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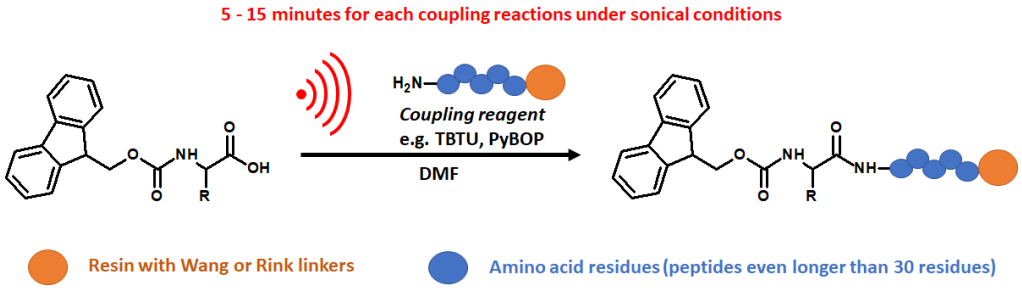

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The faster peptide synthesis on the solid phase using ultrasonic agitation

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ABSTRACT

SOLID PHASE-SUPPORTED SYNTHESIS IS A WIDELY USED STRATEGY IN PEPTIDE CHEMISTRY. THE FACTOR WHICH LIMITS THE PURITY OF THE PRODUCT IS THE INDIVIDUAL STAGES YIELDS. HERE, WE REPORTED THAT THE USE OF ULTRASONIC AGITATION ALLOWS TO REDUCE TENFOLD THE TIME OF THE SYNTHESIS IN THE FMOC STRATEGY, AND IMPROVE THE PURITY OF THE FINAL PRODUCT. OUR METHOD IS A PROMISING ALTERNATIVE TO TRADITIONAL SYNTHETIC METHODS AND MICROWAVE SYNTHESIZERS.

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Introduction

Similarly to microwaves, ultrasound is regarded as a green activation technique. However, in contrast to microwaves, influence of ultrasounds on chemical systems is still poorly understood. Ultrasonic irradiation (consisting of pressure waves that are not quantized) is not absorbed by single molecules, though it is partially converted into heat due to the nonlinear phenomenon of cavitation [1]. In recent years, interest in the use of ultrasound in organic synthesis has increased [2]. The use of ultrasound allows in some cases to reduce drastically the reaction time and implement of processes not available in other ways, and may give a better selectivity. The early peak of interest in the use of ultrasound in chemistry took place in the late 70s and 80s, and at this time Shimonishi *et al.* reported the first solid phase peptide synthesis using ultrasonic waves [3]. Later, Shaw *et al.* demonstrated a new SPPS protocol for sporopollenin (the outer, highly resistant exine of *Lycopodium clavatum* spores) using a sonical agitation [4]. Other applications of sonical agitation in broadly understood peptide chemistry have been also reported, *e.g.* attaching of the first residue to the Merrifield resin [5], cleavage from the resin [6] or synthesis of various kinds of peptidomimetics in solution [7]. Despite a drastic reduction of the reaction times, to the best of our knowledge Shimonishi's and Shaw's works still remain the only readily available data on the direct use of ultrasound in the solid phase peptide synthesis. The reasons of the lack of interest in the sonical solid phase peptide synthesis can be explained by focusing of the scientific community on the chemical aspects of the Fmoc/^{t}Bu strategy when the two works were published and the later development of microwave techniques. The results obtained more than 30 years ago require a reminder and re-validation today. Here, we demonstrate the solid phase peptide synthesis with the Fmoc/^{t}Bu strategy on commercially available solid supports, accelerated by the ultrasonic agitation. We used a common ultrasonic bath available in most laboratories all over the world, what makes our method readily accessible for everyone interested.

Results and discussion

The temperature of water in the ultrasonic bath was maintained between 25 and 30 °C, and the room temperature was at similar level. The cavitation phenomenon causes a lot of local temperature increasing, which provides to heating the whole solution. Therefore, the temperature of the water bath was monitored after each cycle of sonication. If the temperature was higher than 30 °C, we exchanged water in the bath but you can use any type of a circulator with a cooling instead of doing that. All of the sonical peptide syntheses experiments were performed in the sonical cleaning bath obtained from Bandelin (Germany) - SONOREX DIGITEC DT 103 H with ultrasonic peak output: 560 W (it corresponds to 4 times ultrasonic nominal output); and ultrasonic frequency: 35 kHz.

At the first stage, we optimized the times of both coupling reaction and Fmoc deprotection at sonical conditions. As we found by spectrophotometric determination of piperidine-dibenzofulvene adduct, the Fmoc group was fully removed from the Fmoc-Val-Wang polystyrene resin after 1 min of sonication in 25% piperidine in DMF. The coupling reaction was checked by coupling of Fmoc-Val-OH (3 eq.) using TBTU (3 eq.) and DIEA (6 eq.) to the H-Val-Wang resin. The conditions were optimized for 20 mg of resin with loading of 0.7 mmol/g. A portion of the Fmoc-Val-Wang resin was placed in 2 mL syringe reactor and swelled for 5 min with sonication before Fmoc deprotection. The syringe reactors were equipped with a ring made of polyurethane foam enables to float like a bobber fishing. Concentration of Fmoc-Val-OH in the coupling mixture was 0.1M. Yield of the reaction was determined spectrophotometrically by measuring the amount of the dibenzofulvene adduct released from the Fmoc-Val-Val-Wang

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resin. The spectra measured for samples with the coupling time longer than 5 min have the same intensities (after normalization) in the measured range between 270 and 320 nm, what means that 5 minutes of sonication is enough for a successful coupling.

To determine the influence of sonical agitation on the efficiency of solid phase peptide synthesis, we carried out a comparative studies on a series of model peptides, including so called difficult sequences. During synthesis of longer peptides we increased the coupling time using sonical agitation to 10 minutes, and the time of sonication in 25% piperidine in DMF to 2 minutes. Between subsequent synthetic steps (after coupling or Fmoc deprotection), the resin was washed successively with DMF. As a standard protocol we used a procedure, commonly used in our research team, with the coupling time of 2 hours and Fmoc deprotection performed by incubation of the resin in 25% piperidine for 25 minutes at room temperature in a syringe reactor placed on the rotator. In all the experiments, the concentration of Fmoc amino acid derivatives in the coupling mixture was 0.15-0.20 M.

One of the simplest model peptides chosen for comparative studies was Met-enkephalin (H-YGGFM-OH). This choice was made to determine if the sonication conditions can cause oxidation of Met to Met(O), as it is known that during a sonication free radicals may be formed (especially at higher frequencies) [1,2,8]. Both syntheses were performed on a Fmoc-Met-Wang polystyrene resin with loading of 0.7 mmol/g. Met-enkephalin synthesized ultrasonically does not differ from that obtained in the classical way. In both cases we found traces of the oxidized form in the ESI-MS spectra, but the oxidized compound peak intensities in the cases of sonical and classical synthesis are only 2.95 and 2.86%, respectively, of those of the desired product which was derived from the absolute intensities of the monoisotopic peaks. Thus, the sonication does not influence the Met residue oxidation in the peptide. Additionally, at the sonical conditions we obtained the product with a better purity (64%) than in the case of classical synthesis (51%).

As difficult sequences, we chose three peptides described earlier as models during validation of the microwave-assisted solid phased peptide synthesis [9]. One of them is the TAT peptide (Fmoc-GRKKRRQRRRPPQ-NH_2), a cationic cell-penetrating 48-60 fragment of the HIV-1 TAT protein [10], which is difficult to synthesize due to the multiple arginine residues. The peptide was cleaved from the resin with the N-terminal Fmoc group as a chromophore for HPLC analysis with UV detection at 210 nm. During optimization of the TAT peptide synthesis we found that longer time of the coupling reaction at sonical conditions is needed – 20 minutes of agitation. This sequence was the most difficult of our models. Using both an H-Rink amide ChemMatrix® resin and a poly-styrene based Fmoc-Rink-MBHA resin we obtained a much better purity of the TAT peptide synthesized under sonical conditions than using the classical approach (Figure 1).

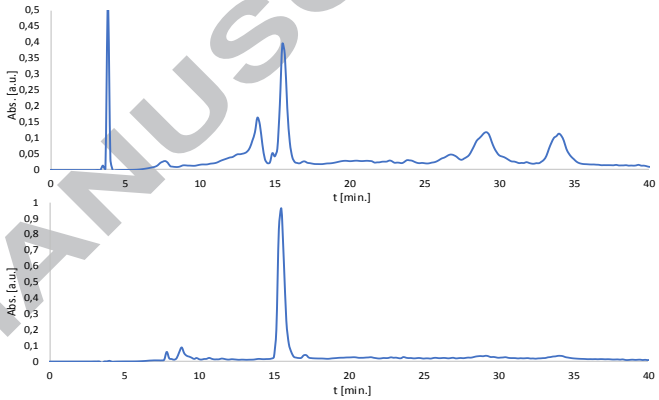

Figure 1. HPLC chromatograms (detection at 210 nm) of crude Fmoc-TAT-NH_2 peptide synthesized classically (top) and sonically (down) on H-Rink amide ChemMatrix® resin.

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Table 1. Comparison between the sonical and the classical solid phase peptide synthesis.

Peptide	Method*	Solid support	Time*[min.]	Purity**[%]
Met-enkephalin	Classical:	Fmoc-Met-Wang, polystyrene, 0.72 mmol/g	600	51,1
Met-enkephalin	Sonical	Fmoc-Met-Wang, polystyrene, 0.72 mmol/g	48	64,4
Fmoc-UB_{33-43}-OCam	Classical	H-Rink ChemMatrix®, 0.4-0.6 mmol/g	1860	71,3
Fmoc-UB_{33-43}-OCam	Sonical	H-Rink ChemMatrix®, 0.4-0.6 mmol/g	237	89,9
Fmoc-TAT_{48-60}-NH_2	Classical	H-Rink ChemMatrix®, 0.4-0.6 mmol/g	1920	24,2
Fmoc-TAT_{48-60}-NH_2	Sonical	H-Rink ChemMatrix®, 0.4-0.6 mmol/g	284	79,4
Fmoc-TAT_{48-60}-NH_2	Classical	Fmoc-Rink MBHA, polystyrene, 0.68 mmol/g	1920	21,8
Fmoc-TAT_{48-60}-NH_2	Sonical	Fmoc-Rink MBHA, polystyrene, 0.68 mmol/g	284	60,6
Ac-$B2m_{58-69}$-NH_2	Classical	H-Rink ChemMatrix®, 0.4-0.6 mmol/g	1860	69,7
Ac-$B2m_{58-69}$-NH_2	Sonical	H-Rink ChemMatrix®, 0.4-0.6 mmol/g	204	82,1
Ac-$B2m_{58-69}$-NH_2	Classical	Fmoc-Rink MBHA, polystyrene, 0.68 mmol/g	1860	39,1
Ac-$B2m_{58-69}$-NH_2	Sonical	Fmoc-Rink MBHA, polystyrene, 0.68 mmol/g	204	51,2
H-ACP_{65-74}-NH_2	Classical	H-Rink ChemMatrix®, 0.4-0.6 mmol/g	1500	64,4
H-ACP_{65-74}-NH_2	Sonical	H-Rink ChemMatrix®, 0.4-0.6 mmol/g	120	74,6
H-ACP_{65-74}-NH_2	Classical	Fmoc-Rink MBHA, polystyrene, 0.68 mmol/g	1500	50,7
H-ACP_{65-74}-NH_2	Sonical	Fmoc-Rink MBHA, polystyrene, 0.68 mmol/g	120	56,8
β-endorphin (31 amino acids)	Sonical	Fmoc-Glu(^{t}Bu)-Wang, polystyrene, 0.64 mmol/g	360	47,8

[a] In all cases the TBTU/DIEA coupling method was used. Classical and sonical method are described in the supplementary materials. Under sonical conditions the syringe with the resin was sonicated in a ultrasonic cleaning bath; in classical conditions the syringe was shaked using rotary shaker.

[b]The total time of synthesis including coupling reactions and Fmoc-deprotections steps, without washing and peptides cleavage from the resin

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The acetylated B2m peptide derived from amyloidogenic human β-2-microglubulin (fragment 58-69) [11] was chosen as a second model. This peptide was synthesized with the 82.1% (ChemMatrix) and 51.2% (MBHA) purity using a sonical agitation in comparison to 69.7% and 39.1%, respectively, using classical SPPS. We also synthesized the acyl carrier protein (ACP) fragment 65-74 (H-VQAAIDYING-NH_2), which is widely used as a standard to demonstrate the solid phase peptide efficiency [9,12]. The ACP peptide was synthesized with a better purity and about ten times faster using a sonical agitation than with the classical approach using both types of resins, H-Rink ChemMatrix® and Fmoc-Rink MBHA. Results of the syntheses are summarized in Table 1.

To demonstrate general applicability of the sonical agitation in solid phase peptide synthesis, we synthesized caboxamidomethyl (Cam) ester of the ubiquitin fragment UB(33-43) cleaved from the resin with *N*-terminal Fmoc group. The peptide Cam esters may be used potentially in the synthesis of larger peptides through peptide fragments enzymatic ligation reactions and head-to-tail cyclizations [13]. The Cam ester was introduced by coupling of bromoacetic acid to the H-Rink ChemMatrix resin using a known method [14]. Then, 3 equiv. Fmoc-Leu-OH and 3 equiv. DIEA were dissolved in DMF and incubated with the resin overnight or sonicated for 15 minutes. However, the synthesis times given in Table 1 include only the time of a peptide synthesis using the TBTU/DIEA method (in this case 9 residues instead of 10). The UB(33-43) sequence contains two Pro residues, which may induce turns hindering attachment of contiguous residues. It makes this synthesis difficult. Although both syntheses were performed on a ChemMatrix® resin dedicated for difficult sequences, the purity of the peptide synthesized sonically was almost twice better than of that obtained with the classical approach.

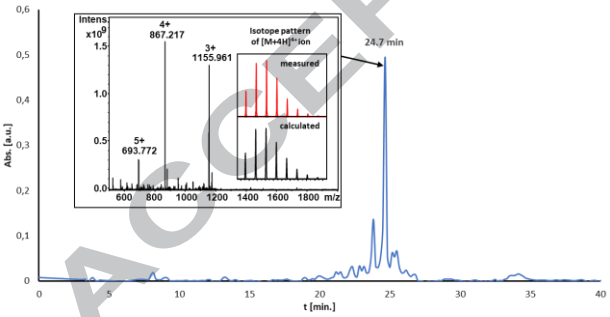

Figure 2. HPLC chromatogram and ESI-MS spectrum of crude β-endorphin synthesized using the sonical solid phase peptide synthesis method.

β-Endorphin was synthesized sonically as an example of longer peptides. The synthesis was performed on a Fmoc-Glu(^{t}But)-Wang polystyrene resin with loading of 0.65 mmol/g, using 3- fold excess of reagents during each coupling step. The purity of a crude peptide (synthesized using 10 minutes of sonical agitation *per* each residue coupling reaction) is around 50% (Figure 2). This peptide was obtained using our sonical manual solid phase peptide synthesis method in about 8 hours, showing that peptides containing 30 amino acid residues may be synthesized manually in one workday. Nowadays, we do not have automatic peptide synthesizers based on the ultrasound technology. However, if one reviews carefully the development of microwave peptide synthesis in recent years, it can be expected that the automation of the sonical synthesis of peptides will lead in the future to equally drastic reduction of synthesis times and increase of the purity of raw products.

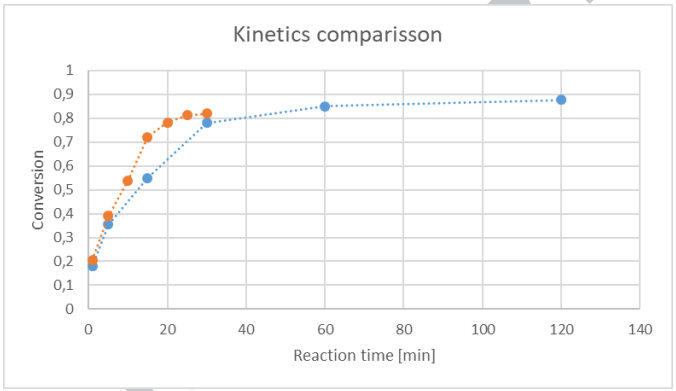

Figure 3. Direct comparison of the coupling reaction kinetics using a sonical agitation (orange) and a classical approach (blue).

Direct comparison of the coupling efficiency using standard mixing and ultrasound agitation was carried out on example of one of the TAT peptide synthesis steps. The choice was dictated by difficulties during synthesis of this peptide. The coupling of Fmoc-Arg(Pbf)-OH to the H-Arg(Pbf)-Pro-Pro-Gln(Trt)-Rink MBHA resin was chosen as a model coupling reaction. 300 mg of the H-Arg(Pbf)-Pro-Pro-Gln(Trt)-Rink MBHA resin with loading of 0.68 mmol/g were prepared using a sonical agitation. After drying, the resin was splitted into 20 mg portions. Then, the samples were incubated with a coupling mixture (3 equiv. Fmoc-Xaa-OH – 0.08M, 3 equiv. TBTU, 6 equiv. DIEA in 500μL DMF) for various time periods using the rotator as a shaker or sonical agitation. After a coupling step, the resin was treated immediately with 25% piperidine in DMF to quench the reaction and remove the Fmoc group. Then, the peptidyl-resin was derivatized using 2,4-dinitrofluorobenzen[§]. After cleavage from the resin with a subsequent evaporation under nitrogen and lyophilization, the samples were dissolved in 10mL of acetonitrile/water (50:50) and analysed using HPLC with the UV detection at 360 nm. The time dependence of coupling yields shows directly that the sonical agitation accelerates the reaction (Figure 3). However, the coupling was not quantitative even after 24 hours of coupling at room temperature and plateau of conversion obtained using sonical agitation is lower than for the classical approach. It may be explained by two factors. First, the competition between the coupling reaction and formation of lactam from the activated arginine derivative surely have a significant contribution to a lower "sonical" yield. Second, sonication may increase both reactions rates, causing a faster reduction of the active arginine derivative concentration. This disadvantage may be omitted by coupling repetitions for difficult amino acid

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derivatives. Nonetheless, we obtained good purities of the TAT peptides synthesized sonically. However, during the synthesis of model peptides we used twice more concentrated solutions of coupling reagents which is easier to do with a larger portion of a resin.

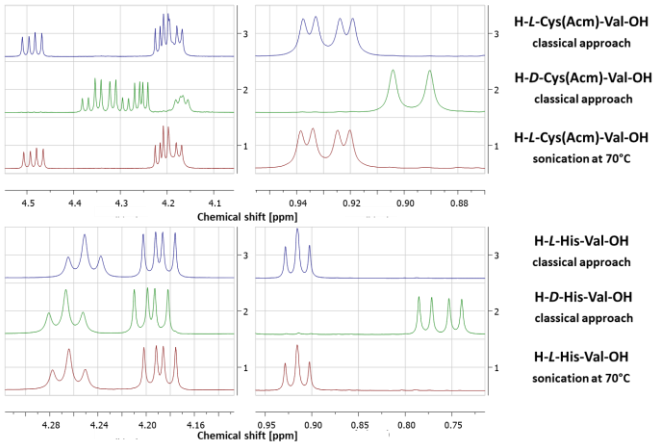

Figure 4. The investigation of racemization. Comparison of significant ^{1}H NMR spectra of models peptides synthesized manually using the classical approach at room temperature *vs* sonical method at elevated temperature. Chemical shifts of His and Cys α-protons and methylene group of Acm (left panels), and γ-methyl protons of Val (right panels) show that sonication at 70°C does not cause racemization.

One of the most important factors describing the efficiency of solid phase peptide synthesis is lack of racemization during coupling reactions, leading to only one desired diastereoisomer of the peptide. We investigated the influence of sonical agitation at room and higher temperatures on racemization. The racemization of His and Cys, the most susceptible residues [15], was tested during dipeptides H-Cys(Acm)-Val-OH and H-His(Trt)-Val-OH syntheses. The peptides were synthesized on a Fmoc-Val-Wang polystyrene resin. Both diasteroisomers were synthesized using the classical approach as reference samples. The temperature of water in the ultrasonic bath was stabilized at the desired level (30, 50 or 70°C). Then, a freshly prepared coupling mixture was mixed with the portion of the H-Val-Wang resin in a syringe reactor and placed immediately in the ultrasonic bath. Peptides were partially purified using Sep-Pak columns. The racemization was examined using ^{1}H NMR spectroscopy, and the assignment of signals was based on information from the COSY spectra. We did not find any evidence of racemization even at 70°C, for both Cys and His residues (Figure 4). As in the microwave assisted solid phase peptide synthesis, we do not observe an increase of racemization in our method, even in the case of susceptible residues. Nonetheless, a comparison between influences of the sonication and the microwave heating on racemization during coupling reactions using various activation methods needs more detailed studies.

Conclusions

As an ultrasonic source, we used a typical ultrasound bath widespread in today's laboratories. We obtained a reduction of the reaction time and improvement of the product purity in comparison to the standard procedure. Our method is easily accessible to all those interested without the need to purchase an additional equipment. We showed also that a sonication even at the elevated temperature (70°C) is free of racemization. The sonical solid phase peptide synthesis began to be used in our team as a routine method of synthesis of peptides and peptide conjugates (examples are available in Supplementary Material, section *6. Additional experimental data*). Its main advantage in relation to the microwave synthesis is the ability to run many parallel syntheses using a simpler source of energy (ultrasonic bath).

Conflicts of interest

There are no conflicts to declare.

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§ 2,4-dinitrophenyl (DNP) derivatives of peptides are commonly used in HPLC analysis with UV detection.

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- Sonication accelerates couplings in solid phase peptide synthesis
- Sonical peptide synthesis gives products with higher purity than classical approach
- Sonication do not cause the racemization of sensible residues (Cys, His)

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