RSC Advances

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2026, **16**, 18073

Nanoassembly-enabled aqueous solid-phase peptide synthesis (ASPPS): a practical DMF-free approach based on the Fmoc strategy

Keiko Hojo, *[ab] Ayumi Nonaka,[a] Yuki Manabe,[a] Cédric Rentier,[c] Amit Mehrotra, [d] Kazuhito Hioki[ab] and Munetaka Kunishima *[ab]

Received 27th January 2026
Accepted 27th March 2026

DOI: 10.1039/d6ra00715e

rsc.li/rsc-advances

The urgent need for sustainable peptide manufacturing has accelerated efforts to replace conventional *N*,*N*-dimethylformamide (DMF)-based solid-phase peptide synthesis (SPPS) with greener alternatives. Here, we present a practical SPPS protocol that uses water as the reaction medium and eliminates hazardous organic solvents. The method leverages nanoassemblies formed from Fmoc-protected amino acids, coupling reagents, and bases, which create reactive interfacial microenvironments that enhance local concentration and promote efficient peptide bond formation under aqueous conditions. These nanoassemblies are readily prepared without specialized equipment and are compatible with microwave-assisted coupling, enabling scalability and semi-automation using existing SPPS platforms. Using this approach, we synthesized various peptides, including β-endorphin (31 residues), with yields and purities comparable to those obtained using conventional DMF-based SPPS. By integrating DMF-free chemistry with nanoassembly-driven reactivity, this work introduces a reaction-field-based strategy for peptide synthesis and provides a simple and eco-friendly platform aligned with the principles of green chemistry.

1. Introduction

Regulatory restrictions on the use and disposal of organic solvents have become increasingly stringent in recent years, driven by growing environmental and health concerns. Within the framework of green and sustainable chemistry,[1] the development of safer, non-hazardous alternatives to conventional solvents is urgently needed.[2] Peptides have attracted considerable attention across diverse fields, particularly in pharmaceutical development,[3–5] where their chemical synthesis is well established. However, conventional peptide synthesis methods were not designed with environmental sustainability in mind and continue to rely heavily on *N*,*N*-dimethylformamide (DMF) as a solvent. DMF is known to cause soil contamination and was classified as a hazardous substance under the EU's REACH regulation in 2021, leading to drastic restrictions and exposure limits for DMF in the EU, as of December 2023.[6]

Against this backdrop, there is a strong demand for environmentally friendly peptide synthesis technologies that eliminate the use of DMF. This has spurred increased efforts to develop water-based peptide synthesis methods. However, none of the reported water-based approaches have achieved broad applicability, primarily due to limitations in efficiency and compatibility with standard amino acid derivatives.

There are several methods of chemical peptide synthesis, including liquid-phase synthesis, solid-phase synthesis and tagged liquid-phase synthesis. Among these, solid-phase peptide synthesis (SPPS)[7] is the most widely used, owing to its simplicity, ease of automation, and suitability for diverse peptide sequences.[8,9] Therefore, we focused our efforts on developing an environmentally friendly SPPS protocol using water, aiming to provide a practical and sustainable alternative to conventional DMF-based methods. However, efficient peptide synthesis in aqueous SPPS (ASPPS) is challenging due to the poor water solubility of commonly used protected amino acids, such as Fmoc-amino acids.[10] To overcome this limitation, water-soluble protected amino acids have been developed and applied in aqueous synthesis.[11–18] Unfortunately, these derivatives often suffer from low stability and slow reaction rates, which restrict the range of peptides that can be synthesized and result in low product crude purity.

Although Fmoc-protected amino acids are generally considered unsuitable for aqueous reactions due to their poor solubility, recent studies have begun to explore their use in water-based systems. Several peptide chemists have focused on developing new methodologies for aqueous peptide synthesis, often by dissolving Fmoc-amino acids in water using organic co-

[a]*Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe 650-8586, Japan. E-mail: hojo@pharm.kobegakuin.ac.jp; kunisima@pharm.kobegakuin.ac.jp*
[b]*Cooperative Research Center of Life Sciences, Kobe Gakuin University, Kobe 650-8586, Japan*
[c]*Biotage Japan Ltd., Tokyo 136-0071, Japan*
[d]*Biotage Sweden AB, Uppsala 753 18, Sweden*

solvents or surfactants, as reported by Albericio and co-workers.[19] While these methods enable aqueous peptide synthesis, they still require significant amounts of organic additives and exhibit slow reaction kinetics, limiting their environmental benefits.

To overcome these challenges, we adopted a different strategy: instead of dissolving protected amino acids in water, we leveraged their inherent insolubility. Previously, we proposed the use of water-dispersible nanoparticles composed of Fmoc-protected amino acids for ASPPS.[20–24] This approach enabled efficient peptide synthesis under aqueous conditions while maintaining compatibility with the Fmoc strategy. Notably, we observed a unique reaction acceleration phenomenon: when protected amino acids were used as nanoparticles without dissolving in water, the reaction proceeded more efficiently than with water-soluble derivatives. Additionally, this method suppressed racemization during coupling. Despite these advantages, the nanoparticle-based method has limitations that hinder its general applicability. The preparation of nanoparticles requires wet grinding using a planetary ball mill, and the resulting particles are relatively large (~300 nm), leading to transient dispersion and eventual aggregation in water. These characteristics make the method unsuitable for automation and large-scale synthesis.

Here, we report an ASPPS method that utilizes nanoassemblies formed by mixing water-insoluble Fmoc-protected amino acids with water-soluble coupling reagents and bases. These nanoassemblies provide reactive interfacial microenvironments, exhibiting a reaction-acceleration effect comparable to nanoparticle-mediated systems, and enabling rapid and efficient peptide synthesis without hazardous DMF or energy-intensive milling. We demonstrate applicability across various peptides, including β-endorphin (31 residues). Recent studies[25] have demonstrated ASPPS *via* salt-based solubilization of Fmoc building blocks; in contrast, our approach operates in a nanoassembled, non-molecularly dissolved state (supported by Tyndall effect and DLS analysis; see SI), which constitutes a fundamentally different reaction environment. This interfacial nanoassembly is critical for maintaining both high reactivity and automation robustness, including line compatibility in flow or microwave-assisted systems. A concise comparison is provided in Table S1. While we avoid assigning a single definitive mechanism, the observed behavior is consistent with on-water-like interfacial reactivity. Overall, this nanoassembly-based approach achieves high efficiency while eliminating hazardous DMF and avoiding specialized equipment or additional additives, offering a scalable and sustainable platform for next-generation peptide synthesis in line with green chemistry principles.

2. Experimental

2.1 Material and methods

Fmoc-amino acids were obtained from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Reagents and solvents were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Particle size was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZSP (Malvern Panalytical Ltd., Malvern, U.K.). Microwave (MW) reactions were performed using a Biotage® Initiator + SP Wave system (Biotage AB, Uppsala, Sweden). Reversed-phase HPLC was carried out on a Waters Alliance e2695 system (Waters Corp., Milford, MA, U.S.A.) equipped with a COSMOSIL 5C18-AR-II column (Nacalai Tesque Inc., Kyoto, Japan) using a gradient of acetonitrile/water containing 0.1% trifluoroacetic acid (TFA). Mass spectra were recorded on an electrospray ionization quadrupole time-of-flight mass spectrometer (ESI-Q-TOF-MS), micrOTOF-Q (Bruker Daltonik GmbH, Bremen, Germany). Detailed HPLC analysis, integration criteria, and ESI-MS acquisition protocols are provided in the Supporting Information.

2.2 General procedure for preparation of nanoassemblies of Fmoc-amino acids containing coupling reagents and bases

Nanoassemblies of Fmoc-Phe-OH prepared with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM)[26,27] and NMM (Example): Fmoc-Phe-OH (39 mg, 100 μmol) and 4-methylmorpholine (NMM) (22 μl, 200 μmol) were mixed in a small amount of water using a vortex mixer. After dilution with 5 mL of water, 1 mL of an aqueous solution of DMT-MM (28 mg, 100 μmol) was added and mixed vigorously using a vortex mixer to form aqueous nanoassemblies. Particle size (mean diameter): 12.8 ± 3.24 nm.

2.3 MW assisted in-water coupling reaction study using nanoassemblies of Fmoc-amino acids with coupling reagents and bases

H-Gly-Rink amide-TentaGel resin[28] (46 mg, Gly-content, 25 μmol) was swelled with water, then MW assisted coupling reactions were performed using an Initiator + SP Wave system employing several types of aqueous nanoassemblies of Fmoc-Phe-OH (100 μmol) combined with a coupling reagent and a base. DMT-MM, 1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide tetrafluoroborate (TBTU),[29,30] 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide tetrafluoroborate (TATU),[29,31,32] O-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TCTU)[33] or 1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HBTU)[29,34,35] were evaluated as the coupling reagent (see SI Section 2). *N*,*N*-diisopropylethylamine (DIEA) or NMM were used for the base. Each reaction mixture was heated to 75 °C by microwave irradiation and kept for 1–10 min. After MW irradiation, the resins were washed with water and 2-propanol for 3 times. Each resin was checked using the ninhydrin (Kaiser) test. These test results are summarized in Table 1.

2.4 General procedure for microwave-assisted in-water solid-phase peptide synthesis

2.4.1 Leu-enkephalin amide. ASPPS was carried out according to the protocol shown in Table 3. H-Leu-Rink amide-TentaGel resin (100 mg, amino group content, 25 μmol) was swollen in water, and aqueous nanoassemblies (6 mL, 16.7 mM with respect to Fmoc-amino acid) prepared from Fmoc-amino

Table 1 Aqueous coupling study using nanoassemblies of Fmoc-amino acids with coupling reagents

Entry	Coupling reagent	Base	MW temp (°C)	Time (min)	Ninhydrin test
1	DMT-MM	NMM	75	10	—
2	DMT-MM	NMM	75	5	—
3	DMT-MM	NMM	75	3	Slightly+
4	TBTU	DIEA	75	10	—
5	TBTU	DIEA	75	5	—
6	TBTU	DIEA	75	3	—
7	TBTU	DIEA	75	1	Slightly+
8	TATU	DIEA	75	5	—
9	TATU	DIEA	75	3	—
10	TATU	DIEA	75	1	+
11	TCTU	DIEA	75	5	—
12	TCTU	DIEA	75	3	—
13	TCTU	DIEA	75	1	+
14	HBTU	DIEA	75	5	+

acid (100 μmol), TBTU (100 μmol), and DIEA (200 μmol) were coupled sequentially onto the resin. MW-assisted coupling reactions were performed at 75 °C for 10 min using an Initiator$^+$ SP Wave system. Fmoc deprotection was carried out with 20% piperidine–ethyl acetate (EtOAc) solution.[24] After completion of the synthesis, the peptide resin (H-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-PEG-grafted Rink amide resin) was washed with ethanol and dried *in vacuo*. The resin was treated with TFA–triisopropylsilane (TIPS)–water (92 : 4 : 4, 15 mL) for 2 h at room temperature. The resin was removed by filtration, and TFA was evaporated under N_2 stream. Diethyl ether was added to the residue, yielding a white precipitate of crude peptide. This crude peptide was subjected to HPLC analysis (220 nm). A dominant peak was observed at a retention time of 18.4 min with a calculated purity of 96.0%. ESI-MS (TOF) *m*/*z*: 555.3737 $[M + H]^+$ (calcd. for $C_{28}H_{39}N_6O_6$, 555.2931).

2.4.2 Dermorphin. ASPPS was carried out according to the protocol shown in Table 3. H-Ser(*t*Bu)-Rink amide-TentaGel resin (100 mg, amino group content, 25 μmol) was swollen in water, and aqueous nanoassemblies (6 mL, 16.7 mM with respect to Fmoc-amino acid) prepared from Fmoc-amino acid (100 μmol), TBTU (100 μmol), and DIEA (200 μmol) were coupled sequentially onto the resin. MW-assisted coupling reactions were performed at 75 °C for 10 min using an Initiator$^+$ SP Wave system. Fmoc deprotection was carried out with 20% piperidine–EtOAc solution. After completion of the synthesis, the peptide resin (H-Tyr(*t*Bu)- DAla-Phe-Gly-Tyr(*t*Bu)-Pro-Ser(*t*Bu)-Rink amide-TentaGle resin) was washed with ethanol and dried *in vacuo*. The resin was treated with TFA–TIPS–water (92 : 4 : 4, 15 mL) for 2 h at room temperature. The resin was removed by filtration, and TFA was evaporated under N_2 stream. Diethyl ether was added to the residue, yielding a white precipitate of crude peptide. This crude peptide was subjected to HPLC analysis (220 nm). Two peaks at 17.8 and 18.6 min were observed and assigned to *cis*/*trans* conformers, and their combined purity was calculated to be 97%. ESI-MS (TOF) *m*/*z*: 803.3705 $[M + H]^+$ (calcd. for $C_{40}H_{51}N_8O_{10}$, 803.3728).

2.4.3 β-Endorphin. ASPPS was carried out according to the protocol shown in Table 3. H-Glu(*t*Bu)-Rink amide-TentaGel resin (100 mg, amino group content, 25 μmol) was swollen in water, and aqueous nanoassemblies (6 mL, 16.7 mM with respect to Fmoc-amino acid) prepared from Fmoc-amino acid (100 μmol), TBTU (100 μmol), and DIEA (200 μmol) were coupled sequentially onto the resin. MW-assisted coupling reactions were performed at 75 °C for 10 min using an Initiator$^+$ SP Wave system. Fmoc deprotection was carried out with 20% piperidine–EtOAc solution. After completion of the synthesis, the protected peptide resin was washed with ethanol and dried *in vacuo*. The resin was treated with TFA–TIPS–water–thioanisole–2,2′-(ethylenedioxy)diethanethiol (92 : 2:2 : 2 : 2, 5 mL) for 2 h at room temperature. The resin was removed by filtration, and TFA was evaporated under N_2 stream. Ether was added to the residue, yielding a white precipitate of crude peptide. This crude peptide was subjected to HPLC analysis (220 nm). The major peak was observed at a retention time of 26.3 min with a calculated purity of 56%. After HPLC purification, 3.2 mg of pure peptide (TFA salt) was obtained (3.1% isolated yield). Due to partial co-elution with impurities, repeated HPLC purification was required, resulting in a reduced isolated yield. ESI-MS (TOF): *m*/*z* 1733.0737 ($[M + 2H]^{2+}$) and *m*/*z* 1155.7189 ($[M + 3H]^{3+}$) which corresponds to $C_{158}H_{253}N_{40}O_{45}S$ (calculated monoisotopic mass: 3463.8537).

3. Results and discussion

Selecting the most suitable amino acid derivatives is one of the most critical - and occasionally challenging – aspects of peptide synthesis. Currently, Fmoc-protected amino acids are widely used as building blocks in SPPS due to their broad applicability and commercial availability. However, Fmoc-amino acids exhibit poor solubility in water. Meanwhile, micelle-mediated synthesis has gained attention as a promising strategy for aqueous-phase reactions in industrial chemistry.[36] In most reported cases, surfactants form micellar systems that either provide a reaction interface in aqueous media or enhance the solubility of hydrophobic reactants.[37–39] Structurally, Fmoc-amino acids consist of a hydrophobic Fmoc group and a hydrophilic carboxyl group, giving them amphiphilic properties similar to those of surfactants. Inspired by this surfactant-

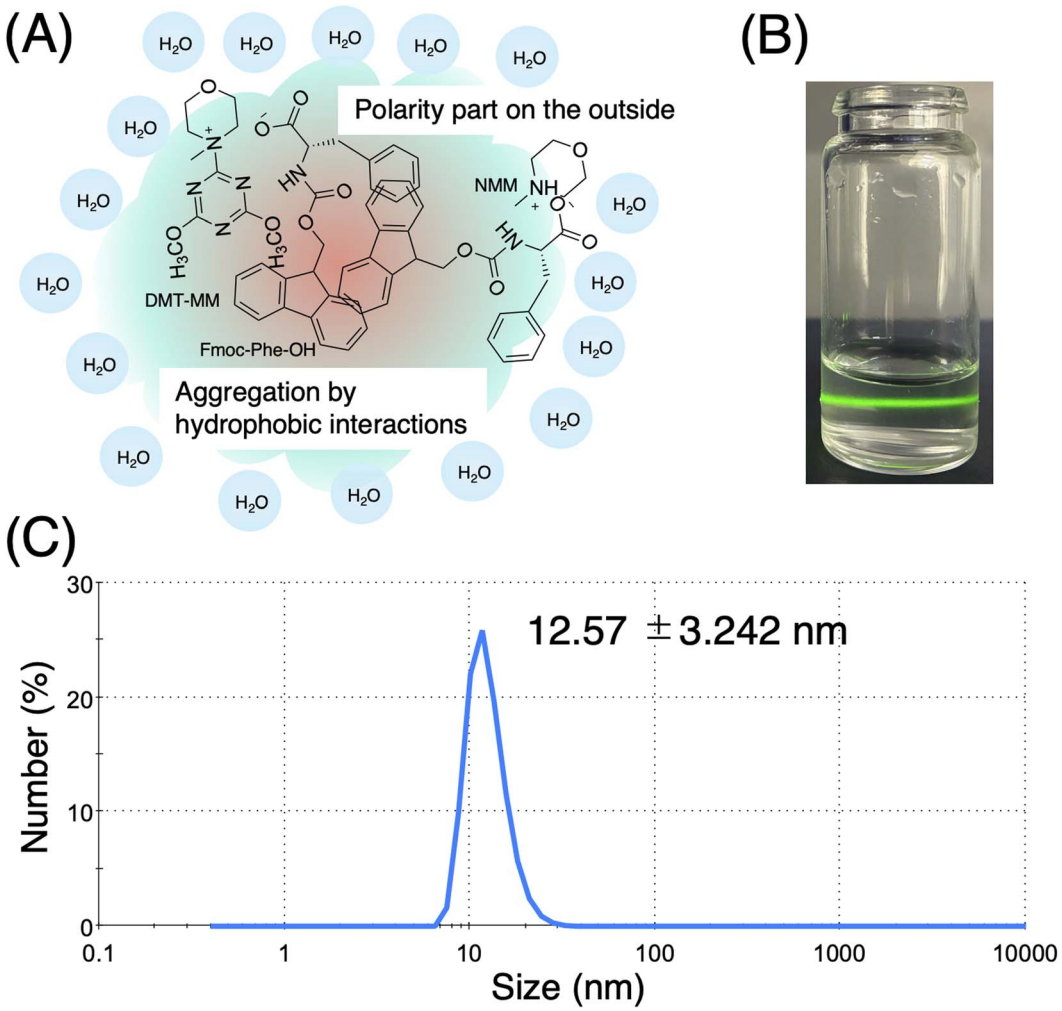

Fig. 1 Aqueous nanoassemblies of Fmoc-Phe-OH with DMT-MM and NMM. (A) Schematic diagram of nanoassemblies; (B) photo image of Tyndall effects by nanoassemblies; (C) particle size distribution (number-weighted) for nanoassemblies showing a sharp peak at 12.57 nm (*Z*-average = 17.96 nm; PdI = 0.085).

like nature, we began exploring peptide synthesis methods that leverage the intrinsic properties of Fmoc-amino acids.

We discovered that mixing water-insoluble Fmoc-protected amino acids with water-soluble coupling reagents and bases in specific ratios leads to the spontaneous formation of nanoassemblies. For example, powdered Fmoc-Phe-OH was blended with two equivalents of NMM, followed by the addition of an aqueous solution of DMT-MM, resulting in the formation of Fmoc-Phe-OH/DMT-MM nanoassemblies (Fig. 1A). Although the solution appeared clear to the naked eye, the Tyndall effect was observed upon laser irradiation, confirming the presence of colloidal particles (Fig. 1B). DLS analysis revealed that these

Table 2 Comparative study of coupling reactions using the DMT-MM/NMM method at room temperature: nanoassemblies of Fmoc-amino acids *versus* water-soluble protected amino acids.[19,21]

Entry	Component	Solvent	Time (min)	Coupling yield (%)	Ninhydrin test
1	Nanoassemblies of Fmoc-Phe-OH	Water	15	Quant.	—
2	Water-dispersible nanoparticles of Fmoc-Phe-OH	Water	15	Quant.	—
3	Sps-Phe-OH[a]	Water (2.0% Triton aq.)	60	ND	+
4	Sps-Phe-OH	Water (2.0% Triton aq.)	180	32	+
5	Sps-Phe-OH	50% ethanol aq.	60	47	+
6	Sps-Phe-OH	50% ethanol aq.	180	Quant.	—

[a] Sps: 2-(4-sulfophenylsulfonyl)ethoxy carbonyl; categorized as a water-soluble and base-lability protecting group for amino acids.

nanoassemblies had an average diameter of 12.8 ± 3.24 nm (Fig. 1C).

To evaluate the feasibility of using these nanoassemblies as building blocks in aqueous peptide synthesis, we conducted a microwave-assisted solid-phase coupling reaction between the Fmoc-Phe-OH nanoassembly (prepared with DMT-MM and NMM) and H-Gly-Rink amide-TentaGel resin. The reaction temperature was maintained between 75 °C using a semi-automated peptide synthesizer (Biotage® Initiator$^+$ SP Wave). Additionally, we prepared nanoassemblies of Fmoc-Phe-OH using various coupling reagents[29] which are highly active and suitable for use *in situ*—TBTU, TATU, TCTU, and HBTU—in combination with appropriate bases. The nanoassembly composed of Fmoc-Phe-OH, (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylaminomorpholinocarbenium hexafluorophosphate (COMU)[29,40] and DIEA could not be prepared because COMU exhibits poor solubility in water. These assemblies were subjected to microwave-assisted coupling reactions, and the results are summarized in Table 1.

All aqueous coupling reactions using nanoassemblies, except those prepared with HBTU, proceeded quantitatively and were completed within 5 minutes. Notably, the nanoassembly formed with TBTU and DIEA exhibited the highest reaction rate, comparable to conventional DMF-based conditions. Also, good results were obtained even when using TATU and TCTU.

The observed acceleration of reactions using nanoassemblies should be attributed to several factors: the local concentration effect, where reactants are confined within microenvironments, the preorientation effect, in which reactive sites are brought into close proximity through hydrophobic and electrostatic interactions, and the dehydration of polar groups, such as carboxyl and amino groups in a hydrophobic environment, which facilitates peptide bond formation. These effects collectively may account for the enhanced reaction efficiency observed in aqueous nanoassembly-based systems. The acceleration observed in our coupling reactions is likely related to the "on-water" mechanism, where water-insoluble substrates react more rapidly at the water–organic interface.[41,42] As discussed by Cortes-Clerget and colleagues, on-water reactions exploit

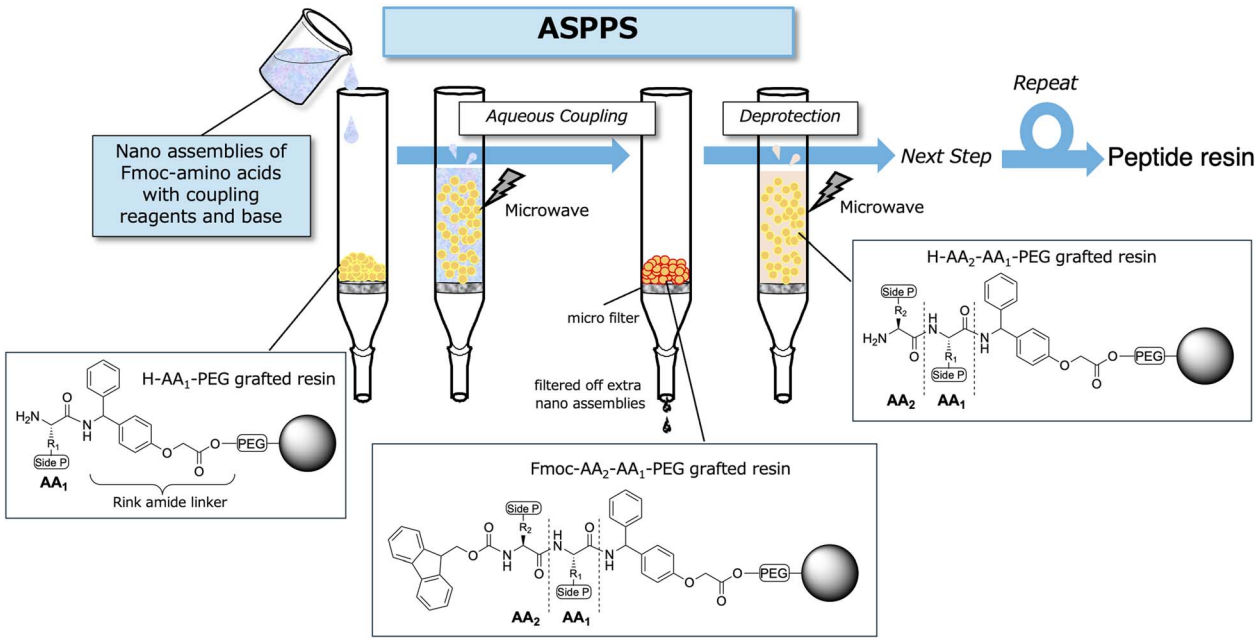

Fig. 2 ASPPS using aqueous nanoassemblies of Fmoc-amino acids.

Table 3 The protocols for ASPPS using nanoassemblies of Fmoc amino acids

Step	Reagents	Volume[a] (mL)	Temp (°C)	Time
Wash	Water	5	25	3 min × 5
Coupling reaction	Nanoassemblies of Fmoc-amino acids with coupling agent and base	6	75	10 min
Wash	Water	5	25	3 min × 5
Wash	EtOAc	5	25	3 min × 3
Deprotection	20% pip-EtOAc	5	25	20 min
Wash	EtOAc	5	25	3 min × 3

[a] The solvent volumes per 100 mg resin.

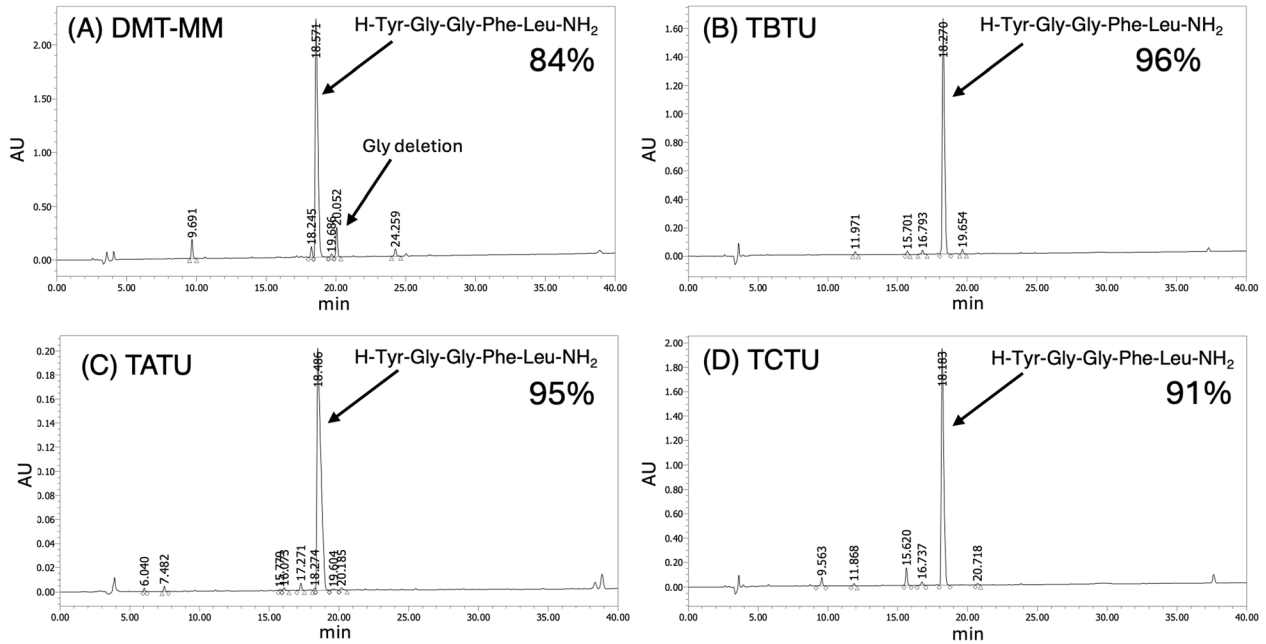

Fig. 3 HPLC profiles of crude Leu-enkephalin amide by ASPPS using nanoassemblies Fmoc-amino acids with (A) DMT-MM and NMM; (B) TBTU and DIEA; (C) TATU and DIEA; (D) TCTU and DIEA. Elution was carried out over 40 min at a flow rate of 1 mL min^{-1} with a linear gradient from 90 : 10 to 50 : 50 mixture of 0.1% aqueous TFA and 0.1% TFA in acetonitrile.

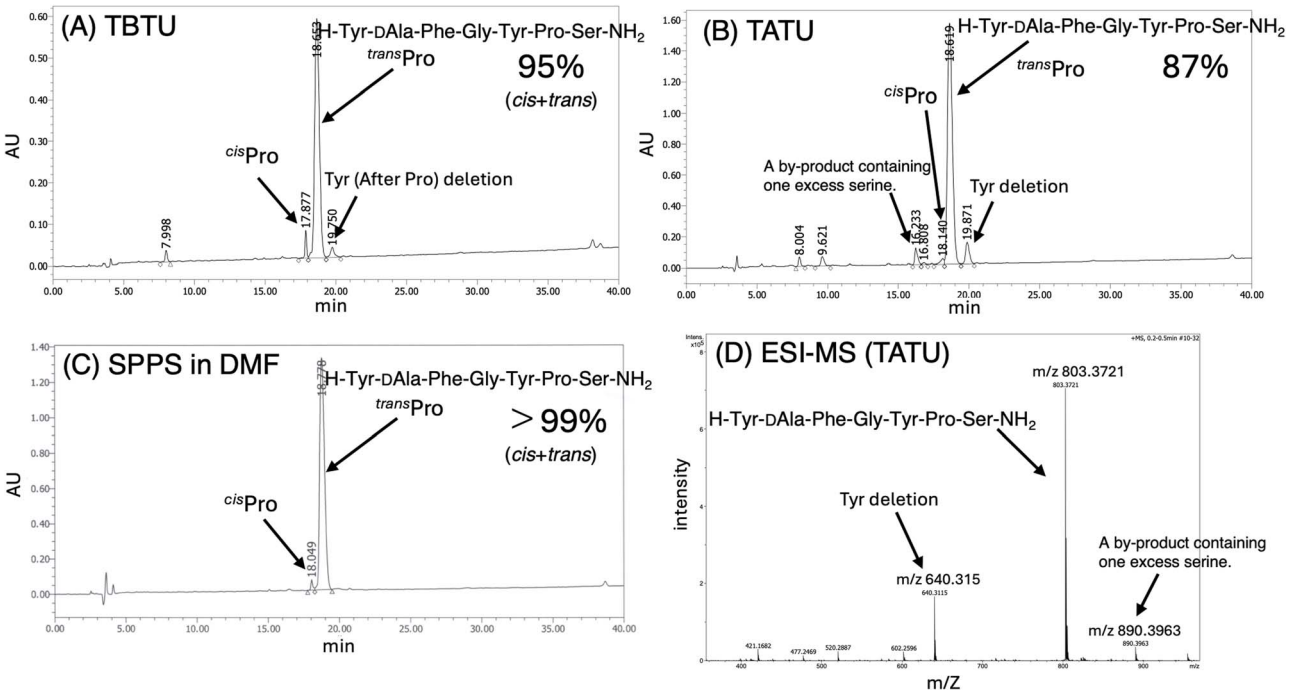

Fig. 4 (A) HPLC profiles of crude dermorphin by ASPPS using nanoassemblies Fmoc-amino acids with TBTU and DIEA. Elution was carried out over 40 min at a flow rate of 1 mL min^{-1} with a linear gradient from 90 : 10 to 50 : 50 mixture of 0.1% aqueous TFA and 0.1% TFA in acetonitrile.; (B) HPLC profiles of crude dermorphin by ASPPS using nanoassemblies Fmoc-amino acids with TATU and DIEA; (C) HPLC profiles of crude dermorphin by conventional SPPS using DIC/HOBt method in DMF. (D) ESI-MS (TOF) spectra of crude dermorphin by ASPPS using nanoassemblies Fmoc-amino acids with TATU and DIEA. The observed mass was *m/z* 803.3705 ($[M + H]^+$), which corresponds to $C_{40}H_{51}N_8O_{10}$ (calculated: 803.3728).

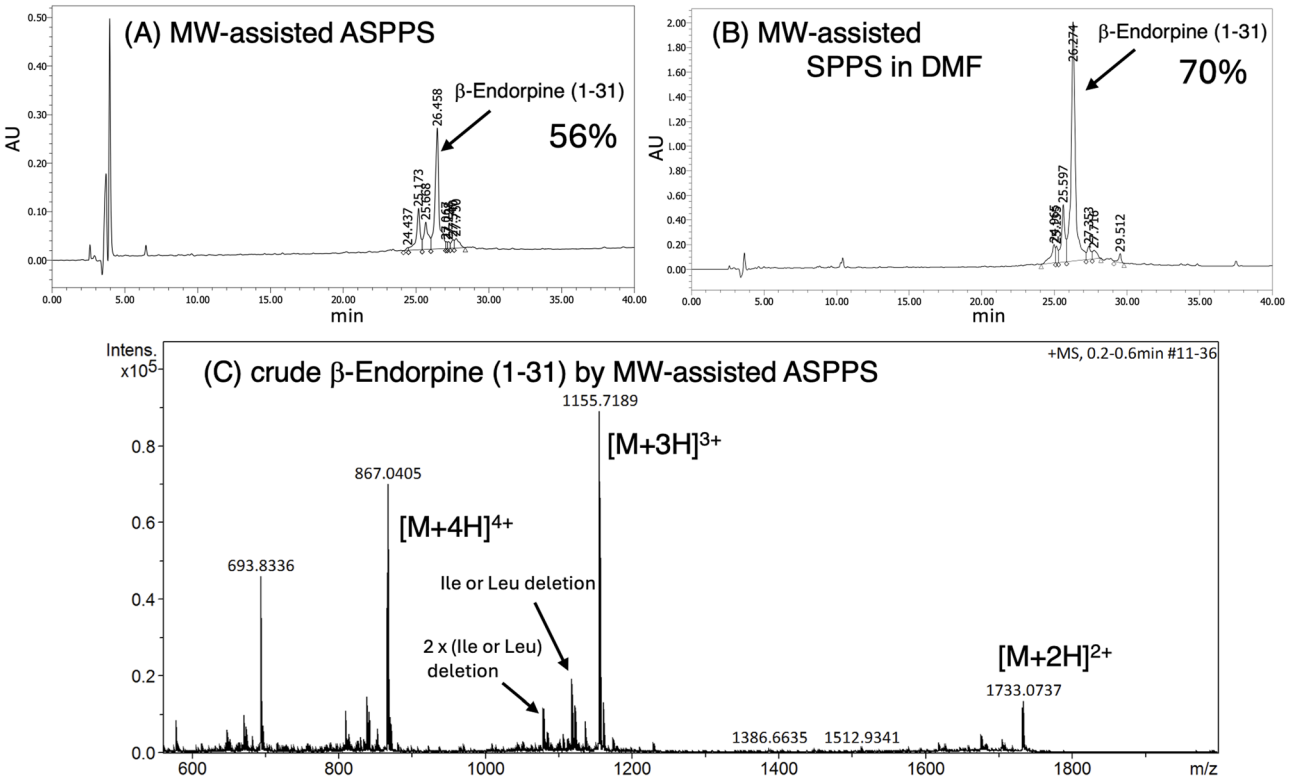

Fig. 5 HPLC profiles of crude β-endorphin (1–31) (A) obtained by ASPPS employing nanoassemblies. Elution was carried out over 40 min at a flow rate of 1 mL min^{-1} with a linear gradient from 90 : 10 to 50 : 50 mixture of 0.1% aqueous TFA and 0.1% TFA in acetonitrile; (B) obtained by conventional SPPS employing Fmoc strategy in DMF; (C) ESI-MS (TOF) spectra of crude β-endorphin (1–31) obtained by ASPPS. The observed doubly charged species was *m/z* 1733.0737 ($[M + 2H]^{2+}$), which corresponds to $C_{158}H_{253}N_{40}O_{45}S$ (calculated monoisotopic mass: 3463.8537). In addition, a triply charged species was detected at *m/z* 1155.7189 ($[M + 3H]^{3+}$), consistent with the same molecular formula.

interfacial hydrogen bonding and hydrophobic interactions to stabilize transition states, in contrast to homogeneous in-water systems where substrates are fully solvated.[43,44] Similarly, the group led by Kunishima demonstrated that micellar interfaces dramatically accelerate bimolecular coupling reactions - up to 2000-fold - through local concentration and preorientational effects.[45,46] While our system does not rely on conventional surfactant-based micelles, the nanoassemblies described here provide analogous hydrophobic microdomains that maintain reactants in a partially dehydrated and spatially localized state. This interfacial nanoassembly environment is therefore considered a key factor underlying the markedly enhanced reaction rates compared to conventional aqueous systems employing fully dissolved, water-soluble protected amino acids (Table 2).[16,21]

Next, we performed ASPPS of a short-chain model peptide, Leu-enkephalin amide (H-Tyr-Gly-Gly-Phe-Leu-NH_2),[47] to evaluate the effectiveness of the nanoassembly-based method (Fig. 2). For the aqueous coupling reactions, several types of nanoassemblies were prepared using water-soluble coupling reagents, including DMT-MM, TBTU, TATU, and TCTU. The model peptide was synthesized according to the aqueous synthesis protocol summarized in Table 3. Pre-loaded resins were employed to decouple sequence-dependent variables from the evaluation of nanoassembly-mediated coupling efficiency (see SI Section 3). Coupling reactions were conducted under microwave irradiation at 75 °C for 10 min. Fmoc deprotection was carried out using 20% piperidine in EtOAc for 20 min at room temperature. EtOAc is considered a green solvent, as it readily hydrolyzes into acetic acid and ethanol in the environment.[48,49] Thus, this protocol eliminates the use of DMF entirely, relying only on water and green solvents, making it more environmentally friendly. However, piperidine is still required in the deprotection step. The nanoassemblies of Fmoc-amino acids with coupling reagents and bases were sequentially coupled onto H-Leu-Rink amide-TentaGel resin. Upon completion of the SPPS cycle, the peptide was cleaved from the resin using TFA. The HPLC analysis results for H-Tyr-Gly-Gly-Phe-Leu-NH_2 are shown in Fig. 3. Chromatographic integration was performed using a uniform protocol (see SI Section 5-1).

In the case of the nanoassembly formed with TBTU, the chromatogram of the crude peptide displayed a single sharp peak (Fig. 3B), indicating excellent purity—comparable to or even exceeding that of conventional SPPS in DMF. Similarly, using nanoassemblies formed with TATU and TCTU also yielded crude peptides with single, well-defined peaks (Fig. 3C and D). In Fig. 3A, using DMT-MM-based nanoassembly produced a chromatogram with a dominant peak corresponding to the target peptide, accompanied by minor byproduct peaks in the latter part of the retention time. The peak at 20.05 min is consistent with YGFL, arising from a glycine-deleted sequence. If the activated form of the protected amino acid becomes

partially dissolved in water, hydrolysis may become more pronounced. The relatively lower reactivity observed for Gly, which lacks a hydrophobic side chain, is consistent with the hypothesis that the reaction proceeds primarily within hydrophobic interfacial microdomains, although we refrain from further mechanistic interpretation at this stage. Importantly, no significant side reactions were detected during microwave-assisted SPPS using our environmentally friendly nanoassembly-based protocol. Furthermore, nanoassemblies formed with uronium-type coupling reagents enabled high-purity peptide synthesis in aqueous conditions. We also synthesized a 7-residue neuropeptide, dermorphin amide (H-Tyr-DAla-Phe-Gly-Tyr-Pro-Ser-NH_2)[50] using nanoassemblies of Fmoc-amino acids formed with TBTU and TATU. In both cases, the HPLC profiles of the crude peptides showed a dominant peak with only minor impurities and no significant detectable by-products or deletion sequences (Fig. 4A and B).

Finally, we attempted the synthesis of a 31-residue peptide, β-endorphin (H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-NH_2),[51] using the same protocol as described above (Table 3) and nanoassemblies formed with TBTU. All coupling reactions were performed as double couplings for 10 minutes at 75 °C. Although minor deletion peaks were observed, β-endorphin was obtained as the main product (Fig. 5A). The MS spectra (Fig. 5C) suggest that these deletion peaks can be attributed to residues such as Ile and Leu, based on the observed mass differences. Some of these residues follow sterically hindered amino acids and correspond to positions known to be challenging for peptide bond formation. These tendencies are consistent with those observed in conventional SPPS. Supporting MS data are provided in Fig. S32 (SI). The chromatogram shows a dominant peak at 26.4 min with a calculated purity of 56%.

To our knowledge, this represents the first successful reported synthesis of a peptide longer than 30 residues using an aqueous coupling method. These results demonstrate that our nanoassembly-based approach is highly effective and broadly applicable, including for the synthesis of long-chain peptides.

4. Conclusion

In this study, we developed a nanoassembly-based approach that utilizes Fmoc-protected amino acids without dissolving them in water as individual molecules, instead forming reactive nanoassemblies with coupling reagents and bases. This strategy leverages the inherent insolubility of protected amino acids to create interfacial reaction fields, which play a key role in accelerating coupling reactions under aqueous conditions. The resulting protocol enables rapid and environmentally friendly SPPS, completely eliminating DMF. Nanoassemblies are easily prepared without specialized equipment and allow efficient microwave-assisted coupling reactions in water. Using this method, we successfully synthesized various peptides, including β-endorphin (31 residues), marking the first demonstration of a peptide exceeding 30 residues synthesized *via* an aqueous coupling strategy. Compared to nanoparticle- or micelle-based approaches, this method avoids the use of surfactants and energy-intensive processes such as wet-milling, and is based on a nanoassembly-driven interfacial reaction environment rather than micellar solubilization, thereby offering advantages in energy efficiency and waste reduction. Importantly, this protocol eliminates hazardous solvents such as DMF and relies only on water and green solvents, aligning with the principles of green chemistry. Although quantitative environmental metrics such as E-factor were not determined in this study, the significant reduction in organic solvent usage strongly indicates improved sustainability compared to conventional SPPS. Future work will include comprehensive green chemistry assessments to further validate these benefits. Overall, the nanoassembly-based approach provides a simple, scalable, and environmentally benign platform for peptide synthesis, with strong potential to advance sustainable manufacturing in peptide chemistry.

Author contributions

K. Hojo: writing-original draft, conceptualization, investigation, and funding acquisition. A. Nonaka: peptide synthesis and analytical measurement. Y. Manabe: peptide synthesis and analytical measurement. C. Rentier: scientific and technical input, review & editing. A. Mehrotra: scientific and technical input, review & editing. K. Hioki: investigation and review & editing, M. Kunishima: review & editing and supervision.

Conflicts of interest

C. Rentier is an employee of Biotage Japan Ltd. A. Mehrotra is an employee of Biotage Sweden AB. The authors declare that related patent applications (WO2024-143052 and JP Application No. 2025-149977) exist concerning the aqueous nanoassembly methodology. K. Hojo is named as an inventor on these applications.

Data availability

All data supporting the findings of this study are available within the article and its supplementary information (SI). Supplementary information: particle size of nanoassemblies (DLS analysis data) and peptide characterization (ESI-Q-TOF-MS spectra and analytical HPLC profiles). See DOI: https://doi.org/10.1039/d6ra00715e.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number JP24K09739. This work was also supported by Adaptable and Seamless Technology transfer Program through Target-driven R&D (A-STEP) from Japan Science and Technology Agency (JST) Japan Grant Number JPMJTR25TD. The authors would like to thank Kirara Mizoguchi and Suzuko Fujiwara for their help with the execution of the chemical reactions performed in this study. We thank Dr Toru Ando and Prof. Hideki Ichikawa for

kindly allowing us to use the Zetasizer Nano ZSP spectrophotometer.

References

1 P. Anastas and N. Eghbali, Green Chemistry: Principles and Practice, *Chem. Soc. Rev.*, 2010, **39**, 301–312, DOI: 10.1039/B918763B.
2 E. C. Thompson, P. Anastas, H. Bialk, D. D'Alessandro, V. P. Hoven, T. J. Kedwards, Z. Liu, A. V. Mudring, K. Saito, V. Z. Zeidler and G. Daher, Advancing green chemistry performance assessment: the Estée Lauder Companies' continuing journey towards meaningful transparency, *Green Chem.*, 2025, **27**, 5015–5026, DOI: 10.1039/D4GC04670F.
3 W. Xiao, W. Jiang, Z. Chen, Y. Huang, J. Mao, W. Zheng, Y. Hu and J. Shi, Advance in peptide-based drug development: delivery platforms, therapeutics and vaccines, *Signal Transduction Targeted Ther.*, 2025, **10**, 74, DOI: 10.1038/s41392-024-02107-5.
4 L. Wang, N. Wang, W. Zhang, X. Cheng, Z. Yan, G. Shao, X. Wang, R. Wang and C. Fu, Therapeutic peptides: current applications and future directions, *Signal Transduction Targeted Ther.*, 2022, **7**, 48, DOI: 10.1038/s41392-022-00904-4.
5 M. Muttenthaler, G. F. King, D. J. Adams and P. F. Alewood, Trends in peptide drug discovery, *Nat. Rev. Drug Discovery*, 2021, **20**, 309–325, DOI: 10.1038/s41573-020-00135-8.
6 J. Sherwood, F. Albericio and B. G. de la Torre, *N,N*-Dimethyl formamide European restriction demands solvent substitution in research and development, *ChemSusChem*, 2024, **17**, e202301639, DOI: 10.1002/cssc.202301639.
7 R. B. Merrifield, Solid phase peptide synthesis. I. The synthesis of a tetrapeptide, *J. Am. Chem. Soc.*, 1963, **85**, 2149–2154, DOI: 10.1021/ja00897a025.
8 R. Behrendt, P. White and J. Offer, Advances in Fmoc solid-phase peptide synthesis, *J. Pept. Sci.*, 2016, **22**, 4–27, DOI: 10.1002/psc.2836.
9 R. Sheppard, The fluorenylmethoxycarbonyl group in solid phase synthesis, *J. Pept. Sci.*, 2003, **9**, 545–552, DOI: 10.1002/psc.479.
10 L. A. Carpino and G. Y. Han, The 9-fluorenylmethoxycarbonyl amino-protecting group, *J. Org. Chem.*, 1972, **37**, 3404–3409, DOI: 10.1021/jo00795a005.
11 R. B. Merrifield and A. E. Bach, 9-(2-Sulfo) fluorenylmethyloxycarbonyl chloride, a new reagent for the purification of synthetic peptides, *J. Org. Chem.*, 1978, **43**, 4808–4816, DOI: 10.1021/jo00419a021.
12 K. Hojo, M. Maeda and K. Kawasaki, A new water-soluble *N*-protecting group, 2-(phenyl(methyl)sulfonio) ethyloxycarbonyl tetrafluoroborate, and its application to solid phase peptide synthesis in water, *J. Pept. Sci.*, 2001, **7**, 615–618, DOI: 10.1002/psc.361.
13 K. Hojo, M. Maeda, Y. Takahara, S. Yamamoto and K. Kawasaki, A new reagent, 2-[phenyl(methyl)sulfonio] ethyl-4-nitrophenyl carbonate tetrafluoroborate (Pms-ONp), for preparing water-soluble *N*-protected amino acids, *Tetrahedron Lett.*, 2003, **44**, 2849–2851, DOI: 10.1016/S0040-4039(03)00538-0.
14 K. Hojo, M. Maeda and K. Kawasaki, Solid-phase peptide synthesis in water. Part 3: A water-soluble N-protecting group, 2-(phenyl(methyl)sulfonio)ethoxycarbonyl tetrafluoroborate, and its application to solid phase peptide synthesis in water, *Tetrahedron*, 2004, **60**, 1875–1886, DOI: 10.1016/j.tet.2003.12.036.
15 K. Hojo, M. Maeda and K. Kawasaki, 2-(4-Sulfophenylsulfonyl)ethoxycarbonyl group: a new water-soluble N-protecting group and its application to solid phase peptide synthesis in water, *Tetrahedron Lett.*, 2004, **45**, 9293–9295, DOI: 10.1016/j.tetlet.2004.10.095.
16 K. Hojo, M. Maeda, N. Tanakamaru, K. Mochida and K. Kawasaki, Solid phase peptide synthesis in water VI: evaluation of water-soluble coupling reagents for solid phase peptide synthesis in aqueous media, *Protein Pept. Lett.*, 2006, **13**, 189–192, DOI: 10.2174/092986606775101607.
17 K. Hojo, M. Maeda, T. J. Smith, E. Kita, F. Yamaguchi, S. Yamamoto and K. Kawasaki, Peptide synthesis in water IV. Preparation of *N*-ethanesulfonylethoxycarbonyl (Esc) amino acids and their application to solid phase peptide synthesis, *Chem. Pharm. Bull.*, 2004, **52**, 422–427, DOI: 10.1248/cpb.52.422.
18 S. Knauer, N. Koch, C. Uth, R. Meusinger, O. Avrutina and H. Kolmar, Sustainable peptide synthesis enabled by a transient protecting group, *Angew. Chem., Int. Ed.*, 2020, **59**, 12984–12990, DOI: 10.1002/anie.202003676.
19 A. Phungula, A. Kumar, M. Kaushal, C. Tucker, L. Chen, B. G. de la Torre and F. Albericio, Aqueous solid-phase peptide synthesis (ASPPS) using standard Fmoc/tBu-protected amino acids, *ACS Sustainable Chem. Eng.*, 2025, **13**, 19833–19848, DOI: 10.1021/acssuschemeng.5c09191.
20 K. Hojo, H. Ichikawa, A. Hara, M. Onishi, K. Kawasaki and Y. Fukumori, Aqueous microwave-assisted solid-phase peptide synthesis using Fmoc strategy: in-water synthesis of "difficult sequences", *Protein Pept. Lett.*, 2012, **19**, 1231–1236, DOI: 10.2174/092986612803217114.
21 K. Hojo, H. Ichikawa, M. Maeda, S. Kida, Y. Fukumori and K. Kawasaki, Solid-phase peptide synthesis using nanoparticulate amino acids in water, *J. Pept. Sci.*, 2007, **13**, 493–497, DOI: 10.1002/psc.874.
22 K. Hojo, N. Shinozaki, A. Hara, M. Onishi, Y. Fukumori and H. Ichikawa, Aqueous microwave-assisted solid-phase peptide synthesis using Fmoc strategy. II. Racemization studies and water based synthesis of cysteine-containing peptides, , *Protein Pept. Lett*, 2013, **20**, 1122–1128, DOI: 10.2174/0929866511320100006.
23 K. Hojo, N. Shinozaki, K. Hidaka, Y. Tsuda, Y. Fukumori, H. Ichikawa and J. D. Wade, Aqueous microwave-assisted solid-phase peptide synthesis using Fmoc strategy. III: Racemization studies and water-based synthesis of histidine-containing peptides, *Amino Acids*, 2014, **46**, 2347–2354, DOI: 10.1007/s00726-014-1779-y.
24 K. Hojo, Y. Manabe, T. Uda and Y. Tsuda, Water-based solid-phase peptide synthesis without hydroxy side chain

protection, *J. Org. Chem.*, 2022, **87**, 11362–11368, DOI: 10.1021/acs.joc.2c00828.
25 D. A. Wellings, J. Greenwood, I. T. C. Hughes, W. Li, F. Lin, M. A. Hossain, A. Lanza, M. Meldal and J. D. Wade, *Nat. Sustain.*, 2026, **9**, 101–112, DOI: 10.1038/s41893-025-01761-z.
26 M. Kunishima, C. Kawachi, J. Morita, K. Terao, F. Iwasaki and S. Tani, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride: An efficient condensing agent leading to the formation of amides and esters, *Tetrahedron*, 1999, **55**, 13159–13170, DOI: 10.1016/S0040-4020(99)00809-1.
27 M. Kunishima, C. Kawachi, K. Hioki, K. Terao and S. Tani, Formation of carboxamides by direct condensation of carboxylic acids and amines in alcohols using a new alcohol- and water-soluble condensing agent: DMT-MM, *Tetrahedron*, 2001, **57**, 1551–1558, DOI: 10.1016/S0040-4020(00)01137-6.
28 E. Bayer and W. Rapp, Verfahren zur Darstellung monodisperser, vernetzter Polymerkügelchen, German Pat. DOS 3714258, Patent No.: DE3714258A1 (1988-11-10), 1988.
29 F. Albericio and A. El-Faham, Choosing the right coupling reagent for peptides: A twenty-five-year journey, *Org. Process Res. Dev.*, 2018, **22**, 760–772, DOI: 10.1021/acs.oprd.8b00159.
30 G. R. Pettit and S. R. Taylor, Synthesis of the marine sponge cycloheptapeptide stylopeptide 1, *J. Org. Chem.*, 1996, **61**, 2322–2325, DOI: 10.1021/jo951986b.
31 L. A. Carpino, 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive, *J. Am. Chem. Soc.*, 1993, **115**, 4397–4398, DOI: 10.1021/ja00063a082.
32 L. A. Carpino, A. El-Faham and F. Albericio, Racemization studies during solid-phase peptide synthesis using azabenzotriazole-based coupling reagents, *Tetrahedron Lett.*, 1994, **35**, 2279–2282, DOI: 10.1016/0040-4039(94)85198-0.
33 O. Marder, Y. Shvo and F. Albericio, New coupling reagents: Development and industrial aspects, *J. Pept. Sci.*, 2003, **9**, 59–67, DOI: 10.1002/chin.200332258.
34 R. Knorr, A. Trzeciak, W. Bannwarth and D. Gillessen, New coupling reagents in peptide chemistry, *Tetrahedron Lett.*, 1989, **30**, 1927–1930, DOI: 10.1016/S0040-4039(00)99616-3.
35 V. Dourtoglou, J. C. Ziegler and B. Gross, L'hexafluorophosphate de *O*-benzotriazolyl-*N,N*-tetramethyluronium: Un réactif de couplage peptidique nouveau et efficace, *Tetrahedron Lett.*, 1978, **19**, 1269–1272, DOI: 10.1016/0040-4039(78)80103-8.
36 N. Fleck, F. Roschangar and A. M. Haydl, API syntheses in aqueous media: assessing the environmental footprint en route from academic discovery to industrial applications as "green opportunity" for process chemistry, *Org. Process Res. Dev.*, 2023, **27**, 822–830, DOI: 10.1021/acs.oprd.3c00037.
37 M. Cortes-Clerget, N. R. Lee and B. H. Lipshutz, Synthetic chemistry in water: applications to peptide synthesis and nitro-group reductions, *Nat. Protoc.*, 2019, **14**, 1108–1129, DOI: 10.1038/s41596-019-0130-1.
38 F. Bordignon, A. Scarso and A. Angelini, Challenges and achievements of peptide synthesis in aqueous and micellar media, *ChemBioChem*, 2025, **26**, e202500099, DOI: 10.1002/cbic.202500099.
39 S. Hazra, F. Gallou and S. Handa, Water: An underestimated solvent for amide bond-forming reactions, *ACS Sustainable Chem. Eng.*, 2022, **10**, 5299–5306, DOI: 10.1021/acssuschemeng.2c00520.
40 A. El-Faham, R. Subirós-Funosas, R. Prohens and F. Albericio, COMU: a safer and more effective replacement for benzotriazole-based uronium coupling reagents, *Chem. Eur. J.*, 2009, **B**, 9404–9416, DOI: 10.1002/chem.200900615.
41 S. Narayan, J. Muldoon, M. G. Finn, V. V. Fokin, H. C. Kolb and K. B. Sharpless, "On water": Unique reactivity of organic compounds in aqueous suspension, *Angew. Chem., Int. Ed.*, 2005, **44**, 3275–3279, DOI: 10.1002/anie.200462883.
42 R. N. Butler and A. G. Coyne, Organic synthesis reactions on-water at the organic–liquid water interface, *Org. Biomol. Chem.*, 2016, **14**, 9945–9960, DOI: 10.1039/C6OB01724J.
43 B. H. Lipshutz, S. Ghorai and M. Cortes-Clerget, The hydrophobic effect Aapplied to organic synthesis: Recent synthetic chemistry "in water", *Chem. Eur. J.*, 2018, **24**, 6672–6695, DOI: 10.1002/chem.201705499.
44 M. Cortes-Clerget, J. Yu, J. R. A. Kincaid, P. Walde, F. Gallou and B. H. Lipshutz, Water as the reaction medium in organic chemistry: from our worst enemy to our best friend, *Chem. Sci.*, 2021, **12**, 4237–4266, DOI: 10.1039/D0SC06000C.
45 M. Kunishima, K. Kikuchi, Y. Kawai and K. Hioki, Substrate-selective dehydrocondensation at the interface of micelles and emulsions of common surfactants, *Angew. Chem., Int. Ed.*, 2012, **51**, 2080–2083, DOI: 10.1002/anie.201107706.
46 M. Kunishima, H. Imada, K. Kikuchi, K. Hioki, J. Nishida and S. Tani, Unusual rate enhancement of bimolecular dehydrocondensation to form amides at the interface of micelles of fatty acid salts, *Angew. Chem., Int. Ed.*, 2005, **44**, 7254–7257, DOI: 10.1002/anie.200502594.
47 J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgan and H. R. Morris, Identification of two related pentapeptides from the brain with potent opiate agonist activity, *Nature*, 1975, **258**, 577–580, DOI: 10.1038/258577a0.
48 V. Martin, P. H. G. Egelund, H. Johansson, S. Thordal Le Quement, F. Wojcik and D. S. Pedersen, Greening the synthesis of peptide therapeutics: an industrial perspective, *RSC Adv.*, 2020, **10**, 42457–42492, DOI: 10.1039/D0RA07204D.
49 M. Vergaelen, B. Verbraeken, J. F. R. Van Guyse, A. Podevyn, A. Tigrine, V. R. de la Rosa, B. D. Monnery and R. Hoogenboom, Ethyl acetate as solvent for the synthesis of poly(2-ethyl-2-oxazoline), *Green Chem.*, 2020, **22**, 1747–1753, DOI: 10.1039/C9GC03872H.
50 M. Broccardo, V. Erspamer, G. F. Erspamer, G. Improta, G. Linari, P. Melchiorri and P. C. Montecucchi, Pharmacological data on dermorphins, a new class of potent opioid peptides from amphibian skin, *Br. J. Pharmacol.*, 1981, **73**, 625–631, DOI: 10.1111/j.1476-5381.1981.tb16797.x.
51 C. H. Li and D. Chung, Primary structure of human β-lipotropin, *Nature*, 1976, **260**, 622–624, DOI: 10.1038/260622a0.