR (300 MHz, D_2O): 8.43 (d, *J* = 6.3 Hz, 4H), 7.66 (d, *J* = 6.0 Hz, 4H), 4.40 (s, 4H), 3.73 (d, *J* = 6.9 Hz, 2H), 2.80 (d, *J* = 26.4 Hz, 4H), 2.40 (s, 8H), 1.78 (dd, *J* = 13.8, 7.2 Hz, 2H), 1.14 (s, 4H), 0.92 (s, 8H), 0.62 (d, *J* = 6.4 Hz, 12H).

^{13}C-NMR (75 MHz, D_2O): 175.3 (x2), 174.8 (x2), 173.2 (x2) (C=O), 160.3 (x2) (C), 140.6 (x4), 124.8 (x4), 59.7 (x2) (CH), 42.3 (x2), 39.0 (x2), 30.18 (d, *J* = 12.7 Hz, x4) (CH_2), 29.6 (x2) (CH), 27.9 (x4), 25.6 (x2) (CH_2), 18.2 (x2), 17.2 (x2) (CH_3).

HR ESMS: *m/z*: calculated for $C_{38}H_{58}N_8O_6$: 722.447; found: 722.447 [M - H^+].

5.3 pK_a determination

Potentiometric titrations to determine acid-base constants were carried out at 298 K for three times. In the experiments, 7 mL of acid solution of the $(PyrSucVal)_2Oct$ (12 mg) and HCl 0.05M were titrated with normalized dissolution NaOH 0.1M commercially available and vigorous stirring. The base was added with an NE-300 "Just Infusion" TM Syringe Pump (0,033 mL/min-inside diameter 14.57 mm) using a SGE Analytical Science syringe 10 mL which had connected a needle of stainless steel cono. Luer. Look 0.7mm x 300mm. The pH was monitored every 8s (in a S220 Seven Compact pHmeter, Mettler Toledo).
Thermodynamic constants for the species in solution could be calculated with HYPERQUAD using titration data previous to the experimental aggregation onset. Then the solubility product was calculated iteratively with HYSS2009, adjusting its value to fit the calculated and experimental pH.

5.4 Gelation methods

5.4.1 Gelation by heating-cooling

To determine the minimum gelation concentration of $(PyrSucVal)_2Oct$ in water by heating-cooling, a quantity of $(PyrSucVal)_2Oct$ was weighted inside vials, 1 mL of distilled water was added and the vials were sonicated to dissolve the compound. After that, the vials were heated with a hot air gun at 200ºC, and they were allowed to cool down.
To determine if gels are resettable, the vials that formed a gel were heated again until a liquid was obtained and they were allowed to cool down again.
To determine if gels are thixotropic, the vials that formed a gel were vortexed and allowed to rest.
Finally, to determine the gelation temperature of the gels, vials were heated in Drybath Standard, 1 Block, Thermo Scientific at different temperatures, starting at 30ºC and increasing the temperature by 10ºC until 50ºC and from there by 5ºC.

5.4.2 Gelation by abrupt pH change

To determine the minimum gelation concentration of $(PyrSucVal)_2Oct$ in water by abrupt pH change, a quantity of $(PyrSucVal)_2Oct$ was weighted inside vials, 200 µL of HCl

0.1M were added and the vials were sonicated to dissolve the compound. After that, 800 µL of buffer Tris 0.5M were added quickly.

To determine if gels are thixotropic, the vials that formed a gel were vortexed and allowed to rest.

Finally, to determine the gelation temperature of the gels, vials were heated in Drybath Standard, 1 Block, Thermo Scientific at different temperatures, starting at 30ºC and increasing the temperature by 10ºC until 50ºC and from there by 5ºC.

5.4.3 Gelation by smooth pH change

To determine if $(PyrSucVal)_2Oct$ can form gels in water by smooth pH change, 2 mg of $(PyrSucVal)_2Oct$ were weighted inside vials, 1 mL of HCl 0.1M was added and the vials were sonicated to dissolve the compound. After that, the open vials were introduced into a larger closed vial containing 2 mL of NH_3 25% and they were allowed to rest.

To determine if gels are thixotropic, the vials that formed a gel were vortexed and allowed to rest.

Finally, to determine the gelation temperature of the gels, vials were heated in Drybath Standard, 1 Block, Thermo Scientific at different temperatures, starting at 30ºC and increasing the temperature by 10ºC until 50ºC and from there by 5ºC.

5.5 Mass Spectrometry

Mass spectra was run by the electro-spray mode (ESMS). Masses spectra was recorded at Mass Spectrometry triple Quadrupole Q-TOF Premier (Waters) with simultaneous Electrospray and APCI Probe.

5.6 Transmission Electron Microscopy (TEM)

Transmission electron microscopy micrographs were taken on a JEOL 2100 microscope equipped with a camera CCD (11 MP). The corresponding fresh gels were applied directly onto a 200 mesh carbon coated copper grids. Excess solvent was carefully removed by capillary action using filter paper.

5.7 Rheology

Rheological measurements were carried out on Discovery Hr-1 Rheometer from TA Instruments performing oscillatory experiments with a plate-plate configuration (40 mm diameter). The gap distance was fixed at 0.5 mm. The hydrogels were prepared under the desired conditions and aged for 24h. Typically, 3 mL of hydrogel were spread with a spatula on the rheometer plate. After 5 minutes, frequency and stress sweep steps were performed at 25˚C. All the measurements were carried out within the linear viscoelastic regime. For this purpose the experimental conditions to achieve a linear viscoelastic regime (LVR) were determined by running a stress sweep step (oscillatory Stress 0.5-500 Pa at 1 Hz) and a frequency sweep step (0.1-10 Hz at 1 Pa). The storage and loss modulus independence with frequency and oscillatory stress applied defined the linear viscoelastic regime. All the measurements were at least duplicated.

REFERENCES

6. REFERENCES

[1] Panja, S., & Adams, D. J. *Chem. Soc. Rev.*, 2021, 50, 5165

[2] Yu, G., Yan, X., Han, C., & Huang, F. *Chem. Soc. Rev.,* 2013, 42, 6697

[3] Mayr, J., Saldías, C., & Díaz Díaz, D. *Chem. Soc. Rev.*, 2018, 47, 1484

[4] Falcone, N., & Kraatz, H. B. *Chem. Eur. J.,* 2018, 24, 14316

[5] Ibrahim, M. M., Hafez, S. A., & Mahdy, M. M. *Asian J. Pharm. Sci.*, 2013, 8, 48

[6] Choe, R. & Il Yun, S. *e-Polymers*, 2020, 20, 458

[7] Chakroun, R. W., Wang, F., Lin, R., Wang, Y., Su, H., Pompa, D., & Cui, H. *ACS Nano*, 2019, 13, 7780

[8] Yang, Z., Liang, G., Wang, L., & Xu, B. J. *Am. Chem. Soc.,* 2006, 128, 3038

[9] Panja, S., Fuentes-Caparros, A. M., Cross, E. R., Cavalcanti, L., & Adams, D. J. *Chem. Mater.,* 2020, 32, 5264

[10] Isidro-Llobet, A., Álvarez, M., & Albericio, F. *Chem. Rev.,* 2009, 109, 2455

[11] Omprakash Rathi, J., & Subray Shankarling, G. *ChemistrySelect*, 2020, 5, 6861

[12] El-Faham, A., & Albericio, F. *Chem. Rev.,* 2011, 111, 6557

[13] Han, S.-Y., & Kim, Y.-A. *Tetrahedron,* 2004, 60, 2447

[14] Bastiaans, H. M., van der Baan, J. L., & Ottenheijm, H. C. *J. Org. Chem.,* 1997, 62, 3880

[15] Vrettos, E. I., Sayyad, N., Mavrogiannaki, E. M., Stylos, E., Kostagianni, A. D., Papas, S., Mavromoustakos, T., Theodorou, V., & Tzakos, A. G. *RSC Adv.,* 2017, 7, 50519

[16] Yan, C., & Pochan, D. J. *Chem. Soc. Rev.,* 2010, 39, 3528

[17] Hashemnejad, S. M., Badruddoza, A. Z. M., & Zarket, B., *et. al. Nat Commun*, 2019, 10, 2749

[18] Nebot, V. J., Armengol, J., Smets, J., Prieto, S. F., Escuder, B., & Miravet, J. F. *Chem. Eur. J.,* 2012, 18, 4063

[19] Angulo-Pachón, C. A., & Miravet, J. F. *Chem. Commun.,* 2016, 52, 5398

[20] Angulo-Pachón, C. A., Díaz-Oltra, S., Ojeda-Flores, J. J., Falomir, E., Galindo, F., & Miravet, J. F. *ChemNanoMat*, 2018, 4, 769

ANNEX

7. ANNEX

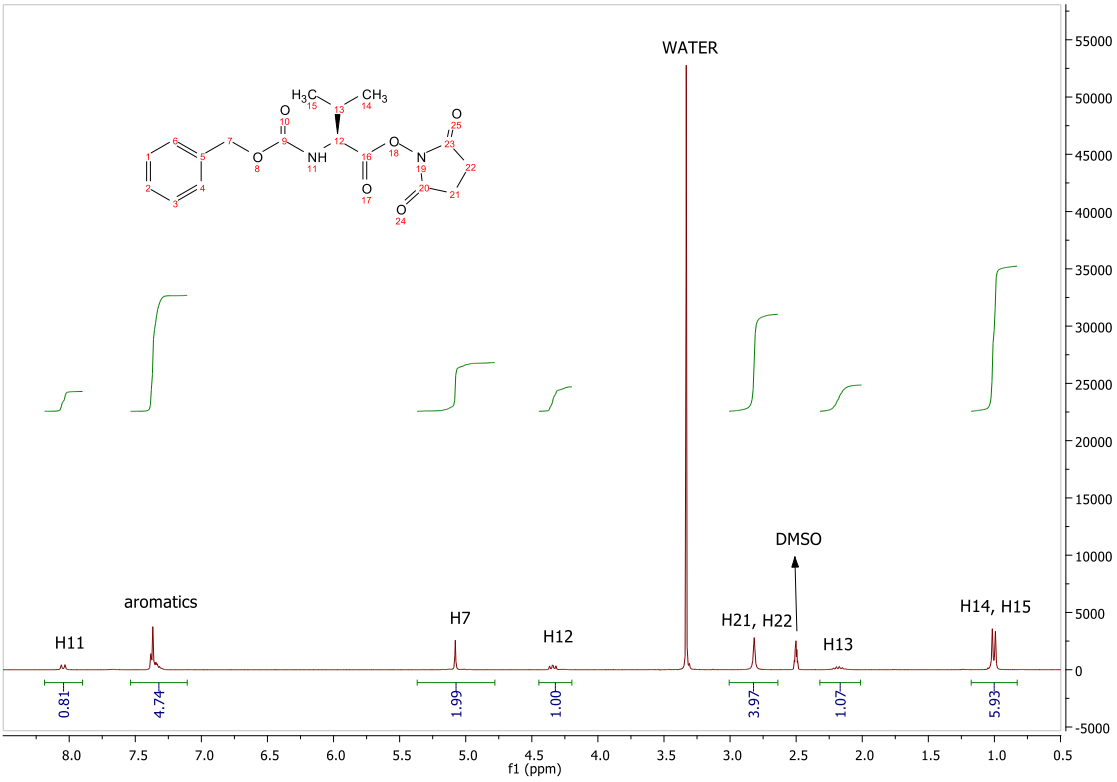


Figure 27. *^{1}H-NMR spectra of recrystallized ZValOSu.*

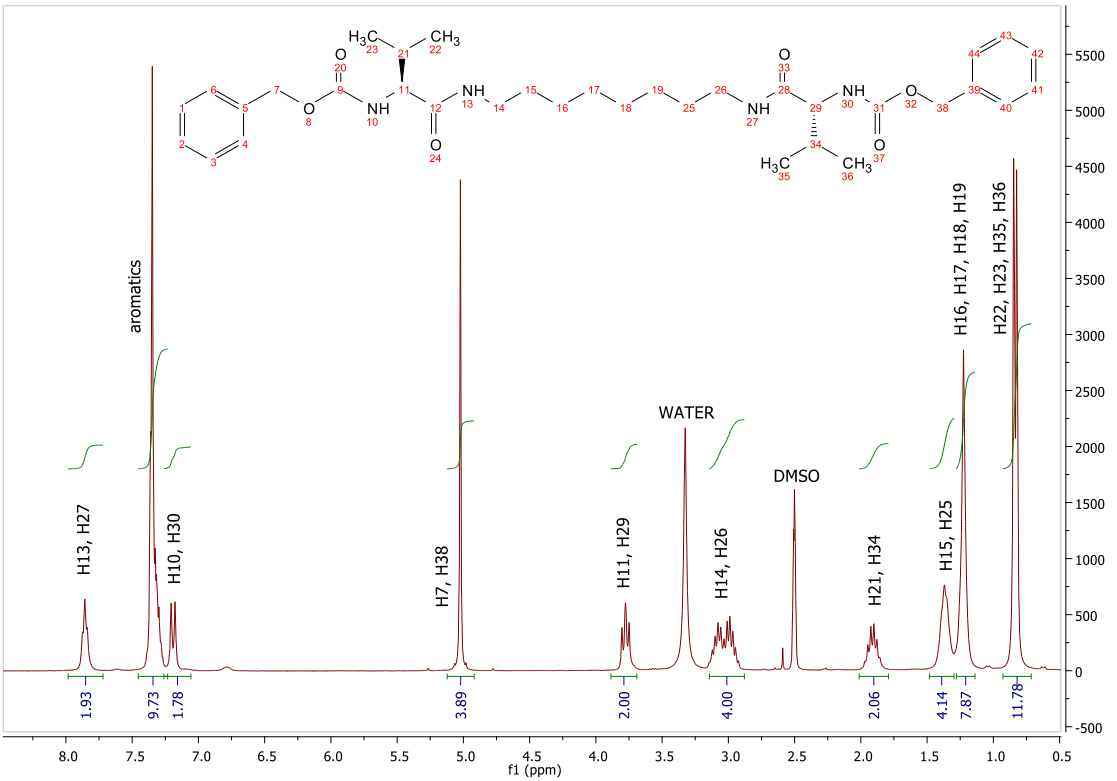


Figure 28. *^{1}H-NMR spectra of (ZVal)$_2$Oct.*

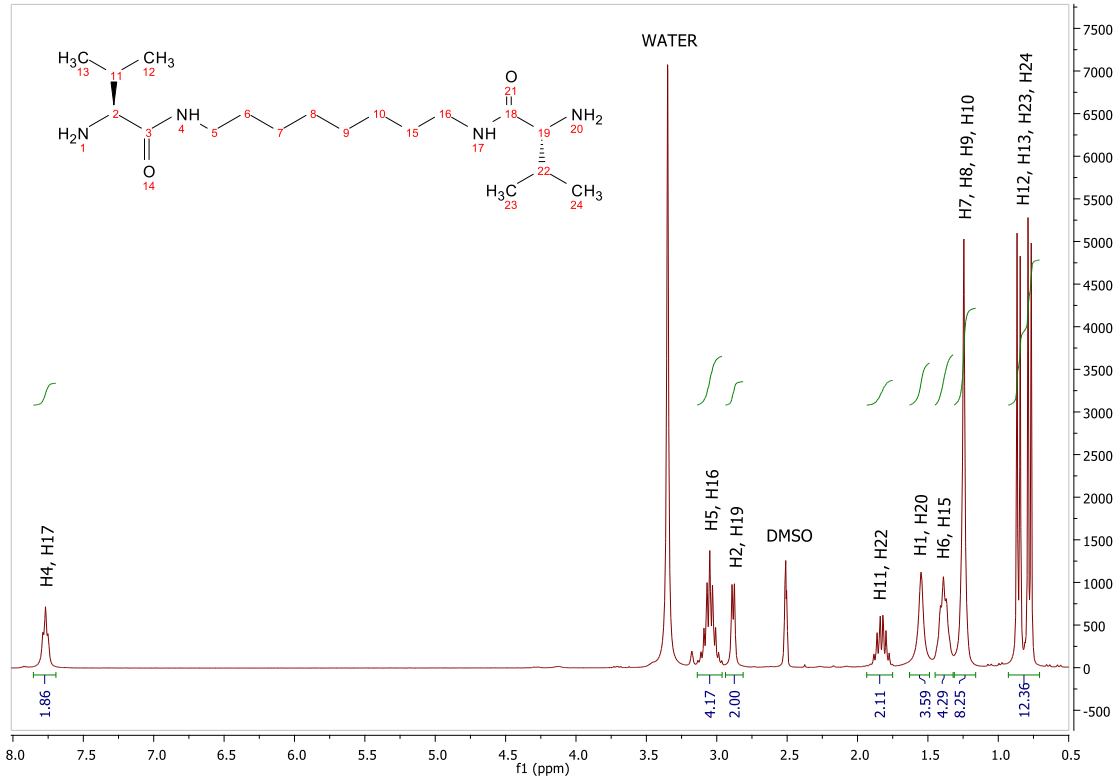


Figure 29. *^{1}H-NMR spectra of $(HVal)_2Oct$.*

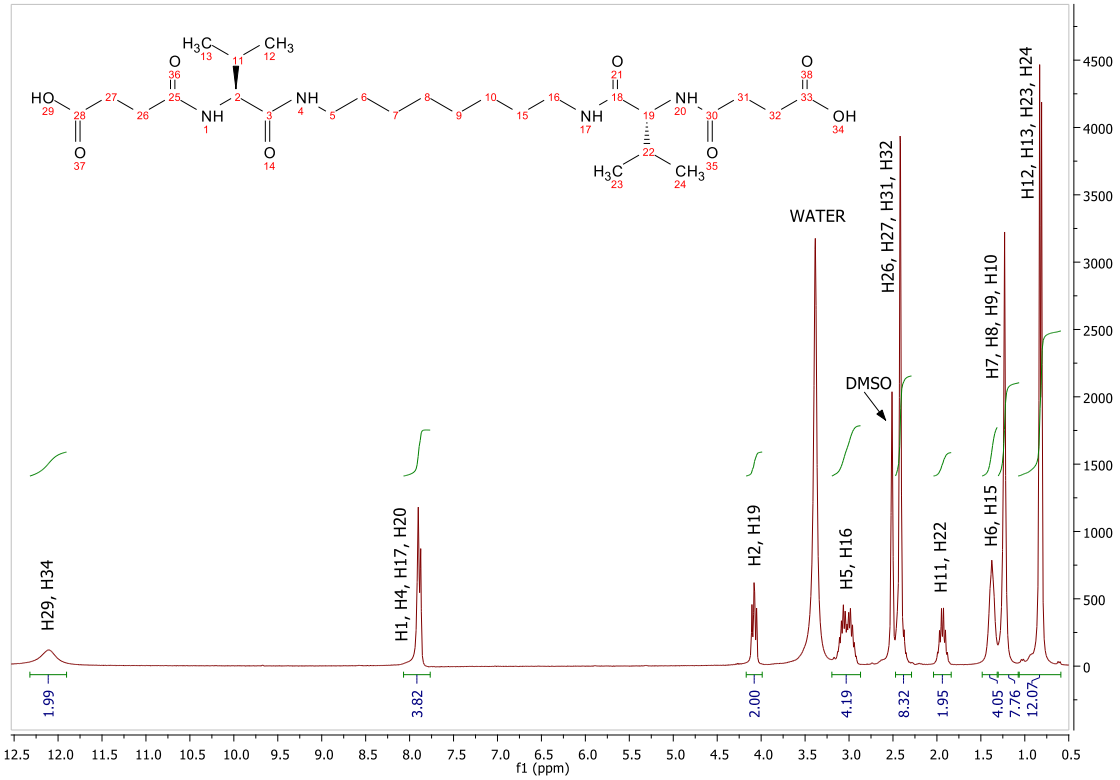


Figure 30. *^{1}H-NMR spectra of $(SucVal)_2Oct$.*

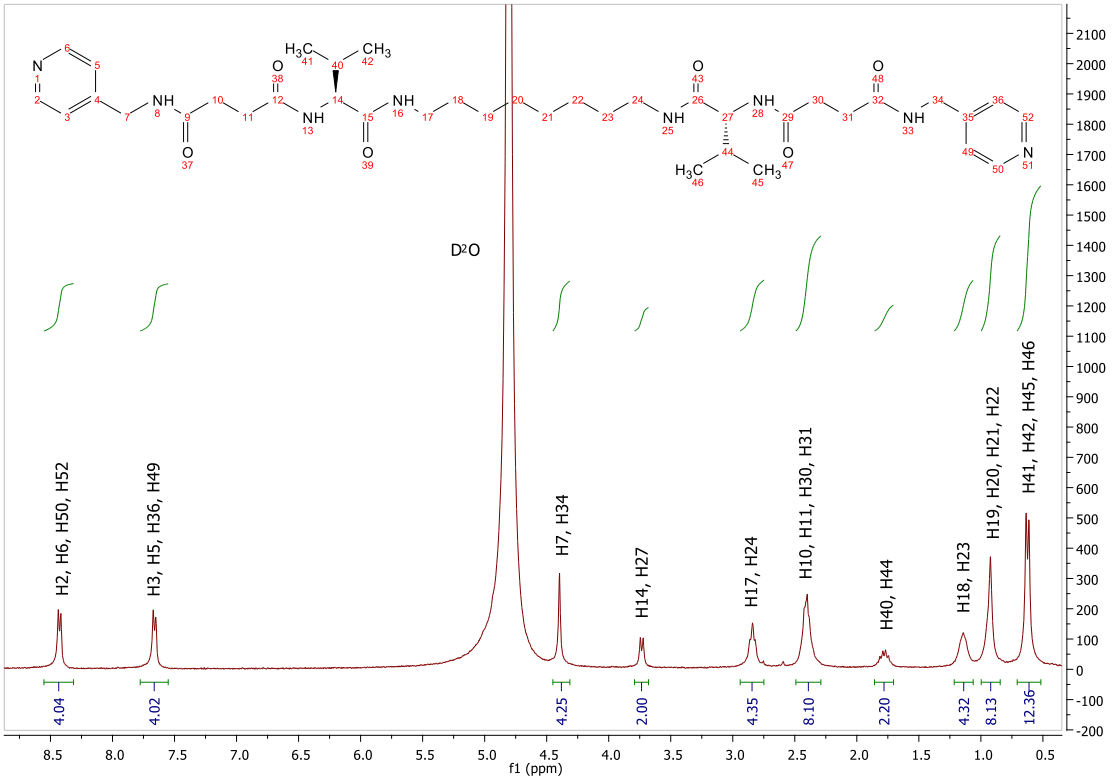


Figure 31. *^{1}H-NMR spectra of $(PyrSucVal)_2Oct$.*

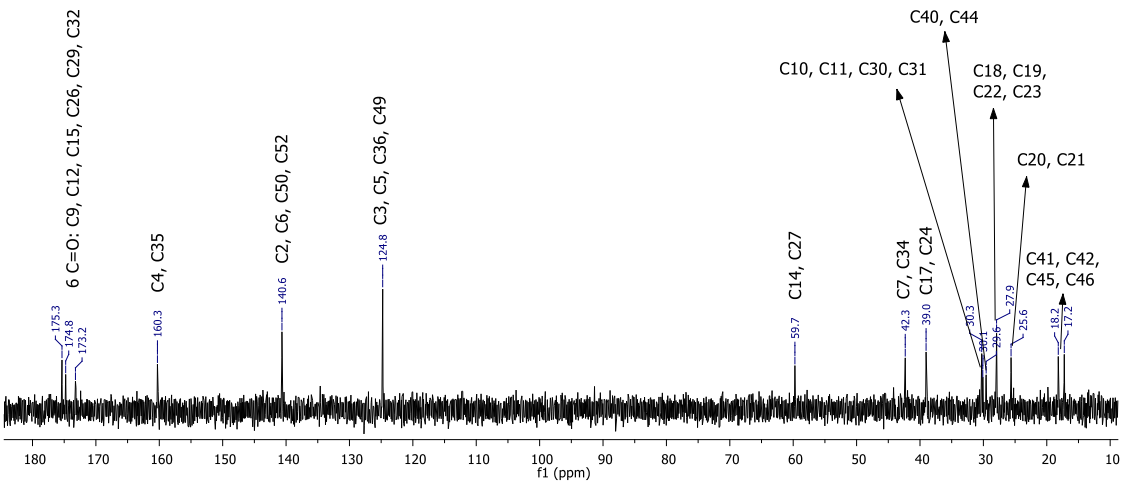


Figure 32. *^{13}C-NMR spectra of $(PyrSucVal)_2Oct$.*

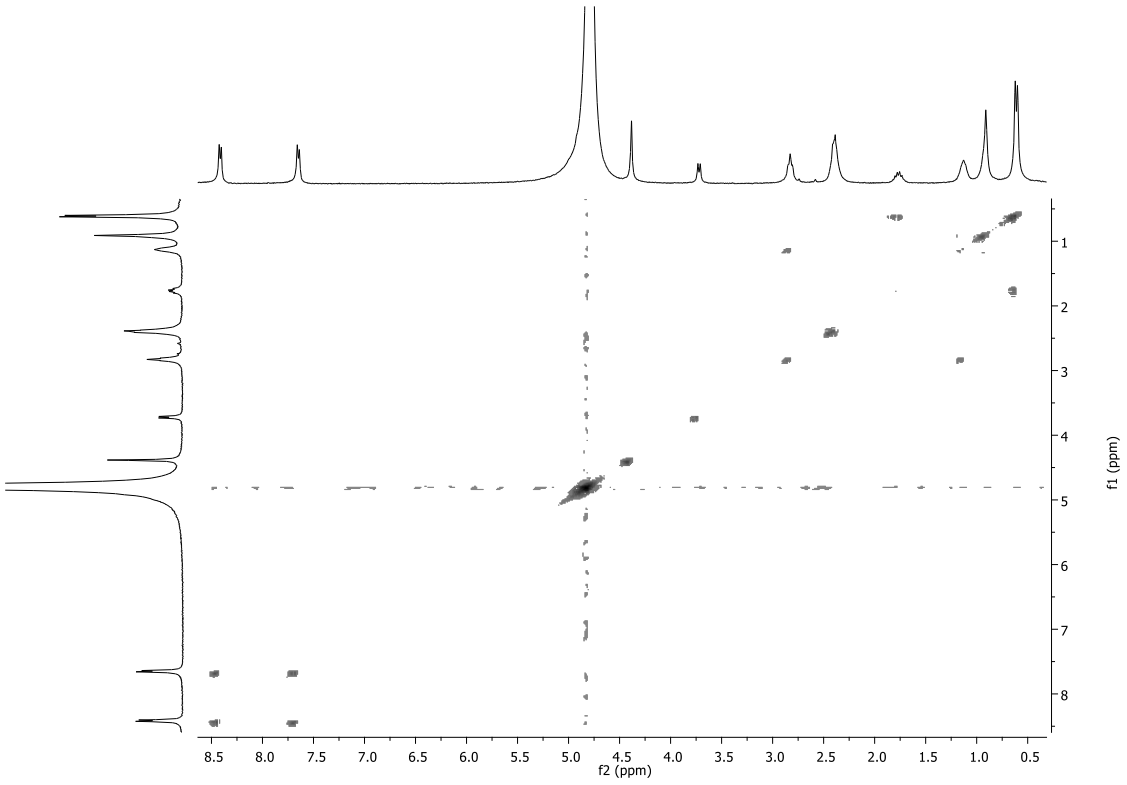


Figure 33. *COSY-NMR spectra of (PyrSucVal)$_2$Oct.*

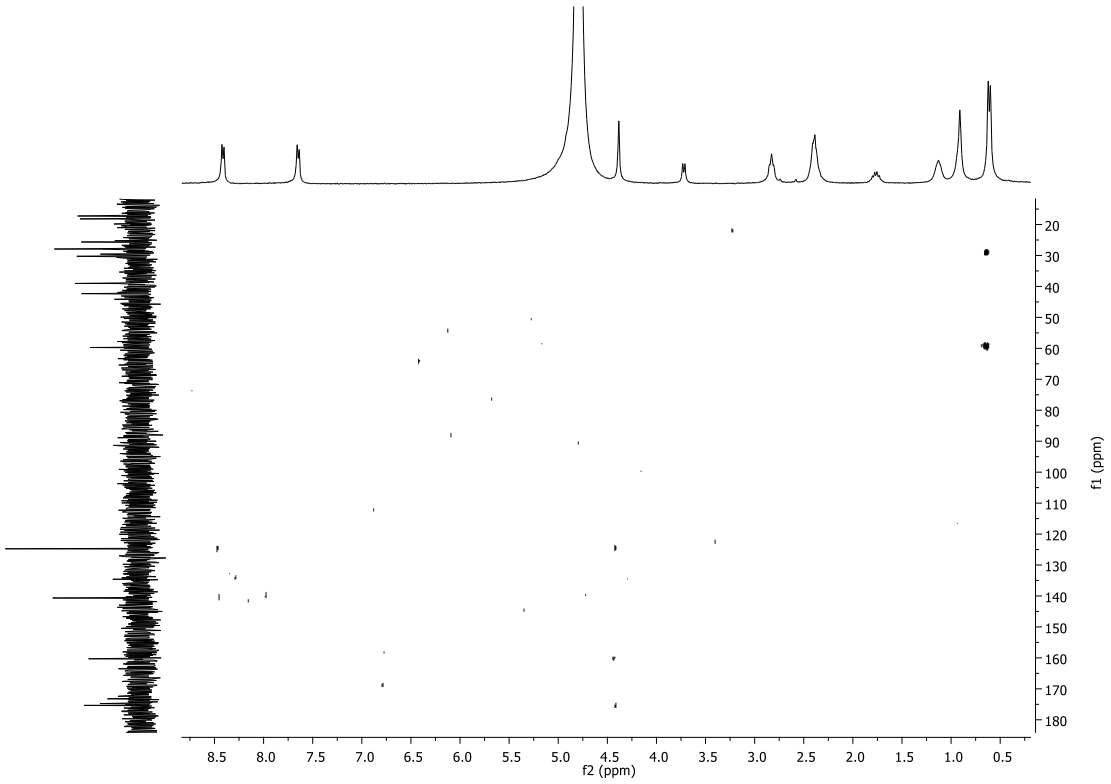


Figure 34. *HSQC-NMR spectra of (PyrSucVal)$_2$Oct.*

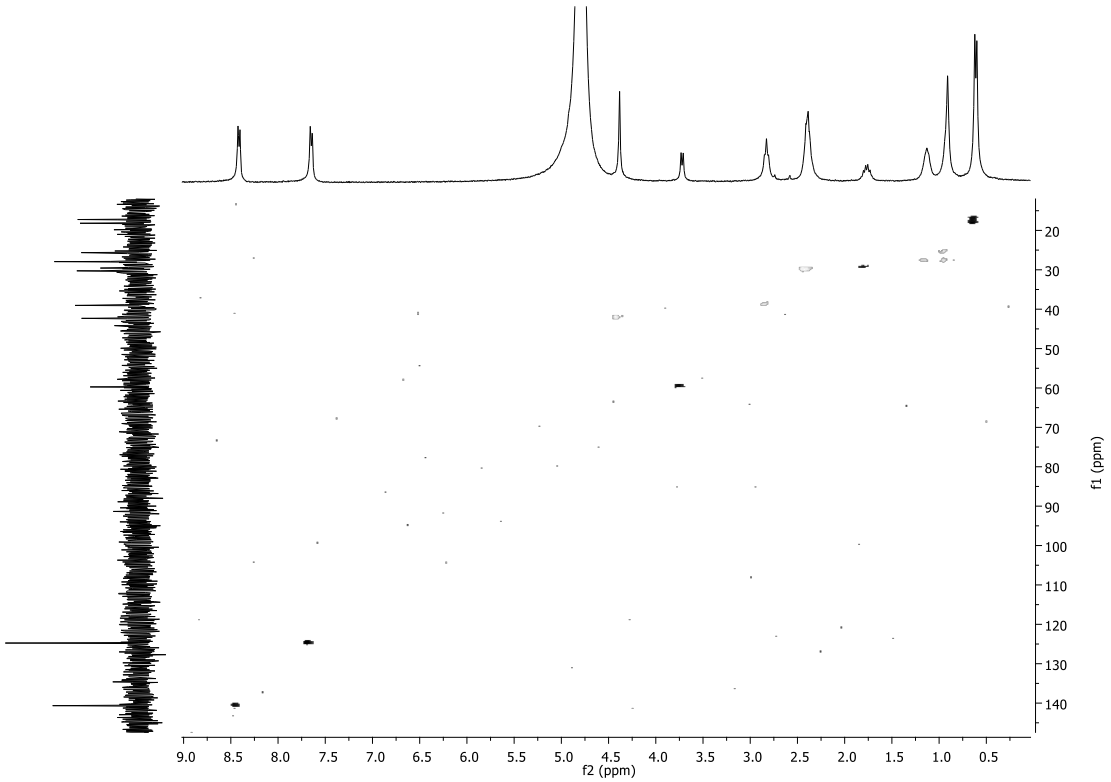


Figure 35. *HMBC-NMR spectra of $(PyrSucVal)_2Oct$.*