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Short communication

Predicting milk-derived hydrogel-forming peptides with TANGO

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ABSTRACT

The uncovering of single peptides derived from food sources that can form hydrogels is of great relevance for several applications. However, identifying single peptide hydrogels from food is a daunting task given the complex nature of the food systems. The proof of concept of the applicability of TANGO, a statistical mechanical-based algorithm that predicts the β-aggregate propensity of peptides, as a tool to uncover peptides derived from milk that can form hydrogels is reported. Using TANGO in conjunction with a set of defined criteria we discovered that from a group of thirteen peptides derived from milk proteins, seven formed hydrogels at a concentration of 2 wt% and pH 7 at room temperature. Three more peptides formed aggregates and appeared to go through the syneresis process, and three additional peptides remained liquid under the experimental conditions. This result sets the basis of a simple methodology for unveiling peptide hydrogels from food and other natural sources.

1. Introduction

Peptide supramolecular hydrogels (PSHGs) constitute an important class of biomaterials with potential applications in the life and food sciences. Foods are an attractive source of PSHGs because of their wide availability and their generally recognized safe status. Milk, in particular, is a known source of proteins, which themselves or their hydrolysate mixtures can form gels. However, examples of single PSHGs derived from milk are scarce (De Leon Rodriguez & Hemar, 2020). A unique example of such peptide is f1-8 obtained by the tryptic digestion of β-lactoglobulin (Guy, Tremblay, Voyer, Gauthier, & Pouliot, 2011). Given that experimental identification of peptide fragments from protein hydrolysates that could form gels is a daunting work, the discovery of PSHGS of natural origin has been serendipitous. In the literature one can find experimental and computational methods that allow one to discover and design PSHGs. However, these methods are based on the synthesis of extensive peptide libraries and/or computationally demanding calculations (molecular dynamics simulations and machine learning methods) (Awhida, Draper, McDonald, & Adams, 2015; Corradini & Rogers, 2016; Frederix et al., 2015; Gupta, Adams, & Berry, 2016; Hu, Zhang, Hu, Euston, & Pan, 2020; Moreira, Scott, Ulijn, & Tuttle, 2019; Pappas et al., 2016; Saracino et al., 2018; Xu et al., 2023) making them restrictive for the case of peptides longer than 4 amino acids. To the best of our knowledge there is no simple predictive method available for identifying peptide sequences that exceed a length of 4 amino acids and that can form supramolecular hydrogels. Peptides longer than 4 amino acids offer a greater structural and functionality diversity, thus translating in a richer landscape for bioactivity and functionality targeting, which is of importance for the potential applications of hydrogels in biomedical and biotechnological areas.

Several algorithms (available as free downloadable software or hosted on an online server) have been used for the prediction of protein aggregation. Among these methods, TANGO offers the possibility of predicting the β-aggregate propensity (not necessarily amyloid) of segments in polypeptides (equal or longer than 5 residues). Importantly, TANGO also allows to consider experimental conditions, such as pH, temperature, ionic strength, and concentration; and *N*- and C-terminal modification options in the predictions (Meric, Robinson, & Roberts, 2017). The TANGO algorithm is based on statistical mechanical arguments where the constituent

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energy terms are from empirical and/or statistical analysis of a protein structure database and incorporate four conformational states and energetic contributions considering H-bonding, electrostatics interactions, side chain-side chain interactions, hydrophobicity and solvation energetics (Fernandez-Escamilla, Rousseau, Schymkowitz, & Serrano, 2004). While predicting aggregation of proteins or peptides is not necessarily a direct marker of potential hydrogel formation, one can posit that aggregation-prone sequences can potentially lead to formation of hydrogels, granted that the kinetic parameters required to achieve the metastable gelation state are identified (De Leon Rodriguez & Hemar, 2020).

In this short communication we report on a feasibility study, where we first searched the published literature for known milk-derived peptide sequences. Second, we ran the TANGO algorithm on these peptides, and in combination with additional selection criteria, we identified the peptides that are likely to form PSHGs. Third, we acquired a set of the selected peptides, synthetically made by solid phase peptide synthesis, and assessed their gel formation visually by the inversion tube test and dynamic oscillatory rheological measurements. This study aims to demonstrate if it is possible to predict theoretically the gel formation of known dairy peptides.

2. Materials and methods

Eleven milk-derived peptides were randomly selected from a set of peptides that satisfied all the selection criteria for hydrogel formation thoroughly discussed in the Results and Discussion section. Two milk-derived peptides that did not satisfy one of the selection criteria for hydrogelation were also randomly selected. Additionally, a well-known hydrogel-forming synthetically designed peptide, MAX1, was included as a positive control. The selected peptides (50 mg) were obtained with a $\geq$90% purity from Toppeptide Co., Ltd, Shanghai, China. Peptide identity was assessed by the mass spectra provided by the supplier.

A stock buffer solution (pH 7) containing 100 mM Bis-Tris propane (BTP, Sigma Aldrich, China) and 300 mM NaCl was prepared. Peptide gelation was initially assessed by the tube inversion test. The test consists of preparing solutions of the peptides in tubes at room temperature (~25 °C), which are inverted after some time. Peptides that form a gel stay on the top part of the inverted tube, while peptide dispersions that do not gel stay in the bottom. Thus, 10 mg of peptide were initially dissolved in 0.25 mL of milliQ water in 1.5 mL Eppendorf tubes and kept in an ice bath. A solution of BTP buffer 0.25 mL (100 mM, pH 7), also kept in an ice bath, was added to Eppendorf tubes containing the peptides to initiate gel formation, and the resultant mixture was left in an incubator (SPX-70, Zhongyi Guoke Technology Co., Ltd. Beijing, China) preset at 25 °C. The final peptide concentration was 2 wt%. Gelation was assessed visually after a 2 h incubation period by inverting the Eppendorf tubes.

In another experiment, peptides (~28 mg) were initially dissolved in 0.5 mL of milliQ water in a glass vial and kept in an ice bath. A solution of BTP buffer (0.5 mL), also kept in an ice bath, was added to the vials containing the peptides to initiate gel formation, and the resultant solution was left at room temperature (25 °C) for 24 h. The final peptide concentration was ~3 wt%. Gelation was assessed visually by inverting the glass vials.

The β-aggregate propensity (AGG) of each peptide was calculated with TANGO (http://tango.crg.es/protected/academic/calculation2.jsp) at three different pH values (2, 7 and 12) at fixed temperature (298.15 K), ionic strength (0.1 M) and peptide concentration (0.003 M which is ~0.5 wt% for an 11 residues peptide) for the case of the native sequence, the *N*-terminal acetylated peptide (Ac-) and C-terminal amidated peptide (-NH_2).

Isoelectric points, pI, were calculated online (http://isoelectric.org/) (Kozlowski, 2016).

Facial amphiphilicity involves the separation of hydrophilic and hydrophobic units perpendicular to molecular axis. For peptides attaining a β-sheet conformation, the facial amphiphilicity is determined by the hydrophobic/hydrophilic nature of the peptide residues' side chains. In this work, we used the distribution coefficients (log *Kp*) reported by Eisenberg, Weiss, Terwilliger, and Wilcox (1982), as a metric of hydrophobicity. Facial amphiphilicity was calculated in excel by adding the log *Kp* values of the amino acid side chain groups located in odd and even positions from the *N*- to C-terminus. Thus, facial amphiphilicity is present if the sum of log *Kp* values of amino acids in odd positions is negative and the sum of log *Kp* values of amino acids in even positions is positive, or vice versa. The overall hydrophilic facial amphiphilicity was determined by adding the sum of log *Kp* values for amino acids in odd and even positions. A negative value constituted the criteria to establish a hydrophilic facial amphiphilicity.

All calculations were performed in excel using conventional methods.

The viscoelastic properties of selected peptide solutions (2 wt%) were measured on an MCR 302 stress-controlled rheometer (Anton Paar, Graz, Austria) using a 25 mm/1° cone and plate geometry, which was maintained at 25 °C in a water-saturated atmosphere using a Peltier temperature controller. The gelation was measured for 2 h by determining storage and loss modulus, G' and G'', at a frequency f = 1 Hz and strain amplitude γ_0 = 1%, which is in the linear viscoelastic regime, for 2 h, followed by a frequency sweep in the range f = 0.01–1 Hz and γ_0 = 1% as well as a strain sweep at f = 1 Hz covering the deformation range γ_0 = 0.1–1000%.

3. Results and discussion

While as mentioned before there are several available algorithms that have shown promising results to predict aggregating peptide segments, they have mainly focused on amyloid fibril databases, and their performance on peptides that form hydrogels has not been assessed. To determine the applicability of TANGO on predicting peptide hydrogel formation, we evaluated a set of 45 peptides (Table S1), which have been previously characterized in terms of their hydrogelation properties (De Leon Rodriguez, Hemar, Cornish, & Brimble, 2016; Guy et al., 2011; Hauptstein et al., 2018; Medini et al., 2016; Valéry, Pandey, & Gerrard, 2013). Thirty-eight of the peptides were reported to form hydrogels and 7 did not form gels under the studied conditions. The β-aggregate propensity (AGG) of each peptide was calculated with TANGO (Table S1). The *N*-terminal acetylated (Ac-) and C-terminal amidated (-NH_2) modified peptides were also included in the calculations as these are known to prevent unwanted electrostatic interactions derived from the amine and carboxylate terminal groups. Performing calculations at different pH values is important as it is known that hydrogel formation is dependent on the charge of the peptide. For example, peptides with an isoelectric point (pI) close to neutral are more likely to form hydrogels under this condition as repulsion of charged functionalities is minimized.

TANGO yielded a success rate of 78%, correctly predicting hydrogelation for 35 out of 45 peptides, with 2 false positives and 8 false negatives (Table S1). This corresponds to a correlation of 0.37 (Fernandez-Escamilla et al., 2004). The peptide set consisted of 23 peptides containing a perfectly alternating hydrophobic–hydrophilic residue sequence known to favor a β-sheet structure. The rest of the peptides did not adhere to these residue alternance. Peptides of natural origin that can form hydrogels typically do not contain alternating hydrophobic–hydrophilic residues (entry 32–34 and 42 in Table S1). Hence, if one leaves out the TANGO calculations done for

Table 1
Selected milk derived peptides (#2–14), their isoelectric point pI (calculated in http://isoelectric.org/) and the protein source is shown #1 is a standard peptide also called MAX1. The residues highlighted in bold correspond to the β-sheet sequences of high aggregation propensity predicted by TANGO.

#	Native sequences	pI[a]	Protein source	References
1	VKVKVKVK-V(DP)PT-KVKVKVKV	9.96	MAX1	(Branco, Pochan, Wagner, & Schneider, 2009)
2	I**QAFLLYQ**E[b]	3.64	β-casein	(Mane, Ciocia, Beck, Lillevang, & McSweeney, 2019)
3	ENLLR**FFVAP**FPEVFGKEK[c]	6.74	αs_1-casein	(Mane et al., 2019)
4	K**ILLDKVGINYWLAH**K	9.06	α-lactalbumin	(Jiang et al., 2019)
5	QPE**VMGV**SKV	6.60	From β-CN A3	(Fan et al., 2019)
6	V**FKIDAL**NENKV	6.56	β-lactoglobulin	(Jeewanthi, Kim, Lee, Yoon, & Paik, 2017)
7	S**VLSLS**QSKV	9.07	β-casein	(Mane et al., 2019)
8	E**VMGVS**K	6.60	β-casein	(Mane et al., 2019)
9	DIQK**VAGTWYS**L	6.34	β-lactoglobulin	(Jeewanthi et al., 2017)
10	K**VGINYWLAH**K	9.35	α-lactalbumin	(Amorim et al., 2019)
11	IP**AVFKIDAL**NENK	6.56	β-lactoglobulin	(Zanutto-Elgui et al., 2019)
12	D**FGHIQYVAAY**R	7.36	Lactotransferrin	(Amorim et al., 2019)
13	ILDK**VGINYWLAH**K	8.76	α-lactalbumin	(Zanutto-Elgui et al., 2019)
14	RDMP**IQAFLL**Y	6.50	From β-casein	(Ali et al., 2019)

[a] Calculated pI values correspond to the uncapped native sequence.
[b] Peptide with a predicted acidic pI and with an overall hydrophobic facial amphiphilicity.
[c] Peptide containing two prolines.

peptides with residues with perfectly alternating hydrophobicity then the success rate increases to 82%, with 18 out of 22 correctly predicted peptides with 1 false positive and 3 false negatives and a correlation of 0.43. This is an acceptable predictive level, especially if one considers that for some peptides reported as no gelling the proper conditions for hydrogel formation might have not been discovered.

To evaluate the peptide hydrogel predictive capabilities of TANGO in peptides derived from milk, we searched the reported literature in Web of Science and Google Scholar for peptides resulting from the hydrolysis of milk proteins for 2017–2020. The search resulted in 4265 peptides with known sequences. The β-aggregate propensity (AGG) of each peptide was calculated with TANGO at a constant pH value (7.0), temperature (298.15 K), ionic strength (0.1 M) and peptide concentration (0.005 M) for the case of the native sequence, the *N*-terminal acetylated peptide (Ac-) and C-terminal amidated peptide (-NH_2). This calculation yielded 425 peptides with a positive β-aggregating propensity (Table S2). Further selection of peptides that can potentially form hydrogels was performed according to the following criteria: 1) peptides without cysteine, as this amino acid is prone to oxidation and this can yield complex mixtures of inter/intra bonded species; 2) peptides with less than 1 or equal to 9 residues, as the synthesis of longer peptides is costly; 3) peptides predicted to aggregate in the native, acetylated (Ac-), and amidated (-NH_2) state, as we considered this to be a criteria of a robust residue mediated aggregation; 4) peptides with a predicted isoelectric point (pI) between 6.0 and 9.4, as our initial goal is to discover peptides that form hydrogels under neutral conditions; 5) peptides presenting facial amphiphilicity (calculated as reported in the literature by Rani et al., 2020), as this is an essential requirement for peptide assembly driven by hydrophobic interactions to occur; 6) peptides with an overall hydrophilic facial amphiphilicity, since the inclusion of hydrophilic residues has previously been shown to transform insoluble nanofibers to a hydrogel network (Zaccai et al., 2011), thus preventing solubility issues; and 7) peptides containing less than 2 prolines (Pro), as Pro is an amino-acid with a five-member ring, hence it lacks a hydrogen on the α amino group and presents a sterically restricted rotation around the *N*–C(α) bond, a feature known to disrupt peptide structure. This selection criteria led to a list of 21 peptides (Marked with an asterisk in Table S2). From this list we randomly selected 11 peptides. Also, for reference, a peptide containing 2 prolines and another peptide with a pI of ~3 and with an overall hydrophobic facial amphiphilicity while satisfying the other criteria listed above, were randomly chosen. Thus, from this exercise, a total of 13 peptides were selected; their sequences and pI calculated values are given in Table 1. Additionally, a set of peptides (not shown), where tango predicted a zero AGG, were randomly selected as negative control.

The 13 selected and the negative control peptide sets with *N*-acetylated and C-amidated termini-to prevent unwanted electrostatic interactions-were acquired from a commercial source (see experimental section). MAX1 a well-known *β*-hairpin peptide that forms hydrogels was used for comparison (#1). Solutions of the peptides in the buffer were prepared and hydrogel formation was assessed by the inverting tube test and rheological measurements. As shown in Fig. 1, 3 peptides (#3, #4, #11) and the control peptide (#1) formed hydrogels; 4 peptides (#6, #12, #13, #14) also formed gels (Fig. 3), but these appeared more turbid and weaker than the hydrogels of #3, #4 or #11 as evidenced by the presence of some liquid dribbling down the tube wall during the tube inversion test; 3 peptides (#2, #7, #9) appeared as aggregates that underwent extensive phase separation (syneresis), and 3 peptides (#5, #8, #10) formed low-viscosity liquid solutions. Phase separation for peptides #2, #7 and #9 was somehow expected as these peptides among the set have pI values farther from neutral (3.6, 9.06 and 6.3 for #2, #7 and #9, respectively), hence will be charged at pH 7.0 where repulsive electrostatic interactions might prevent peptide assembly. Interestingly, peptide #3, containing two Pro, formed a hydrogel, suggesting that the short peptide subsequence predicted to have a high β-aggregation propensity (FFVAP) can compensate the Pro propensity to break secondary structures. Furthermore, the presence of two aromatic phenylalanine residues is known to favor intramolecular π–π interactions and promote hydrogel formation. When compared to the hydrogel of peptide #1 (MAX1), the hydrogels of peptides #3, #4, and #11 were the next most transparent samples. The physical state of a hydrogel (transparent vs turbid) was reported to correspond to different polymorphic states of peptides in the gel state and to be dependent on the peptide concentration and other conditions (e.g. pH) (Baral et al., 2015). Importantly, none of the negative control peptides formed gels, further highlighting the predictive robustness of the method tested in this work.

Dynamic oscillatory rheology was performed to investigate quantitatively the mechanical behavior of the peptides under study. Peptides showing liquid behavior and those showing extensive phase separation are excluded since the former have lower elastic modulus G' and the latter will not provide a meaningful experimental result due to presence of large aggregates. Thus, only peptide hydrogels were measured. The time-sweep measurements show that the elastic modulus G' of peptide hydrogels #3, #4, and

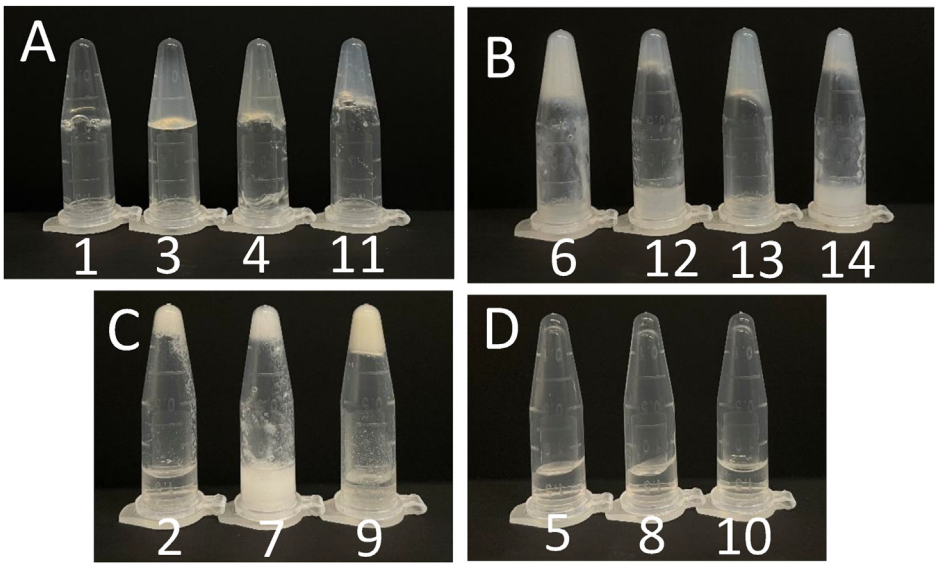

Fig. 1. Inverted Eppendorf tubes containing solutions of the peptides at 2 wt% pH 7 after 2 h incubation at 25 °C. (**A**) Peptide gels, (**B**) viscoelastic peptide solutions, (**C**) phase-separated peptide solutions, (**D**) non-gelled peptide solutions. Numbers are same as indicated in Table 1 to allow peptide sequence identification.

#11 increased with time, except for peptide #4 which plateaued after 1 h (Fig. 2A). The slowest kinetics of hydrogelation corresponded to peptide #3, and this could be attributed to the presence of two Pro, which might decrease the speed under which peptide chains self-assemble. Furthermore, after 2 h the magnitude of G' has the following order #11 > #1 > #4 > #3. A hydrogel of peptide #11 can reach a G' of up ~100,000 Pa, which appoints it as a promising candidate for tissue engineering applications (De Leon Rodriguez et al., 2016). The frequency-sweep measurements (Fig. 2B) confirmed that these peptides form hydrogels since at 1 Hz, G' is higher than G'' under an applied strain γ of $\leq$1%. The hydrogels of peptides #3, #4, and #11 are also brittle as they break

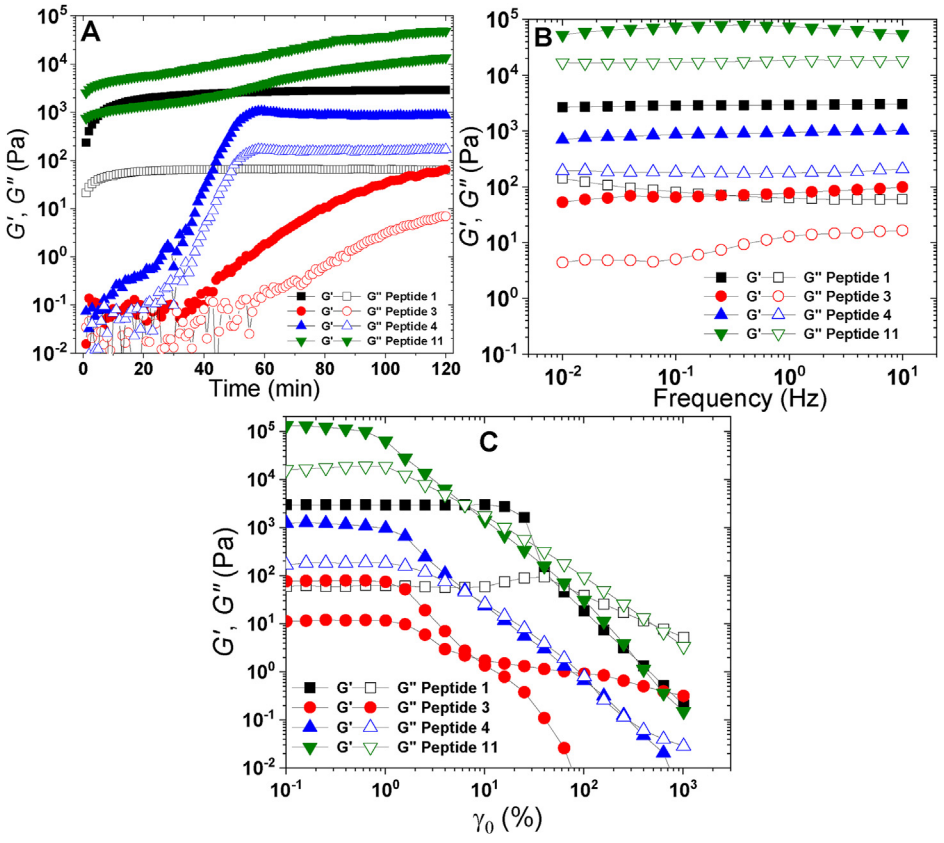

Fig. 2. Elastic modulus G' (solid symbols) and loss modulus G'' (open symbols) of peptide hydrogels #1, #3, #4 and #11 as a function of time (**A**), frequency (**B**), and strain (**C**). Peptide gels were prepared at 2 wt% and pH 7. Peptide numbers are the same as in Table 1.

and flow at relatively smaller deformations (γ <10%), that is when $G' = G''$ (Fig. 2C), when compared to that of hydrogel of peptide #1 (γ ~60%).

In comparison, and with the exception of peptide #6, rheological measurements showed that G' of hydrogels of peptides #12, #13 and #14 do not evolve markedly with time but started with a relatively high value which remained basically constant during the measurement (Fig. 3A). This indicates that peptide assembly and gelation is fast. Interestingly, in average, the G' values after 2 h for hydrogels of peptides #12, #13 and #14 were higher (>1000 Pa) than those of hydrogels of peptides #3 and #4 (Fig. 2A). Gel formation for #6, #12, #13 and #14 is also confirmed by frequency-sweep measurements, where G' was higher than G'', under the measured frequencies at constant strain ($\gamma \leq 1\%$) (Fig. 3B). However, except for peptide hydrogel #14, a marked difference when compared to peptide hydrogels #3, #4, and #11, is that peptide hydrogels #6, #12, and #13 flowed at strains >40%. Overall, this set of peptides formed stronger gels opposite to what the tube inversion test suggested.

Here it is relevant to highlight that the fact that some TANGO-predicted peptides did not form gels might also indicate that the right conditions for hydrogelation have not been discovered, hence further optimization (e.g., peptide concentration, incubation time, salt, pH and temperature) might be required. As proof of this point the tube inversion test was also performed for peptide solutions prepared at ~3 wt% peptide concentration after incubation for 24 h at 25 °C. The results were similar to our previous test performed at a 2 wt% peptide concentration and 2 h incubation time. However, peptides, #7 and #8, which did not form a gel before, formed gels at higher concentrations and longer incubation time (Fig. S1). Therefore, one can conclude that the procedure reported here successfully predicted the gelation of 9 out of 13 peptides, that is a ~70% success rate, which is acceptable considering the simplicity of the method.

Furthermore, it is relevant to indicate that some of the peptides that formed hydrogels are acidic and hence will be negatively charged at neutral pH (peptide hydrogels #6, #11, and #13, with pI values of 6.56, 6.56 and 6.50 as shown in Table 1). Yet, despite being partially charged, which suggests the presence of electrostatic repulsive forces that might prevent hydrogel formation, hydrogels formed. Also, these peptides appear to have fast gelation kinetics, as evidenced by rheology (Fig. 3A). Two of the peptides that formed aggregates, where the solids stayed on the top of the tube inversion test, and that appeared to go through a syneresis process indicated by a considerable amount of water at the bottom of the tube (Fig. 1 and Fig. S1), are also acidic (peptides #2, and #9, with pI values of 3.64, and 6.34 as shown in Table 1). Therefore, one can posit that peptides #2, and #9 quickly formed a hydrogel and then expelled water (syneresis). Syneresis is mostly driven by an increase in hydrophobicity of the self-assembled peptide fibres followed by expulsion of water from the gel network (Panja, Dietrich, & Adams, 2022). Of note, peptide #2 and #9 have a high content of hydrophobic amino acids with these being sequentially placed within the peptide sequence (AFLLY for peptide #2 and VAGTWY for peptide #9 as shown in Table 1). Furthermore, charged amino acids are located at the terminal residues in both peptides. Therefore, one hypothesis is that the hydrophobic peptide subsequence can become more exposed to the aqueous environment during assembly possibly due to repulsive interactions between charged residues. This leads the self-assembled peptide fibres to pack together, which in turn drives the expelling of water from the hydrogel (Castilla et al., 2016). However, further research is needed to confirm this hypothesis. It is important to highlight this point for the reader, as there is a scarcity of reports on peptide hydrogels undergoing spontaneous syneresis in the literature. Moreover, these systems are noteworthy due to their potential applications in developing actuators, smart switchable materials, artificial

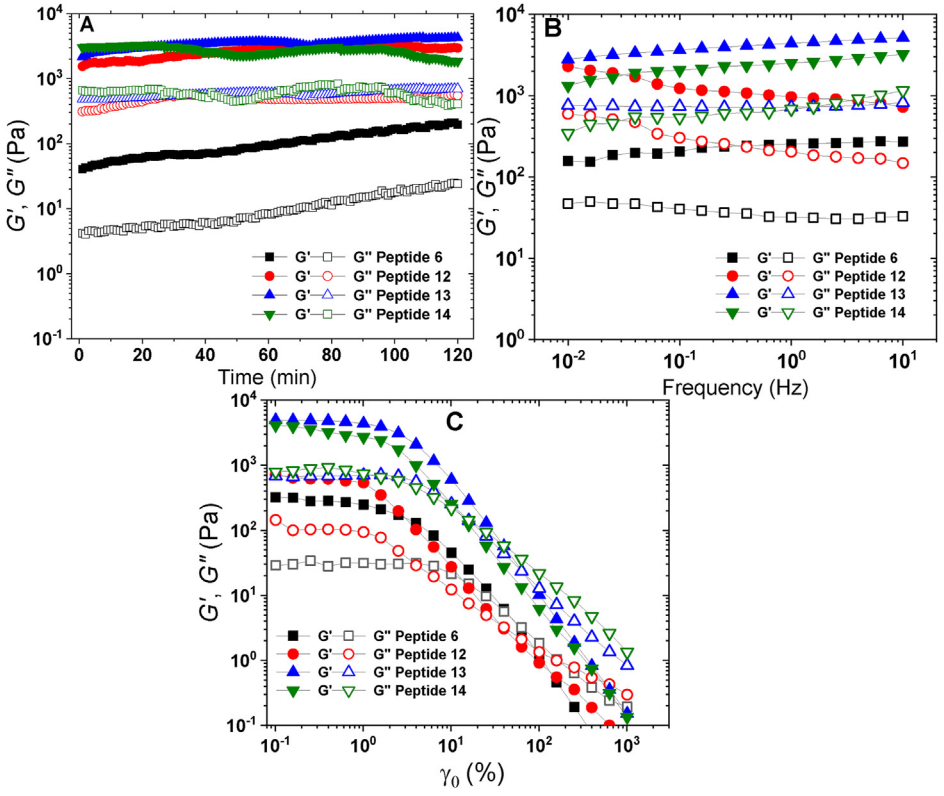

Fig. 3. Elastic modulus G' (solid symbols) and loss modulus G'' (open symbols) of peptide hydrogels #1, #3, #4 and #11 as a function of time (**A**), frequency (**B**), and strain (**C**). Peptide gels were prepared at 2 wt% and pH 7. Peptide numbers are the same as in Table 1.

muscles, drug release, biosensing, microfluidic devices, and water purification (Panja et al., 2022).

4. Conclusion

In summary, this work demonstrates that TANGO can be used as a tool to predict peptides which have potential to form hydrogels. However, further characterization methods, such as FTIR, CD, and X-ray scattering, might be required to determine the fine structure of the gel peptide networks. Peptide #11 (IPAVFKIDALNENK), obtained by the hydrolysis of bovine β-lactoglobulin using fungal proteases from *Aspergillus oryzae* (ATCC 1003) (Zanutto-Elgui et al., 2019), showed a transparent hydrogel with an elastic modulus >100,000 Pa. This peptide would be an ideal candidate to explore for applications in biomedicine and other areas. Finally, it is important to note that some peptides that failed to form uniform gels might also potentially form hydrogels granted further optimization of the peptide concentration, salt, pH and temperature conditions is performed.

CRediT authorship contribution statement

Muhammed Aslam Khan: Writing – review & editing, Investigation, Formal analysis, Data curation. **Yacine Hemar:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ka-Wing Cheng:** Writing – review & editing, Supervision. **Florian J. Stadler:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Luis M. De Leon-Rodriguez:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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