Stirring Peptide Synthesis to a New Level of Efficiency

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Abstract

Accelerating solid-phase peptide synthesis is crucial to access a large number of peptides and proteins in a short time. Peptide synthesis usually done using poor mixing methods with slow diffusion of solid support and reagents hence the acceleration of the process is achieved by elevated temperature and large reagent excess. In this work, a new setup that relies on fast stirring and heating was used to increase the diffusion of both reagents and solid support. We show that the combination of fast mixing and elevated temperature enables the acceleration of solid-phase peptide synthesis without using a large excess of reagents, providing a greener and accessible alternative to the state-of-the-art.

Keywords: SPPS, Peptide synthesis, Fick's second law, diffusion, Green chemistry

Introduction

Solid-phase peptide synthesis (SPPS) is a common methodology for the preparation of peptides.[1–3] The reactions in this synthetic process are performed in a heterogeneous phase, where the sequence chain extends on a solid support and the reagents are dissolved in an organic solvent.[2] This enables the removal of the reagents from the growing peptidyl chains by filtration, which reduces the number of purifications.[1] SPPS, like other processes on insoluble support, is diffusion-dependent.[4–6] The solid phase process is heterogeneous, hence it obeys Ficks' second law of diffusion (Equation 1).[7,8]

Eq. 1: $$\frac{dC_{(x,t)}}{dt} = \nabla^2 \left(D_{(x,T,t)} C_{(x,t)} \right) - k_{(T)} C_{(x,t)}$$

Where $C_{(x,t)}$ is the reactant concentration, D is the diffusion parameter, *k* is the reaction rate coefficient. *T* is the reaction temperature, *t* is reaction time, *x* is the location. $C_{(x,t)}$ depends on distance and time. *D* depends on mixing, time, and temperature. *k* depends on temperature.

For peptide synthesis, both coupling and deprotection follow pseudo-first-order kinetics, as the local concentration of functional groups on the solid phase is very high.[9] This suggests that both the diffusion and the rate coefficient are detrimental to these reactions. Therefore, the conditions that influence the SPPS reaction efficiency are 1) the initial concentration of reagents;[10–12] 2) reaction time;[12–14] 3) temperature;[15,16] and 4) the mixing method.[2,4,5] Increased reaction durations, high reagent concentrations, and elevation of reaction temperature are commonly applied to accelerate reactions on a solid support.[12,17] However, the effect of mixing efficiency on SPPS processes is not commonly discussed.[4,5,16] To perform an efficient SPPS process, one needs to consider all conditions that can affect the parameters related to Fick's second law of diffusion (Equation 1), including mixing, which is setup-dependent.

Different setups are employed for SPPS processes (Fig 1A). The first generations of SPPS setups were based on the rotatory motion of the entire vessel to achieve mixing, due to reports that stirring destroys the integrity of the solid support (Fig 1A).[1,2,18,19] The inefficient diffusion in these slow mixing setups is compensated by extending the reactions and/or by increasing the excess of reagents.[2,15] Microwave-assisted SPPS (Mw-SPPS) which utilizes a stationary reactor with gentle bubbling-based mixing, relies on elevated temperatures for improving reaction efficiency (Fig 1A).[20–22] Mw-SPPS requires a large excess of reagents in addition to the irradiation and temperature effect for the acceleration of reaction cycles, to compensate for the lack of proper mixing. Flow-based SPPS systems gained much attention in the last decades as they allow automation and acceleration of the process.[23–25] A automated flow-based setup for SPPS (AFPS) presented an extremely fast peptides and proteins syntheses process. AFPS relies on elevated temperatures for accelerating amide bond formation (coupling) and Fmoc deprotection reactions. In AFPS, a full cycle for the introduction of

amino acids to the peptide is performed in a clave of 40 seconds, which is extremely fast compared to other setups (Fig 1A).[16,17,26] In AFPS the solid support is stationary and a large excess of reagents (6-60 equivalents) is used for coupling, proving that elevated temperature and flow-based mixing only partially suffice when the short reactions are employed. The above strategies show that for accelerating SPPS processes in reactors with poor mixing (decreasing *t*, Ficks' law), the use of high concentrations is inevitable (increasing C, Ficks' law).

We recently showed that fast overhead stirring does not break the polystyrene beads, confirming that such setups are viable for SPPS.[4,5] High sheer stirring SPPS (Fig 1A, HSS-SPPS) enabled decreasing the concentration of reagents used for both Fmoc deprotection and amino acid coupling steps, albeit with extended reaction times suggesting that improved mixing by itself is not enough for accelerating SPPS.[4,5] After realizing the effect of both mixing (HSS-SPPS) and temperature (Mw-SPPS and AFPS), we hypothesized that their combination should enable accelerated SPPS even at low concentrations by maximizing the effect of diffusion.

Here, a new setup that enables both fast stirring and an elevated temperature was developed for the acceleration of SPPS (Fig 1A, High-Temperature Stirred Peptide Synthesis, HTFSPS). The parameters affecting SPPS acceleration in a narrow reactor with overhead stirring were studied based on Fick's second law of diffusion. The influence of stirring, temperature, and concentration on SPPS efficiency in short reaction times was evaluated. HTFSPS was challenged by synthesizing peptides of different lengths and complexity levels, including ones that are prone to fail or epimerize.

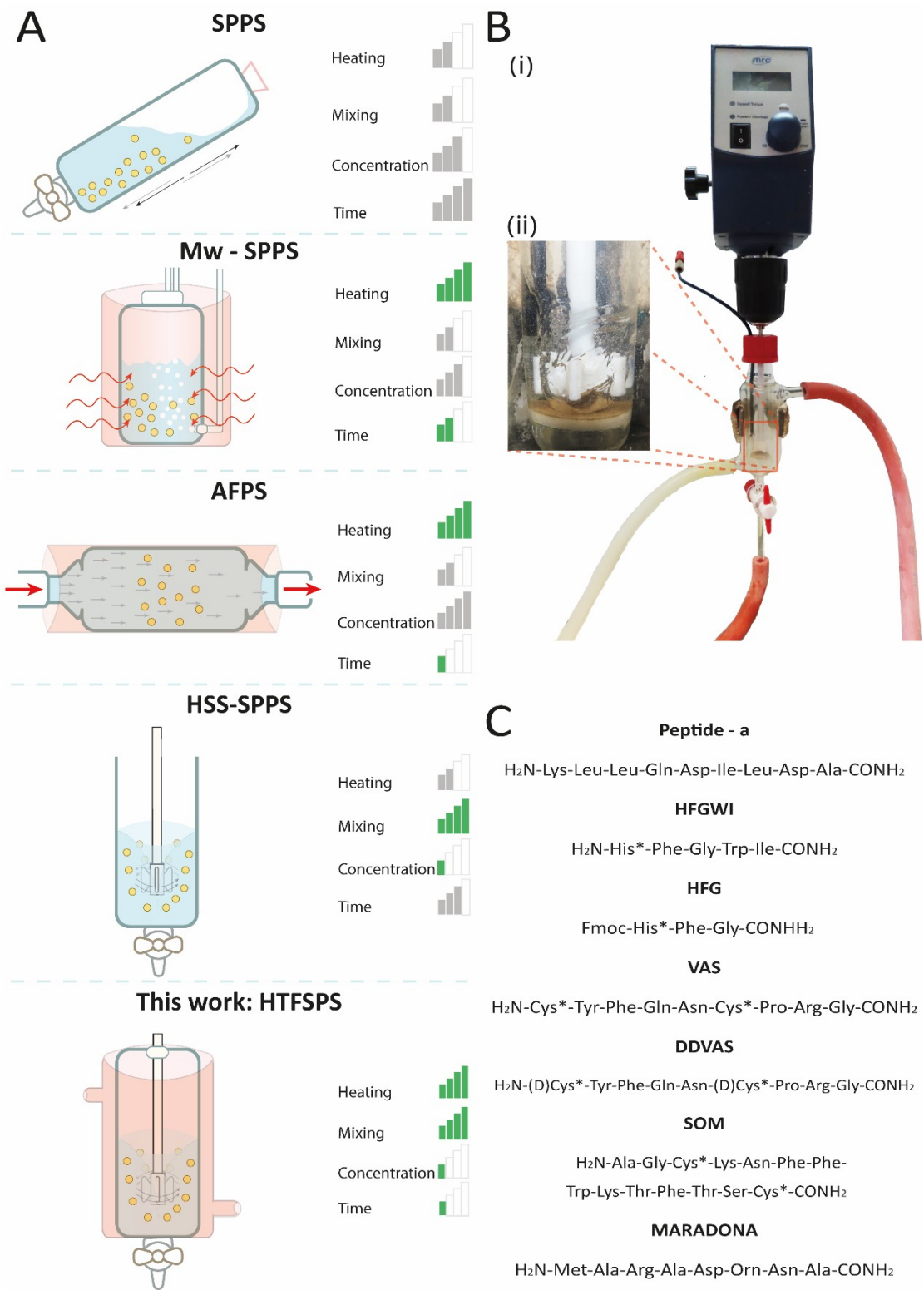


Figure 1: A) Different setups used for SPPS. B) (i) HTFSPS reactor overview (ii) zoom-in of the impeller region taken using short exposure-time while stirring at 1200 rpm. C) The different peptides synthesized in this work.

Results and Discussion

A new reactor was designed to enable a full and continuous Fmoc-SPPS process while maintaining high temperature and fast overhead stirring. A self-prepared reactor with a sintered glass filter and a heating jacket was equipped with a feed insert that acts both as a baffle for vortex relapse and direct delivery of reaction and washing solutions (Fig. 1B, S1, S2). An overhead five-fin turbine agitator PTFE impeller for fast stirring in a narrow dimension was inserted into the reactor. The heating jacket was connected to a circulating water bath. To confirm that this setup does not harm the solid support, microscope images of the beads were taken before and after 7 h of mixing at 1200 rpm with a temperature of 90 °C (ESI section 4). The integrity of the beads suggests that the design setup is feasible for SPPS.

Initial evaluation of HTFSPS

Initial assessment of HTFSPS feasibility was performed by synthesizing a nine-amino acid model **peptide-a**, KLLQDILDA (Fig. 1) at a constant stirring rate of 1200 rpm using the HTFSPS reactor.[16] **Peptide-a** was synthesized in the HTFSPS reactor *via* several routes, which differ in the reaction conditions and the crude purity was determined after each synthesis (Fig. 2, Routes 1-3). Reaction mixtures and washing solvents were added to the reactor by injection from the feed line and drained by vacuum filtration. Coupling mixtures containing only two equivalents of protected amino acid, an activator, and a base were added to the reactor without pre-heating or pre-activation. Fmoc deprotection was performed by inserting a solution of 5% piperidine in DMF without pre-heating. The crude purity of **peptide-a** synthesized *via* Route-1 (5 min reactions, 30 °C) was above 97% (Fig 2). This is in line with our previous observation that high shear stirring and long reaction times lead to high purity.[4] Furthermore, the result proves that given enough time, an almost complete conversion is achieved in each step when using HSS-SPPS even while using low concentrations of reagents. The crude purity of **peptide-a** synthesized *via* Route-2 (30 sec reactions, 30 °C) was 91% (Fig. 2). Since Route-2 applies much shorter reaction times compared to Route-1, it demonstrated that time is a limiting factor in reaching high conversion in each reaction step. Based on Fick's second law,[7,8,16,17] elevated the temperature to 90 ºC while maintaining short

reaction periods, fast stirring, and low reagent concentrations. **Peptide-a** was synthesized *via* Route-3 (30 sec reactions, 90 °C), which is almost identical to Route-2, only that the entire process was performed at 90 °C instead of 30 °C (Fig. 2). The crude purity of **peptide-a** synthesized *via* Route-3 was above 97% and took only 27 min instead of 108 min *via* Route-1 with a similar outcome. This suggested that the combination of fast stirring and elevated temperature, enabled by the HTFSPS reactor, can accelerate the SPPS process at low reagent concentrations. Interestingly, although the sequence contains two aspartic acids and the process was performed at a high temperature,[17] no significant aspartimide formation was observed. We assume that the combination of low piperidine concentration and a short reaction cycle decreases the probability of this side reaction.

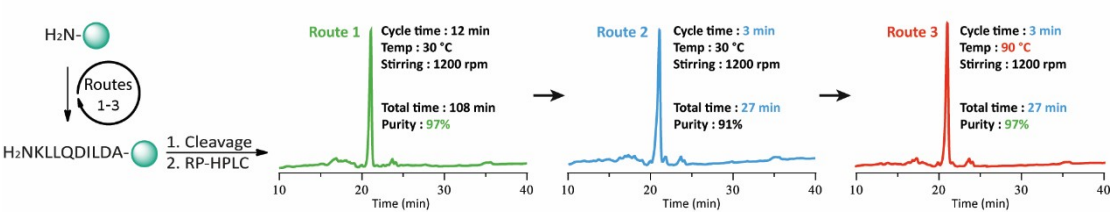


Figure 2: Synthesis of **peptide-a** at HTFSPS reactor via three different synthetic routes.

To check whether the HTFSPS can be used for difficult couplings, a notoriously hard coupling of Fmoc-L-His(Trt)-OH to the tetrapeptide H_2N-Phe-Gly-Trp-Ile was used here as a case study (Fig 1, **HFGWI**).[4] Coupling of Fmoc-L-His(Trt)-OH was performed in the HTFSPS-reactor using the same conditions described in Route-3, and the HPLC analysis confirmed over 85% conversion (ESI section 5.4). The result is very encouraging, as a similar reaction performed for 60 minutes using 700 rpm stirring at room temperature provided 82% conversion.[4] This showed that a combination of elevated temperature and fast stirring can be used instead of extended reaction even for difficult coupling steps, indicating that HTFSPS might surpass other technologies. The joint contribution of elevated temperature and stirring rate in HTFSPS can be used to perform rapid reactions not only for hurdles-free peptide sequences like **peptide-a** but also for difficult-to-synthesize ones.

HTFSPS does not cause significant epimerization

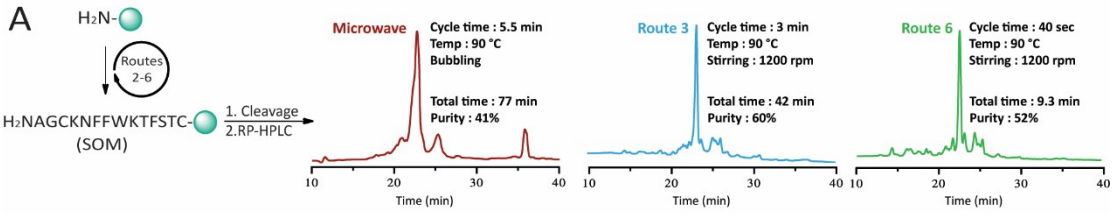
It is well accepted that elevated temperatures during coupling reactions can lead to racemization, especially of histidine, and also of cysteine.[15] To evaluate if HTFSPS results in significant racemization, Fmoc-L-His(Trt)-OH was reacted with the Phe-Gly dipeptide under Route-3 conditions. The HPLC purity of tripeptide Fmoc-**HFG** and Fmoc-**(D)HFG** showed epimerization of less than 3% and 4%, respectively (ESI Sections 5.5-5.7). This specific coupling was chosen since it is prone to epimerization, and is often used as a model case study.[15] Although the prone-to-epimerize Fmoc-L-His(Trt)-OH was used at a high temperature, the degree of epimerization was not significantly higher than in other methods. Two vasopressin-derived peptides **VAS** (with two L-Cys) and **DDVAS** (with two D-Cys) were synthesized using HTFSPS *via* Route-3 to evaluate epimerization of cysteine. HPLC analysis showed that there were no significant traces of **VAS** in **DDVAS** and vice versa (ESI sections 5.8 and 5.9).

These studies indicated that HTFSPS does not result in significant epimerization compared to other methods.[16] We assume that the short time and the absence of pre-heating minimize racemization even at elevated temperatures. The above results confirm that peptides containing His and Cys can be synthesized by HTFSPS without using special building blocks or deviating from the standard cycle protocol maintaining the high temperature.[17]

SOM MODEL

To further push the limits of HTFSPS, the effects of essential parameters (based on Fick's law) were evaluated for the synthesis of a 14-amino acid somatostatin-derived peptide. Somatostatin is an endogenous hormone of the mammalian pituitary gland and is not trivial to synthesize (Fig. 1, **SOM** sequence).[27,28] **SOM** was synthesized here using automated Mw-SPPS at 90 °C by applying 5 equivalents for couplings periods of at least 2 min (Fig 3, Route-Mw). The Mw-SPPS synthesis afforded **SOM** in a purity of 41% indicating that it is a challenging-to-synthesize peptide even using state-of-the-art methods (Fig. 3). Synthesis of **SOM** by HTFSPS *via* Route-3 resulted in a crude purity of 60%, which is significantly higher than by Mw-SPPS (Fig 3). This proves that HTFSPS surpasses state-of-the-art Mw-SPPS even when lower concentrations of reagents and shorter reaction times are used at almost the

same temperature. Since **SOM** proved such a challenging sequence, it was a suitable candidate for evaluating crucial reaction conditions. Synthesizing **SOM** at a low temperature *via* Route-2 did not result in a detectable product (Fig. 3 and ESI section 5.10). Given that the only difference between Route-2 and Route-3 is the temperature, it confirms that using 90 °C is crucial for accelerating the HTFSPS of hard-to-synthesize peptides like **SOM**. It also shows that evaluating the efficiency only with peptides that are easy to synthesize, e.g., **peptide-a**, is not sufficient for optimizing a new strategy. To examine the effect of the mixing rate, **SOM** was synthesized *via* Route-4, which only differs from Route-3 by employing a stirring rate of 100 rpm instead of 1200 rpm (Fig. 3). Synthesis of SOM *via* Route-4 resulted in a 48% purity, which is significantly lower than the purity gains *via* Route-3, showing that stirring rate is also an important factor in addition to the temperature (Fig. 3). Interestingly, synthesizing **SOM** at a low stirring rate via Route-4 was still more beneficial in purity, process time, and reagents quantity, than by Mw-SPPS microwave.



B

SOM conditions	Mixing rate (rpm)	Temperature (°C)	Time per reation (sec)	Equivalents of AA and HATU	Total synthesis duration (min)	Purity (%)
Microwave	0	90	120	5	77	41
Route 2	1200	30	30	2	42	N.D
Route 3	1200	90	30	2	42	60
Route 4	100	90	30	2	42	48
Route 5	1200	90	20	6	18.7	57
Route 6	1200	90	10	6	9.3	52

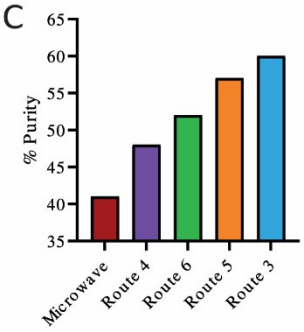


Figure 3: A) analytical HPLC chromatogram of **SOM** synthesis *via* Route-Mw, Route-3, and Route-6. B) The different conditions used in the synthesis of **SOM**. C) Histogram represents the different purities obtained after each synthesis. [a] Routes 2-6 were performed in the HTFSPS reactor.

The above results verify, independently, that the effect of both heating and stirring on **SOM** synthesis outcome is dramatic. It suggests that fast stirring and a high temperature can be used to compensate for low concentration of reagents and/or short the reactions also for peptides that are not easy to synthesize.

After understanding the influence of fast mixing and a high temperature on HTFSPS efficiency, we wanted to check if even shorter reactions are applicable. **SOM** was synthesized *via* HTFSPS Route-5 and Route-6, employing short reaction and washings of twenty and ten seconds, respectively. Six equivalents of amino acids and 20% piperidine were used to compensate for the rapid cycles while the stirring rate, solution volumes, and temperature were identical to Route-3. Synthesis of **SOM** using Route-5 resulted in a crude purity of 57%, which is very close to the one gained using Route-3 (Fig 3). By performing reaction and washing steps at twenty seconds, the entire SPPS process took 1/3 of the time used for the synthesis *via* Route-3, whereas the purity was decreased only by 3%. **SOM** synthesis following Route-6 resulted in a further decrease in purity to 52% (Fig. 3). The synthesis of **SOM** using Route-6 presents the shortest HTFSPS cycles, as the total time required to introduce each amino acid was 40 seconds, while the payment in purity is reasonable. This proved that a combination of fast stirring, high temperature and slightly increasing the reagent concentrations could compensate for the short reaction time. Comparing **SOM** synthesis *via* Route-3 and Route-5 showed that time and concentrations are somehow interchangeable, indicating that all parameters in Ficks' second law must be considered when accelerating SPPS. The reasonable purity obtained in the synthesis of **SOM** *via* Route-5 and Route-6 proved that HTFSPS can be applied for fast synthesis of even difficult-to-synthesize peptides. HTFSPS *via* Route-6 provided accessibility to peptides in record time, offering a reasonably green and cost-efficient strategy.

To confirm that short cycles can be used for other peptides, we selected a completely random peptide, **MARADONA**, and synthesized it *via* Route-6 in a crude purity of above 80% (ESI section 5.16). This model is the ultimate case study since we had no previous knowledge of the complexity or difficulty of the sequence and it is composed of a variety of canonical and

non-canonical amino acids. Route-6 protocol was not optimized for **MARADONA**, yet the octapeptide was obtained in sufficient purity in a process that took less than ten minutes, thus demonstrating the generality of HTFSPS.

The above examples highlight the effect of conditions and setup on the SPPS process, in light of Ficks' second law of diffusion. The results indicate that accelerating SPPS can be done by designing a reactor and process that maximizes the contribution of all parameters and not only by employing a high concentration of reagents. HSS-SPPS and HTFSPS are the only methods reported to date which take advantage of fast overhead mixing (over 600 rpm) of both reagents and support for improving peptide synthesis processes. Compared to HSS-SPPS, HTFSPS benefits from the contribution of heating which allows acceleration of the process. High temperature increases diffusion and reaction kinetics, but might also result in side reactions like epimerization and aspartimide formation. In the examples shown here, these side reactions seem to be subsided in HTFSPS because of the short reactions, the use of low base concentration, and avoiding preactivation at high temperatures (frequently applied in other systems). This suggests that the process can be performed without changing the temperature between steps which is a unique and practical advantage over other setups. The ability to decrease reagent excess, shorten reaction time and avoid undesired side reactions benefits directly from the high efficiency of fast overhead stirring. It is important to note that in all processes described above only standard Fmoc protected amino acids were used. In each HTFSPS example, the same activator, base, mixing setup were used for all steps of the synthesis. No special additives, solvents, or amino acid protecting groups were used to avoid side products. Unlike fixed-bed setups, beads swelling and size increase during peptide elongation does not pose a limitation in HTFSPS hence enabled the use of a high loading resin. Using high-loading resin allowed maximizing the output from each process, using high reagents concentrations without increasing the molar excess and minimizing the volume of solvents.

Conclusion

Our study proves that both temperature and mixing are crucial for accelerating SPPS. Using large quantities of reagents as a default strategy to accelerate SPPS was replaced here by fast mixing, aiming at generating a high local concentration around the beads at a short time. By using the HTFSPS reactor, which employs concomitant fast stirring and heating, we shortened reaction time to seconds. This enabled the synthesis of small-to-medium peptides of various levels of complexity within minutes while maintaining low reagent quantities. A green, cost-efficient, and fast SPPS process was developed, which does not require the use of elaborated machinery or a special set of conditions. Performing all steps at a constant temperature and the stirring rate is key for facilitating the process. HTFSPS setup is generic, which ensures that accelerated SPPS can be done at any standard laboratory.

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