Enzyme-Instructed Self-Assembly of Small Hexapeptides Based on an Immunoreceptor Tyrosine-Based Inhibitory Motif (ITIM)

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# Table of Contents

Table of Contents .................................................................................................................. 2  
Acknowledgement .................................................................................................................. 3  
Introduction ............................................................................................................................ 5  
Materials and Methods ......................................................................................................... 7  
  Experimental materials and instruments .............................................................................. 7  
  Peptide synthesis, purification and characterization ............................................................... 7  
  General procedures for hydrogel preparation (enzymatic gelation) ....................................... 8  
TEM sample preparation ........................................................................................................ 8  
Rheological measurement ...................................................................................................... 9  
Circular Dichroism (CD) measurement .................................................................................. 9  
General procedure for digestion experiment .......................................................................... 10  
Dephosphorylation assay ....................................................................................................... 11  
Cell culture ............................................................................................................................. 11  
MTT cell viability assay .......................................................................................................... 12  
  Live/Dead cell viability/cytotoxicity assay (2D and 3D) ......................................................... 13  
Results and Discussion ........................................................................................................ 15  
Conclusion ............................................................................................................................. 20  
Appendix ................................................................................................................................ 23  
  NMR spectra ....................................................................................................................... 23  
  Liquid Chromatography-Mass Spectrometry (LC-MS) ....................................................... 27
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Abstract

Enzyme-Instructed Self-Assembly of Small Hexapeptides Based on an Immunoreceptor Tyrosine-Based Inhibitory Motif (ITIM)

A thesis presented to the Department of Chemistry

College of Arts and Sciences
Brandeis University
Waltham, Massachusetts

By Natsuko Nina Yamagata

Here we show the first example of an immunoreceptor tyrosine-based inhibitory motif (ITIM), LYYYYL, as well as its enantiomeric or retro-inverso peptide, to self-assemble in water upon enzymatic dephosphorylation. In recent years, supramolecular hydrogels that consist of peptidic nanofibrils have attracted considerable attention due to their versatility in biomedical applications. Previous studies discovered that certain short-sequence peptides self-assemble once the phosphate is cleaved by ectophosphatases overexpressed on the surface of cancer cells (enzyme-instructed self-assembly, or EISA). The hydrogels that form upon EISA can be cytotoxic; however, the cytotoxicity of ITIMs has yet to be demonstrated. Upon synthesizing 5 ITIM hexapeptides using standard solid phase peptide synthesis (SPPS), we characterized the resulting hydrogels by transmission electron microscopy (TEM), rheometry and circular dichroism (CD) spectroscopy. We also quantified the dephosphorylation rate of the precursor peptides and examined the effects of stereochemistry on gelation. We examined the cytotoxicity of the peptides on cancer cells by using cell viability assays such as MTT and Live/Dead Cell Viability Assays (2D and 3D). During our study of EISA of the ITIM-based peptides, we unexpectedly observed the moderate cytotoxicities of the L version peptides. This work illustrates a new approach to design bioinspired soft materials from a less explored, but important pool of functional peptides.
Introduction

Supramolecular hydrogels made of peptides have attracted considerable research interests, particularly, in the biomedical field due to their versatility and applications, such as a scaffold mimicking the native microenvironment necessary for cell–cell and cell–matrix interactions, the medium for controlled drug release, the promoters for nerve regeneration, the adjuvants for immune stimulation, the motif for developing novel anticancer agents, and the model system for understanding the origin of life. These developments indicate that it is beneficial and necessary for exploring the self-assembly of functional peptides for biomedical applications. Based on this notion, we decided to explore the self-assembly of a special and important type of functional peptides, ITIM, which are present in B-cells, T-cells, and natural killer (NK) cells and play a critical role in the inhibitory signaling process of immune response.

Scheme 1. Molecular structures of the hexapeptides based on ITIM and the corresponding phosphopeptides.
We chose to study the self-assembly of ITIM because of its unique role in immune response—when the ITIM is phosphorylated, it has an inhibitory effect; however, when the ITIM is dephosphorylated, it triggers signal activation. Our long-term goal is to develop the assemblies of ITIMs as potential immunomodulation materials in the form of hydrogels, but currently, there is little knowledge on the self-assembling behavior of ITIMs. Based on the previous reports on phosphopeptides and on tyrosine being a naturally-occurring amino acid in the human body, we decided to evaluate the ability of self-assembly and hydrogelation of ITIMs upon enzymatic dephosphorylation. Based on the well-established consensus sequence of ITIM (where X denotes any amino acid): (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val), we chose a sequence of LYYpYYL (L-1P), a peptide consisting of leucine and tyrosine which, when dephosphorylated, becomes a symmetric hexapeptide (L-1). Such a symmetry allows the design of its retro-inverso peptide (RI-1) to be the same as its enantiomeric peptide (D-1), though the relevant phosphopeptides, D-1P and RI-1P, differ. Our studies reveal that, although these peptides form stable hydrogels likely consisting of beta-sheets, the precursors differ considerably in cytotoxicity (i.e., L-1P exhibits higher cytotoxicity than D-1P and RI-1P do). Among D-1P and RI-1P, RI-1P is more cell compatible. As the first case of exploring ITIMs for self-assembly and given that ITIMs are endogenous functional peptides in mammals, this work may ultimately provide a new way to modulate immune responses by combining ITIMs and enzyme-instructed self-assembly (EISA).
Materials and Methods

Experimental materials and instruments

Alkaline phosphatase (ALP) was purchased from Biomatik USA, 2-naphthylacetic acid from Alfa Aesar, N,N-diisopropylethylamine (DIPEA), O-benzotriazole-N,N,N’,N’-tetramethyl-uronium-hexafluoro-phosphate (HBTU) from Acros Organics USA, and all amino acid derivatives from GL Biochem (Shanghai) Ltd. All the solvents and chemical reagents were used directly as received from the commercial sources without further purification. All products (L-1P, D-1P, RI-1P, L-1, and D-1) were purified with Water Delta600 HPLC system, equipped with an XTerra C18 RP column and an in-line diode array UV detector. $^1$H-NMR spectra were obtained on Varian Unity Inova 400, LC-MS spectra on a Waters Acquity ultra performance LC with Waters MICRO-MASS detector, rheological data on TA ARES G2 rheometer with 25 mm cone plate, TEM images on Morgagni 268 transmission electron microscope, confocal microscopy images on Leica TCS SP2 spectral confocal microscope.

Peptide synthesis, purification and characterization

We prepared the precursors and hydrogelators by solid phase peptide synthesis (SPPS) in fair yields (70-80%). The standard SPPS uses 2-chlorotriyl chloride resin (100-200 mesh and 0.3-0.8 mmol/g) and N-Fmoc-protected amino acids with side chains properly protected. Fmoc-PTyr-OH was synthesized using a combination of established procedures by Alewood. Figure 1 illustrates the synthetic procedure of D-1P. The synthetic route of others is the same with that of L-1P, RI-1P, L-1, and D-1.
Figure 1. Synthesis route of D-1P

General procedures for hydrogel preparation (enzymatic gelation)

The precursors (2.5 mg) were dissolved in either distilled water or PBS buffer (500 µL), and the pH of the solution was adjusted carefully by adding 1 M NaOH, monitored by pH paper. After reaching a pH of 7.4, extra distilled water was added to make the final concentration of 0.5 wt%, followed by the addition of alkaline phosphatase (ALP) at the concentration of 10 U/mL.

TEM sample preparation

A negative staining technique was used to study the TEM images. The 400 mesh copper grids coated with continuous thick carbon film (~ 35 nm) were first glow-discharged prior to use to increase the hydrophilicity. After loading samples (4 μL) on the grid, the grid was rinsed with dd-water three times. Immediately after rinsing, the grid containing the sample was stained with 2.0 % w/v uranyl acetate three times. Afterwards, the grid was dried in air to be placed inside the electron microscope.
Figure 2. TEM images of hydrogels formed by control hexapeptides A) L-1 and B) D-1 at the concentration of 0.5 wt %. The two compounds dissolve in PBS buffer and form gels at pH 4.5 and 4.0, respectively, by careful addition of hydrochloric acid (0.1 M). Scale bar is 100 nm.

**Rheological measurement**

Rheological tests were conducted on a TA ARES-G2 rheometer, having a parallel-plate geometry with an upper plate diameter of 25 mm and a 0.4 mm gap. During the measurement, the stage temperature was maintained at 25 °C by Peltier heating/cooling system. The hydrogel was loaded on the stage with a spatula, and then we performed the dynamic strain (0.1–100%) at 6.28 rad/s; the strain for maximum G’ in the linear range of strain sweep test was picked for frequency sweep test (0.1–200 rad/s).

**Circular Dichroism (CD) measurement**

CD spectra were recorded (185–300 nm) using a JASCO 810 spectrometer equipped with nitrogen. The hydrogel (0.5% wt., 400 μL) was placed carefully in a 1 mm thick quartz cuvette and scanned at a 0.5 nm interval three times. The CD spectra in the Results and Discussion section confirm the chirality of enantiomeric pairs of the peptides.
Figure 3. CD analysis of control hexapeptides L-1 (left) and D-1 (right) upon addition of hydrochloric acid (0.1 M). L-1 forms a gel at pH 4.5, while D-1 forms a gel at pH 4.0.

**General procedure for digestion experiment**

3 mL of solution for each compound dissolved in PBS buffer (500 μM, pH = 7.4) were treated with proteinase K (4.0 U/mL) at 37 °C. A 200 μL aliquot of each sample was taken out at the desired time and mixed with 200 μL of methanol. The obtained samples were analyzed by analytical HPLC to determine the amount of compound remaining in solution.

Figure 4. Digestion curve of three phosphopeptides upon treatment with proteinase K (4 U/mL) for 24 h. All compounds are at the concentration of 0.5 wt. %.
Dephosphorylation assay

6 mL of precursor solution in PBS buffer (500 μM, pH = 7.4) was treated with ALP (1 U/mL) at 37 °C. 500 μL of sample was taken out at the desired time, and mixed with 500 μL of methanol. The samples were analyzed by HPLC to determine the ratio of precursor and hydrogelator in each sample.

Figure 5. Increase of hydrogelators with time shows the dephosphorylation process of the precursors L-1P, D-1P, and RI-1P after incubation with ALP (1 U/mL) at 37 °C. The precursors dissolve in pH 7.4 PBS buffer at the concentration of 500 μM.

Cell culture

All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HeLa cells were propagated in Minimum Essential Media (MEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics, in a fully humidified incubator containing 5% CO2 at 37°C. The Saos-2 cells were propagated in McCoy’s 5A, supplemented with 15% FBS and antibiotics, in a fully humidified incubator containing 5% CO2 at 37°C. The HS-5 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with FBS to a final concentration of 10% and antibiotics, in a fully humidified incubator containing 5% CO2 at 37°C.
**MTT cell viability assay**

We followed the protocol available on the ThermoFisher Scientific website. Cells in exponential growth phase were seeded in a 96 well plate at a concentration of $1 \times 10^4$ cell/well. The cells were allowed to attach to the wells for 24 h at 37 °C, 5% CO$_2$. The culture medium was removed and 100 µL culture medium containing compounds (immediately diluted from freshly prepared stock solution of 10 mM) at gradient concentrations (0 µM as the control) was placed into each well. After culturing at 37 °C, 5% CO$_2$ for 48 h, to each well was added 10 µL of 5 mg/mL MTT ((3-(4, 5- Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). The plated cells were incubated in the dark for 4 h. 100 µL of 10% SDS with 0.01M HCl was added to each well to stop the reduction reaction and to dissolve the purple dye. After incubation of the cells at 37 °C overnight, the OD at 595 nm of the solution was measured in a microplate reader.
Figure 6. Cell viability (measured by MTT assay) of HeLa, Saos-2 and HS-5 cells treated with L-1P, D-1P, RI-1P, L-1, and D-1 (pH 7.4) at concentrations from 20 µM to 500 µM for 3 days.

Live/Dead cell viability/cytotoxicity assay (2D and 3D)

We followed the protocol available on the back labels of the LIVE/DEAD® Viability/Cytotoxicity Kit and on the ThermoFisher Scientific website. In a 35 mm disposable petri dish, prior to the assay, we washed the HeLa cells gently with 500–1,000 volumes of Dulbecco’s phosphate-buffered saline (D-PBS). Upon dilution of the calcein AM dye to 1 µM and EthD-1 dye to 2 µM, we added 100–150 µL of the combined LIVE/DEAD® assay reagents to the surface of a 22 mm square coverslip, so that all cells are covered with solution. The cells were incubated in a covered 35 mm disposable petri dish for 30–45 minutes at room temperature.
After incubation, we added approximately 10 μL of the fresh LIVE/DEAD® reagent solution/D-PBS to a clean microscope slide and viewed the labeled cells under a fluorescence microscope.
Results and Discussion

To investigate whether ITIM-based peptides are capable of forming supramolecular nanofibers by EISA of the hexapeptides, we selected a sequence consisting of leucine on both C and N terminals of four tyrosines (one of them having a phosphate group) to generate a phosphopeptide, LYY_pYYL. Such a symmetric design simplifies the synthesis of three phosphopeptides and reduces the number of hydrogelators to two (i.e., the Leu-(Tyr)_4-Leu and DLeu-(DTyr)_4-DLeu form upon dephosphorylation (Scheme 1)). Among the five hexapeptides of interest, L-1P and D-1P, and their dephosphorylated forms, L-1 and D-1, are enantiomers. D-1P and RI-1P are diastereomers, but once dephosphorylated, they produce the same D-enantiomeric hexapeptides (i.e., RI-1 and D-1 are DLeu-(DTyr)_4-DLeu). We prepared the five hexapeptides by standard solid phase peptide synthesis (SPPS) in fair yields (Figure 1). After the use of reversed-phase high performance liquid chromatography (HPLC) for purification, we obtained the desired peptides in good yields (60%).
Figure 7. (A) TEM images of the nanostructures formed by adding ALP (10 U/mL) in the PBS buffered solutions of the precursors (L-1P, D-1P, and RI-1P; all at 0.5 wt%) overnight. Scale bar is 100 nm. (B) Optical images of the hydrogels corresponding the TEM above them. (C) Rheological properties of the hydrogels of the hexapeptides: strain sweep (left); frequency sweep (right).

As shown in Figure 7, transmission electron microscopy (TEM) reveals that, after the addition of alkaline phosphatase (ALP) to the solutions of the precursors, L-1, generated by dephosphorylation of L-1P, self-assembles to form long and straight nanofibers with diameters of 8±1 nm, and D-1, generated from D-1P, forms nanofibers (diameters of 12±2 nm) plus short fibrillar aggregates. However, D-1, generated from RI-1P, produces largely non-fibrillar aggregates. These results not only indicate that the EISA of L-1 results in better assembly than the EISA of D-1 does, but also that the pathway for generating D-1 determines the morphologies of its assemblies. As expected, upon the addition the ALP, the conversion of L-1P, D-1P, or RI-1P to L-1, D-1, or RI-1, respectively, results in a hydrogel (Fig. 1B). Rheological analysis of the hydrogels formed by enzymatic dephosphorylation of the three phosphopeptides (0.5 wt%) confirms that all hydrogels are viscoelastic (Fig. 1C). The strain sweeps show that the critical strains are at 2%, 5%, or 4% for the hydrogel formed by EISA from L-1P, D-1P, or RI-1P, respectively. The frequency sweeps show that immediately after addition of ALP (into the solution of L-1P, D-1P or RI-1P), G’ becomes larger than G”, indicating that L-1, D-1, or RI-1 already reaches its gelation point immediately after mixing. Upon the same addition of ALP to the precursors, the rheological properties of the hydrogel of D-1 differ from those of L-1 and RI-1. For example, the storage modulus (G’) intersects the loss modulus (G”) at 100% for the hydrogels of L-1 and RI-1, while at 50% for the hydrogel of D-1. This observation indicates the rate of the dephosphorylation of D-1P is slower than those of L-1P and RI-1P, agreeing with the results of circular dichroism (CD) analysis (vide infra).
**Figure 8.** Time dependent CD analysis (top panel) and time-intensity graphs (bottom panel) of hydrogels, formed upon the addition of ALP (10 U/mL) to solutions of corresponding precursors at concentration of 0.5 wt% and scanned at desired times. The time-intensity graphs for the EISA of L-1P, D-1P, and RI-1P are 0th order (rate = kθ), with initial apparent rate constants of 1.82, 2.46, and 1.68, respectively.

To determine the secondary structure of the peptides in the hydrogels, we conducted a time-dependent CD analysis of the hydrogels formed by dephosphorylating the phosphopeptides (Fig. 2). The CD spectrum of the hydrogel formed by L-1 shows a negative peak at 195 nm followed by a positive peak at 210 nm. The hydrogels formed by D-1 and RI-1 show the negative band at 210 nm and a positive band at 195 nm, agreeing with the fact that D-1 (or RI-1) is the enantiomer of L-1. These results indicate that the molecular assemblies of these hexapeptides adopt well-defined antiparallel β-pleated sheets to form nanofibrils. The time-dependent changes of the CD signals during the formation of these hydrogels indicate that the dephosphorylation of L-1P and RI-1P and the self-assembly of L-1 and RI-1 occur faster than those of D-1P and D-1. This observation is consistent with the hypothesis that the retro-inverso peptides may have similar properties as the L-peptides.23
To determine the role of EISA from the phosphopeptides for its inhibitory activities and biocompatibility, we tested the cell viabilities of three mammalian cell lines (HeLa, Saos-2, and HS-5) treated by the five hexapeptides. As shown in Figure 9, all of these molecules are nearly innocuous to HS-5 cells even at a concentration as high as 500 μM. With the exception of D-1, the other precursors and hydrogelators exhibit significant cytotoxicities toward Saos-2 cells, with IC₅₀ values from 178 - 1196 μM (2nd day). Aside from the fact that L-1 inhibits the growth of HeLa cells considerably, the other four peptides are almost innocuous to HeLa cells. An interesting trend observed in the viability assay is the moderate cytotoxicities of L-1 and L-1, which may be an inherent feature of the ITIM hydrogels.
Figure 10. Fluorescent images of 2D (cells on gel) and 3D (cells in gel) live/dead cell assay of HeLa cells treated with 300 μL of 0.5 wt.% gels formed by L-1P, D-1P, and RI-1P via EISA for 24 hours.

Figure 10 shows the toxicity of the phosphopeptides on mammalian cell lines at or above the hydrogelation concentration (0.5 wt %) overnight by mimicking the cellular environment with a hydrogel produced by L-1P, D-1P, or RI-1P via EISA. The proliferation of the cells largely follows the results from Figure 9. That is, among the hydrogels produced from the three precursors, the hydrogel formed by EISA from RI-1P is the most compatible to HeLa cells. However, we have little understanding of the different mechanisms of cell death due to peptidic stereochemistry, which needs further investigation.
Conclusion

In conclusion, this work illustrates the first case of EISA of ITIM peptides and investigates their self-assembly and cytotoxicities in different forms. The cytotoxicity of RI-1P (or D-1P) towards Saos-2 cells, but not to HeLa and HS-5 cells, likely originates from the high-level expression of ALPs on the cell surface of Saos-2. There are other possible causes for the difference in cytotoxicity. Although such unanswered questions remain to be addressed, this work establishes the feasibility of forming hydrogels or supramolecular assemblies of ITIM from EISA processes.
References

Appendix

NMR spectra

NMR data of L-1P
NMR data of D-1P

$^{1}$H-NMR

$^{31}$P-NMR
NMR data of RI-1P
NMR data of L-1

NMR data of D-1
Liquid Chromatography-Mass Spectrometry (LC-MS)

L-1P (m/z): C_{48}H_{61}N_{6}O_{14}P, calc. 976.40; observed (M-1)- 975.51.

D-1P (m/z): C_{48}H_{61}N_{6}O_{14}P, calc. 976.40; observed (M-1)- 975.72.

RI-1P (m/z): C_{48}H_{61}N_{6}O_{14}P, calc. 976.40; observed (M-1)- 975.45.

L-1 (m/z): C_{48}H_{60}N_{6}O_{11}, calc. 896.43; observed (M-1)- 895.97.

D-1 (m/z): C_{48}H_{60}N_{6}O_{11}, calc. 896.43; observed (M-1)- 895.90.