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Environmentally Conscious In-Water Peptide Synthesis Using Boc Strategy

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Abstract
Seeking a more environmentally balanced method of peptide synthesis, we are focusing on developing organic solvent-free synthetic methods using water, an environmentally friendly solvent. In current peptide synthesis, the most common building blocks are Boc- and Fmoc-protected amino acids, which are highly soluble in organic solvents. We previously reported a technique for solid-phase peptide synthesis in water that utilizes Fmoc-amino acids converted to water-dispersible nanoparticles. The Boc strategy is well-known to be suitable for the industrial chemistry and green chemistry, because only gases are generated without any other by-products in the deprotection step of Boc group. Here we summarize in-water both liquid and solid-phase method using Boc-amino acids based on MW-assisted coupling reaction of nanosized reactants, nanoparticles and nanomicelles.

Keywords Green sustainable chemistry · Water · Solid-phase peptide synthesis · Liquid-phase peptide synthesis · Boc strategy · Nanoparticles · Nanomicelles · Microwave synthesis

Introduction

In the chemical industry, carrying out reactions in organic solvents has become common place in the past 100 years. In light of the issues of global pollution and climate change, "green sustainable chemistry" has been recently promoted as a high priority in science and technology. In particular, reducing the use of organic solvents, which ultimately undergo combustion for disposal, is sought (Anastas and Warner 1998; Winterton 2001). In this context, there is an urgent need to transition to environmentally conscious chemical synthesis that does not involve organic solvents; in other words, the development and advancement of novel methodologies with low environmental impact.

Meanwhile, peptides are attracting much attention as bio-derived functional substances, and the requirement for peptides is expanding in all fields including the pharmaceutical and biomaterials industry. Although different methods are available for manufacturing peptides, chemical syntheses that yield large quantities of high-purity product are mainly employed in manufacturing to supply the market needs. Indeed, the chemical synthesis of peptides has been more or less established as a technology in its own right with the commercial availability of automated synthesizers. However, it is a multi-step process that involves repeated condensation reactions and consumes large amounts of organic solvent for only small amounts of peptide product. The whole process, including the disposal of spent organic solvent, creates problems from the perspective of environmental impact, resulting in the need to develop techniques that can achieve an organic solvent-free manufacturing process. Substituting organic solvent with water, which has low environmental impact, would substantially solve this problem (Ballini 2009). Although several studies have described the formation of peptide bonds in water, so far the methodology has been limited to the synthesis of short-length peptides (Marco et al. 2013).

The primary reason for using organic solvents in peptide synthesis is that amino acids with protecting groups are poorly soluble in water and thus not feasible for in-water reactions. Although protected amino acids can be chemically converted to water-soluble forms for reaction in water (Hojo et al. 2001, 2004a, b), this adds another step—conversion—to the manufacturing process, which is undesirable from the viewpoint of

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manufacturing costs and material savings. Therefore, a novel peptide synthesis technology that is both environmentally conscious and simplifies the manufacturing process is required.

In standard peptide synthesis, *tert*-butyloxycarbonyl (Boc)- and 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids are commonly used. These two types of protecting group are highly soluble in organic solvent and poorly soluble in water. Both molecules are generally applied to peptide synthesis via one of two approaches, liquid-phase peptide synthesis (LPPS) and solid-phase peptide synthesis (SPPS), which each have needs. Through our research, we have developed a technique that enables SPPS to proceed in water by converting the existing water-insoluble Fmoc-protected amino acids into water-dispersible nanoparticles; furthermore, the reaction time in water is markedly shortened in this method (Hojo et al. 2007, 2008, 2011a). Here, we summarize further development of the method for both LPPS and SPPS in water using Boc-amino acids processed into nanoparticles and nanomicelles in water.

Materials and Methods

Boc-amino acids, resins, and reagents were purchased from Watanabe Chemical Industries, Ltd. Triton X-100 and bases were purchased from Nakalai Tesque, Inc. A planetary ball mill, model pulverisette 7 (Fritsch GmbH) was used for pulverization to prepare water-dispersible nanoparticles. The particle size of nanoparticles and nanomicelles of Boc-amino acids were determined by a dynamic light scattering (DLS) analysis, model LB-500 (Horiba Instruments Inc.). The MW-assisted reaction system used in nanoparticle based SPPS was the μ Reactor Ex (2.45 GHz) (Shikoku Instrumentation Co. Ltd.). The semi-automatic MW reaction system used in nanomicellar based SPPS was Biotage Initiator + SP Wave. Reversed phase HPLC was performed using a Waters model 600 instrument or Alliance system equipped a Waters e2456 with a cosmosil $5C_{18}$-AR-II column and gradient system of acetonitrile / water containing 0.05 or 0.1% trifluoroacetic acid (TFA). Amino acid ratios in an acid hydrolysate were determined with a Waters Pico Tag amino acid analyzer. Reversed phase HPLC was performed using Waters model 600 equipment with a Cosmosil C18 column and a gradient system consisting of acetonitrile / water containing 0.05% TFA. Optical rotations were determined with an automatic polarimeter, model DIP-360 (Japan Spectroscopic Co.). Mass spectra were measured with a Kratos MALDI IV mass spectrometer (Shimadzu Co.) using the TOF technique.

Preparation of Water-Dispersible Nanoparticle Boc-Amino Acids in PEG Solution for Aqueous LPPS (Hojo et al. 2011b)

Boc-Phe-OH Nanoparticles

An aqueous dispersion of nanoparticulate Boc-amino acids was prepared by wet-milling using a planetary ball mill as follows: a 40 ml agate jar was charged with 1.0 mm diameter pre-cleaned zirconium oxide beads (80 g), Boc-Phe-OH (530.6 mg, 2.0 mmol), polyethylene glycol (PEG) (average MW 4000 g/mol, 400 mg, 0.1 mmol), and 20 ml of water. The batch was rolled at 495 rpm for 4 h. After pulverization, the zirconium oxide beads were removed by filtration with 40 ml of water. Particle size = 578 ± 48 nm.

Boc-Gly-OH Nanoparticles

Particle size = 712 ± 36 nm.

Boc-Tyr(tBu)-OH Nanoparticles

Particle size = 687 ± 32 nm.

In-Water Coupling Reaction of Water-Dispersible Boc-Phe-OH Nanoparticle with H-Leu-NH_2 Using Water-Soluble Coupling Reagents (Hojo et al. 2011b)

H-Leu-NH_2•HCl (166 mg, 1.0 mmol) was dissolved in 10 ml of water, and then water-dispersible Boc-Phe-OH nanoparticles (60 ml, 2.0 mmol) were added. Several coupling methods using water-soluble carbodiimide (WSCD, 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide hydrochloride) (382 mg, 2.0 mmol) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (552 mg, 2.0 mmol) were examined. *N*-hydroxy-5-norbornene-*endo*-2,3-dicarboximide (HONB) (450 mg, 2.0 mmol), *N*-hydroxysuccinimide (HOSu) (230 mg, 2.0 mmol) or 3-sulfo-*N*-hydroxysuccinimide (sulfo-HOSu) (436 mg, 2.0 mmol) was used as a coupling additive. *N,N*-diisopropylethylamine (DIEA) (348 μl, 2.0 mmol) was used in the WSCD methods, and N-methylmorpholine (NMM) (192 μl, 2.0 mmol) was used in the DMTMM method. After stirring overnight, the reaction mixture was filtered to collect the precipitates. The precipitates, which contained the crude peptide, were directly applied to analytical HPLC, and the yields and purities were measured. Results were summarized in Table 1.

Table 1 The Study of aqueous coupling reaction between water-dispersible Boc-Phe-OH nanoparticles and H-Leu-NH_2 (Hojo et al. 2011b)

Entry	Reagent	Additive	Base	Yield (%)[a]
1	DMTMM	–	NMM	89
2	WSCD	HONB	DIEA	25
3	WSCD	HOSu	DIEA	32
4	WSCD	Sulfo-HOSu	DIEA	71

[a]The yield of peptides was calculated from analytical HPLC profiles

General Procedure for Synthesis of Peptides Using Water-Dispersible Boc-Amino Acid Nanoparticles, with Boc-Gly-Phe-Leu-NH_2 as an Example (Hojo et al. 2011b)

Boc-Phe-Leu-NH_2 (674 mg, 1.0 mmol) was treated with 20 ml of trifluoroacetic acid (TFA) for 1 h at room temperature. The TFA solution was concentrated to a residue *in vacuo*, to which 1 ml of solution 4.0 mol/L HCl in dioxane was added. The residue was triturated with diethyl ether. The precipitate was collected by filtration and dried over sodium hydroxide pellets *in vacuo*. H-Phe-Leu-NH_2•HCl was thus obtained and dissolved in 20 ml of water. Water-dispersible Boc-Gly-OH nanoparticles (60 ml, 2.0 mmol) were mixed and then DMTMM (552 mg, 2.0 mmol) and NMM (196 µl, 2.0 mmol) were added. After stirring at room temperature overnight, the precipitate was collected by filtration and washed with water. The residue was dried *in vacuo* to obtain crude peptide, which was directly applied to analytical HPLC, and the yields and purities were measured. The yields and characteristics were: Boc-Gly-Phe-Leu-NH_2: Yield 82% (calculated from analytical HPLC), HPLC analytical purity 90%. Crude peptide was crystallized from ethyl acetate and diethyl ether. mp 155–157 °C; $[\alpha]_D^{24}+55.7$ (*c* 1.0 in MeOH); *m/z* (MALDI-MS) 457.54 ($[M+Na]^+$, $C_{22}H_{34}N_4NaO_5$ calculated m/z = 457.52); *Boc-Phe-Leu-NH_2*: white material; mp 176–178 °C; $[\alpha]_D^{24}$-20.2 (*c* 1.0 in MeOH); *m/z* (MALDI-MS) 400.86 ($[M+Na]^+$,$C_{20}H_{31}N_3NaO_4$ calculated m/z = 400.47); *Boc-Gly-Gly-Phe-Leu-NH_2:* white material, mp 137–140 °C; $[\alpha]_D^{24}+64.4$ (*c* 1.0 in MeOH); *m/z* (MALDI-MS) 514.27 ($[M+Na]^+$, $C_{24}H_{37}N_5NaO_6$ calculated m/z = 514.57); *Boc-Tyr(tBu)-Gly-Gly-Phe-Leu-NH_2*: yellowish material; $[\alpha]_D^{24}+68.6$ (*c* 1.0 in MeOH); *m/z* (MALDI-MS) 733.27 ($[M+Na]^+$, $C_{37}H_{54}N_6NaO_8$ calculated m/z = 733.85).

Preparation of Leu-Enkephalinamide (H-Tyr-Gly-Gly-Phe-Leu-NH_2•TFA) (Hojo et al. 2011b)

The protected pentapeptide Boc-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-NH_2 (710 mg, 1.0 ml) was treated with 20 ml of TFA for 1 h at room temperature. The TFA solution was concentrated to a residue *in vacuo*. The residue was purified by preparative HPLC to give an amorphous powder. The yield was 51.8%; $[\alpha]_D^{24}+12.6$ (*c* 1.0 in H_2O); *m/z* (MALDI-MS) 555.31 ($[M+H]^+$. $C_{28}H_{39}N_6O_6$ calculated m/z = 555.64); amino acid analysis: Tyr, 0.94; Gly; 2.03, Phe, 0.92; Leu; 1.01. (average recovery: 92%).

Preparation of Water-Dispersible Boc-Amino Acid Nanoparticles in 0.2% Triton X-100 Solution for Aqueous SPPS (Hojo et al. 2013)

Boc-Phe-OH Nanoparticles

An aqueous dispersion of nanoparticulate Boc-Ala-OH was prepared by wet-milling using a planetary ball mill. An 40 ml agate jar was charged with 0.5 mm diameter pre-cleaned zirconium oxide beads (80 g), Boc-Phe-OH (600 mg, 1.55 mmol), and 20 ml of aqueous 0.2% Triton-X 100 solution. The batch was rolled at 400 rpm for 3 h. After grinding, the beads were removed by filtration with 60 ml of aqueous 0.2% Triton-X 100 solution. Particle size: 362 ± 203 nm.

Boc-Tyr(tBu)-OH Nanoparticles

Particle size: 315 ± 170 nm.

Aqueous MW Assisted Coupling Reaction Study on Solid Phase Using Water-Soluble Coupling Reagents (Hojo et al. 2013)

H-Gly-Rink amide-PEG grafted resin (80 mg, 20 µmol) was swollen with 0.2% Triton X-100 solution, then an aqueous dispersion of nanoparticulate Boc-Phe-OH (5.0 ml, 100 µmol) was coupled onto the resin by WSCD (19 mg, 100 µmol) or DMTMM (18 mg, 100 µmol). WSCD was used in conjugation with sulfo-HOSu (22 mg, 100 µmol). DIEA (17 µl, 100 µmol) or NMM (11 µl, 100 µmol) were used for base catalysts. Each reaction mixture was heated at 70 °C by MW (< 70 W) irradiation and kept for 1–10 min. After MW irradiation, the resins were washed with 0.2% Triton X-100 solution and ethanol. The coupling efficiency was checked by a Kaiser test.

Preparation of H-Leu-HMBA-PEG Grafted Resin

HMBA-PEG grafted resin (181 mg, amino group content, 40 µmol) was swollen with *N,N'*-dimethylformamide (DMF), the first amino acid, Boc-Leu-OH (46 mg, 200 µmol) was coupled onto the resin by *N,N'*-diisopropylcarbodiimide (DIC) (31 µl, 200 µmol) and 4-(dimethylamino)pyridine (DMAP) (10 mg, 80 µmol) in DMF. Boc deprotection was

carried with TFA. The free amino group was neutralized with 0.5 mol/l $NaHCO_3$ solution. The resin was washed with H_2O and used in the following synthesis.

General Procedure for Aqueous MW Assisted SPPS Using Water-Dispersible Boc-Amino Acid Nanoparticles (Hojo et al. 2013)

Leu-Enkephalin as an Example

H-Leu-HMBA-PEG grafted resin (40 µmol) was swelled with 0.2% Triton X-100 solution, then water-dispersible Boc-Phe-OH nanoparticles (200 µmol), Boc-Gly-OH in 0.2% Triton X solution (200 µmol), and water-dispersible Boc-Tyr(*t*Bu)-OH nanoparticles (200 µmol) were serially coupled onto the resin. Aqueous MW coupling reaction was performed by DMTMM (35 mg, 200 µmol), and NMM (22 µl, 200 µmol) at 70 °C using MW (< 70 W) for 5 min. Deprotection was carried out with TFA. The free amino group was neutralized with 0.05 mol/l $NaHCO_3$ solution, followed by washing with H_2O. After completion of the synthetic reaction, the peptide resin (H-Tyr-Gly-Gly-Phe-Leu-HMBA PEG grafted resin) was washed with ethanol and dried *in vacuo*. The resin was treated with 3 ml of 0.5 mol/l NaOH solution for 30 min at room temperature. The resin was removed by filtration and then 1.5 ml of 1.0 mol/l HCl solution was added to filtrates in order to neutralize the base solution. The filtrate solution was lyophilized. The crude product was purified by preparative HPLC to give an amorphous powder. Yield (calculated from the amino group content of the used resin): 17.1 mg, 64%; ESI–MS (TOF) m/z: 556.2790 ($C_{28}H_{40}N_5O_7$ requires with calculated $[M+H]^+$ 556.6306). amino acid analysis: Tyr, 0.94; Gly; 2.02, Phe, 1.00; Leu; 0.97. (average recovery: 94%).

Val-Ala-Val-Ala-Gly-OH

14.2 mg, 53%; ESI–MS (TOF) m/z: 416.2704 ($C_{18}H_{34}N_5O_6$ requires with calculated $[M+H]^+$ 416.4925). amino acid analysis: Val, 2.06; Ala; 2.01, Gly, 1.00 (average recovery: 98%).

Preparation of Aqueous Nanomicelles of Boc-Amino Acid with DMTMM in Water

Boc-Phe-OH Nanoparticles

An aqueous dispersion of nanoparticulate Boc-Ala-OH was prepared by wet-milling using a planetary ball mill. An 40 ml agate jar was charged with 0.5 mm diameter pre-cleaned zirconium oxide beads (80 g), Boc-Phe-OH (600 mg, 1.55 mmol), and 20 ml of aqueous 0.2% Triton-X 100 solution. The batch was rolled at 400 rpm for 3 h. After grinding, the beads were removed by filtration with 60 ml of aqueous 0.2% Triton-X 100 solution. Particle size: 749 ± 272 nm.

Aqueous MW Assisted Coupling Reaction Study on Solid Phase Using Nanomicelles of BocPhe-OH with DMTMM in Water

H-Gly-Rink amide-PEG grafted resin (80 mg, 20 µmol) was swollen with 0.2% Triton X-100 solution, then an aqueous nanomicelles of Boc-Phe-OH (5.0 ml, 100 µmol) with DMTMM and NMM (11 µl, 100 µmol). Each reaction mixture was heated at 75 ℃ by MW irradiation and kept for 1–10 min. After MW irradiation, the resins were washed with water. The coupling efficiency was checked by a Kaiser test.

Synthesis of Leu-Enkephalin by MW Assisted SPPS Using Aqueous Nanomicelles of Boc-Amino Acid with DMTMM

The solid-phase synthesis was carried out according to the protocol shown in Table 3. The H-Leu-HMBA-PEG grafted resin (40 µmol) was swelled with water, then aqueous nanomicelles of Boc-Phe-OH (200 µmol) with DMTMM and NMM. Boc-Gly-OH (200 µmol) solution, and aqueous Boc-Tyr(*t*Bu)-OH nanomicelles (200 µmol) were serially coupled onto the resin. MW-assisted reaction was performed at 75 °C for 5 min in semi-automatic synthesizer. Deprotection was carried out with TFA by a manually method. The free amino group was neutralized with 0.05 mol/l $NaHCO_3$ solution, followed by washing with H_2O. After completion of the synthetic reaction, the peptide resin (H-Tyr-Gly-Gly-Phe-Leu-HMBA PEG grafted resin) was washed with ethanol and dried *in vacuo*. The resin was treated with 3 ml of 0.5 mol/l NaOH solution for 30 min at room temperature. The resin was removed by filtration and then 1.5 ml of 1.0 mol/l HCl solution was added to filtrates in order to neutralize the base solution. The filtrate solution was lyophilized. The crude product was purified by preparative HPLC to give an amorphous powder. Yield (calculated from the amino group content of the used resin): 8.3 mg, 31%; ESI–MS (TOF) m/z: 556.2790 ($C_{28}H_{40}N_5O_7$ requires with calculated $[M+H]^+$ 556.2273).

Results and Discussion

Aqueous LPPS with Water-Dispersible Nanoparticles of Boc-Protected Amino Acids

In industrial synthesis, manufactured peptide products, for example Leuplin® (LH-RH analog for cancer therapy)

among others, are mainly generated by LPPS. At present, the Boc strategy is the most employed LPPS technique. Because Boc-amino acids are sparingly soluble in water, they have not been applied much in aqueous synthesis. However, by converting Boc-amino acids into nanoparticles that are uniformly dispersed in water, we expected to achieve water-based peptide synthesis according to Boc chemistry in the same manner as that of Fmoc-amino acid nanoparticles reported previously (Hojo et al. 2007, 2008, 2011a, b, 2012). Moreover, the Boc strategy is well-suited for environmentally conscious chemistry because, during the deprotection process, the Boc group generates only gases without other side products. Therefore, we tried aqueous LPPS using water-dispersible Boc-amino acid nanoparticles (Hojo et al. 2012).

As with Fmoc-amino acids, we first employed wet grinding in a planetary ball-mill to prepare water-dispersed nanoparticles of Boc-amino acids in aqueous PEG solution (Fig. 1) (Rabinow 2004). The water-dispersible nanoparticles were found by DLS to have a mean diameter of 579 ± 48 nm. To evaluate the feasibility of using these nanoparticles for aqueous LPPS, we then tested aqueous coupling reactions of nanoparticulate Boc-Phe-OH and Leu-NH_2 under different conditions using WSCD (Sheehan and Hlavka 1956) or DMTMM (Kaminski et al. 1998; Kunishima et al. 1999). The results were summarized in Table 1. Coupling reactions using DMTMM were more favorable than those using WSCD in combination with an additive, HONB (Fujino et al. 1974) or sulfo-HOSu. Furthermore, the aqueous reaction using DMTMM with NMM resulted in a satisfactory yield of dipeptide (89%).

Next, we attempted aqueous LPPS of Leu-enkephalinamide using nanoparticles of Boc-amino acids. The aqueous coupling reaction was performed by mixing nanoparticulate Boc-amino acids and amine components with DMTMM, after which the objective protected peptide fragments were obtained by precipitation. The reacting molecules—that is, the water-dispersed nanoparticles—were nano-sized and readily passed through a microfilter, and the reagents were water-soluble (Fig. 2). After only a filtration step, the yield was 80% or higher and the purity was at least 90% (Table 2). Furthermore, the protected peptide fragment of Leu-enkephalinamide showed more or less a single peak on HPLC analysis (Fig. 3d). Non-level diastereomeric peaks were observed in each protected peptide. This means that racemization was almost suppressed under these reaction conditions. After the final deprotection step, the objective Leu-enkephalinamide was obtained in good yield. Thus, our method successfully synthesized objective peptides with simple filtration to remove unreacted nanoparticles and side products after the reaction. Furthermore, even without any purification other than filtration, the objective peptides were obtained in satisfactory yield and high purity.

In general, most manufacturing processes for organic compounds are performed in organic solvent because the reaction intermediates and final compound are sparingly soluble in water. Therefore, chemical synthesis in water has been avoided. Our environmentally friendly method, which uses nanoparticle technology, might improve standard organic synthesis methods in terms of not only efficiency but also a reduced number of synthetic steps.

Aqueous Microwave-Assisted SPPS with Water-Dispersible Nanoparticles of Boc-Protected Amino Acids

Many studies have described advances in the development of SPPS with microwave (MW) irradiation, especially for the synthesis of peptides with a difficult sequence or long length (Olivos et al. 2002; Yu et al. 1992; Polshettiwar and Varma 2008). Because water has high polarity and a high dielectric constant, MW irradiation in aqueous reactions would be expected to accelerate both the rise in temperature and the reaction. A trial of MW-assisted SPPS in water using unprocessed Boc-amino acids has been reported (Galanis et al. 2009). Therefore, we aimed to develop a technique of solid aqueous SPPS using water-dispersible Boc amino acid nanoparticles combined with MW irradiation. We anticipated that combining MW irradiation with a solid-phase reaction using water-dispersed

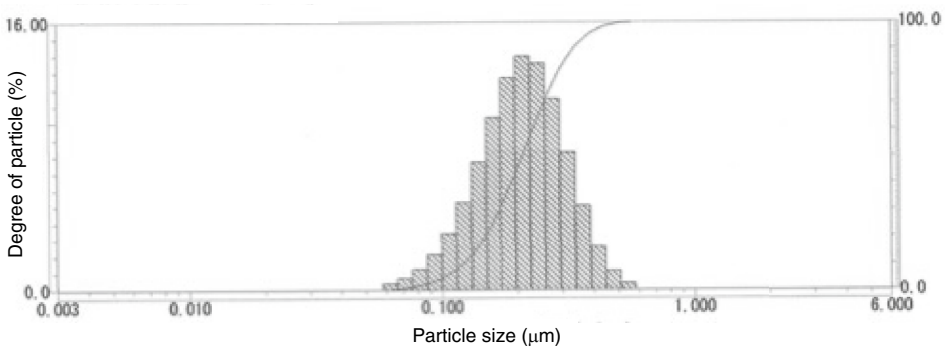

Fig. 1 Particle size distribution of water-dispersible Boc-Phe-OH nanoparticles

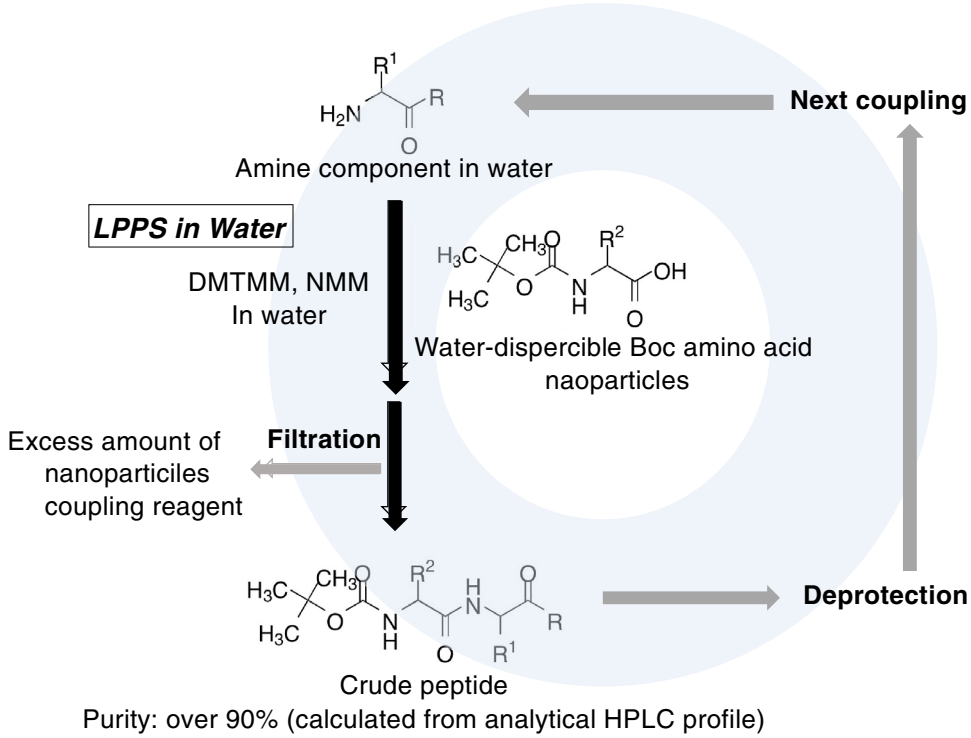

Fig. 2 Aqueous LPPS using water-dispersible Boc-amino acid nanoparticles

Table 2 Yield and purity of peptide fragment obtained by aqueous LPPS

Synthetic peptide	Tof–MS [m/z]	Yield [%][a]	Purity [%][a]
Boc-Phe-Leu-NH_2	$[M+Na]^+$: 400.86	89	90
H-Phe-Leu-NH_2	$[M+H]^+$: 278.11	–[b]	–[b]
Boc-Gly-Phe-Leu-NH_2	$[M+Na]^+$: 457.54	82	90
H-Gly-Phe-Leu-NH_2	$[M+H]^+$: 335.44	–[b]	–[b]
Boc-Gly-Gly-Phe-Leu-NH_2	$[M+Na]^+$: 514.27	84	92
H-Gly-Gly-Phe-Leu-NH_2	$[M+Na]^+$: 414.60	–[b]	–[b]
Boc-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-NH_2	$[M+Na]^+$: 733.27	86	93
H-Tyr-Gly-Gly-Phe-Leu-NH_2	$[M+H]^+$: 555.31	51[c]	> 100[c]

Aqueous coupling reactions were performed with DMTMM

[a]Yield and purity of peptides was calculated from analytical HPLC profiles

[b]Since crude peptide was directly used in next coupling after deprotection of Boc group, yield and purity were not checked

[c]Here shows the results after preparative HPLC purification

nanoparticles would stabilize the dispersion of nanoparticles and at the same time achieve a more efficient reaction in water (Hojo et al; 2013).

To evaluate MW irradiation for aqueous synthesis, we examined a coupling reaction using PEG-grafted resin and water-dispersed Boc-Phe-OH nanoparticles in 0.2% Triton X-100 solution. We tested several coupling methods using WSCD/sulfo-HOSu and DMTMM. This resulted in acceleration of the reaction as expected, with the condensation reaction in water completed in 1 min at 70 °C by the DMTMM method. Thus, MW irradiation promoted an aqueous coupling reaction on the solid phase. Based on this study, we carried out solid-phase synthesis of Leu-enkephalin under MW irradiation in water using DMTMM (Fig. 4), and successfully synthesized the peptide with high purity.

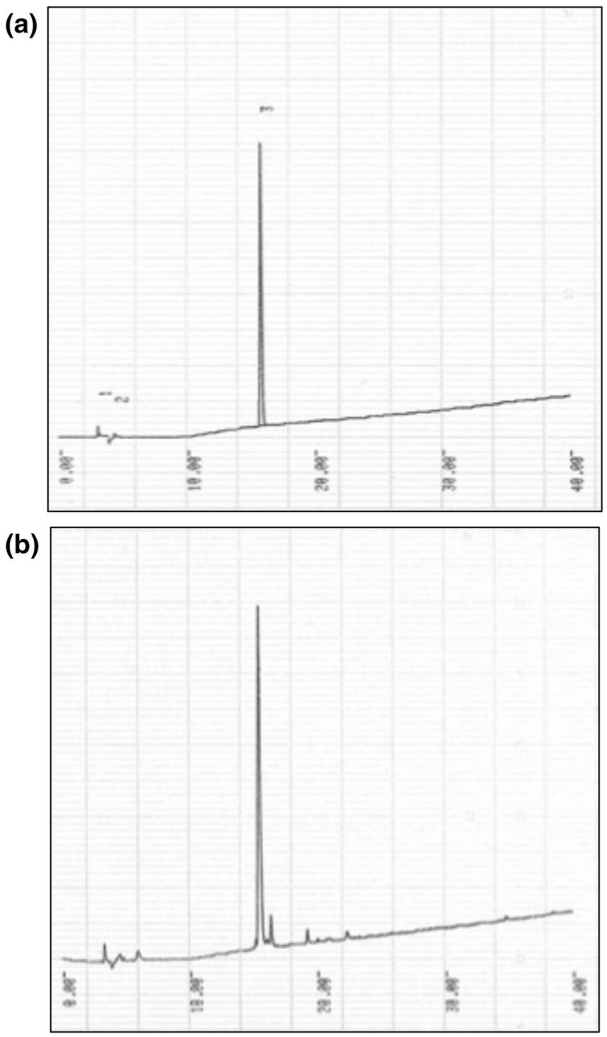

Fig. 3 Analytical HPLC profiles of crude peptides obtained by aqueous LPPS **a** Boc-Phe-Leu-NH_2; **b** Boc-Gly-Phe-Leu-NH_2; **c** Boc-Gly-Gly-Phe-Leu-NH_2; and **d** Boc-Tyr(tBu)-Gly-Gly-Phe-Leu-NH_2. Elution was carried out over 30 min at a flow rate of 1 ml/min with a linear gradient from 9:1 to 3:7 mixture of 0.05% aqueous TFA and 0.05% TFA in acetonitrile. (Hojo et al. 2011b)

Using water as a solvent would be expected to intensify peptide chain aggregation (Sabatino and Papini 2008; Bacsa et al. 2008), making synthesis in water unfeasible. Because peptide backbones are dipolar, it is thought that MW may be effective in disrupting the aggregation of peptide chains. We further demonstrated the application of MW irradiation to synthesis of a difficult pentapeptide, Val-Ala-Val-Ala-Val-Gly-OH, which is known to have a tendency toward aggregation (Ajikumar and Devaky 2001). The coupling reactions in the respective steps of the MW synthesis protocol (Hojo et al. 2013) proceeded smoothly, and we successfully obtained the target pentapeptide with high purity as expected (Fig. 5a). As a result, this method should be useful

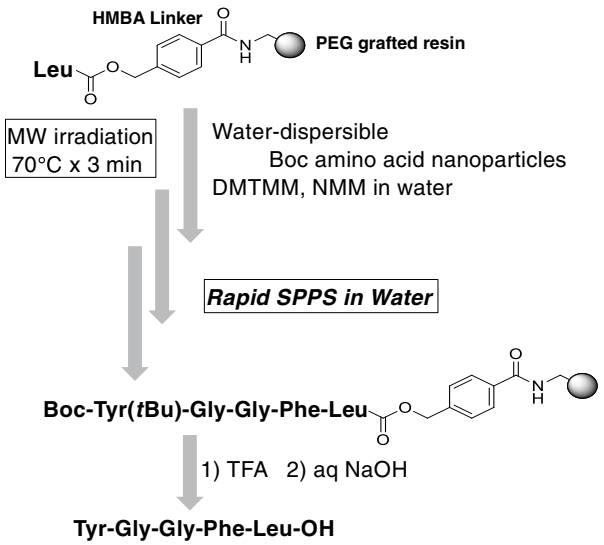

Fig. 4 Aqueous MW-assisted SPPS using water-dispersible Boc-amino acids nanoparticles

for various types of peptides, including those with difficult sequences.

Aqueous MW-Assisted SPPS with Aqueous Nanomicelles of Boc-Protected Amino Acids with a Water-Soluble Coupling Reagent

Recently, micelle-mediated synthesis has attracted attention as a useful method for aqueous synthesis in the field of industrial chemistry. In almost all reported cases, surfactants are used to make a micellar system, which either provides a reaction field with its surface in aqueous medium or acts as a solubilizing agent (Steven 2019). Although a designer surfactant such as TPGS-750-M has been successfully used in aqueous polypeptide synthesis, unfortunately the speed of the coupling reaction using surfactant is not fast (Cirtes-Clerget et al. 2019). With this mind, we considered that a simplified technique without additives such as surfactant is needed to make building blocks soluble or more amenable to water. Because the structure of a Boc-amino acid consists of the Boc protecting group and carboxylic acid of an amino acid, it has both hydrophobic and hydrophilic characters, similar to a surfactant. Here, therefore, we tried to prepare nanomicelles made from Boc-amino acids with a coupling reagent and to apply these nanomicelles for aqueous peptide synthesis.

First, we prepared aqueous nanomicelles of Boc-Phe-OH with the water-soluble coupling reagent DMTMM, which was in the hydrochloride salt form. Powdered Boc-Phe-OH was kneaded with two equivalents of NMM, and then aqueous DMTMM solution was added to obtain nanomicellar Boc-Phe-OH with DMTMM. The nanomicelles in water were found by

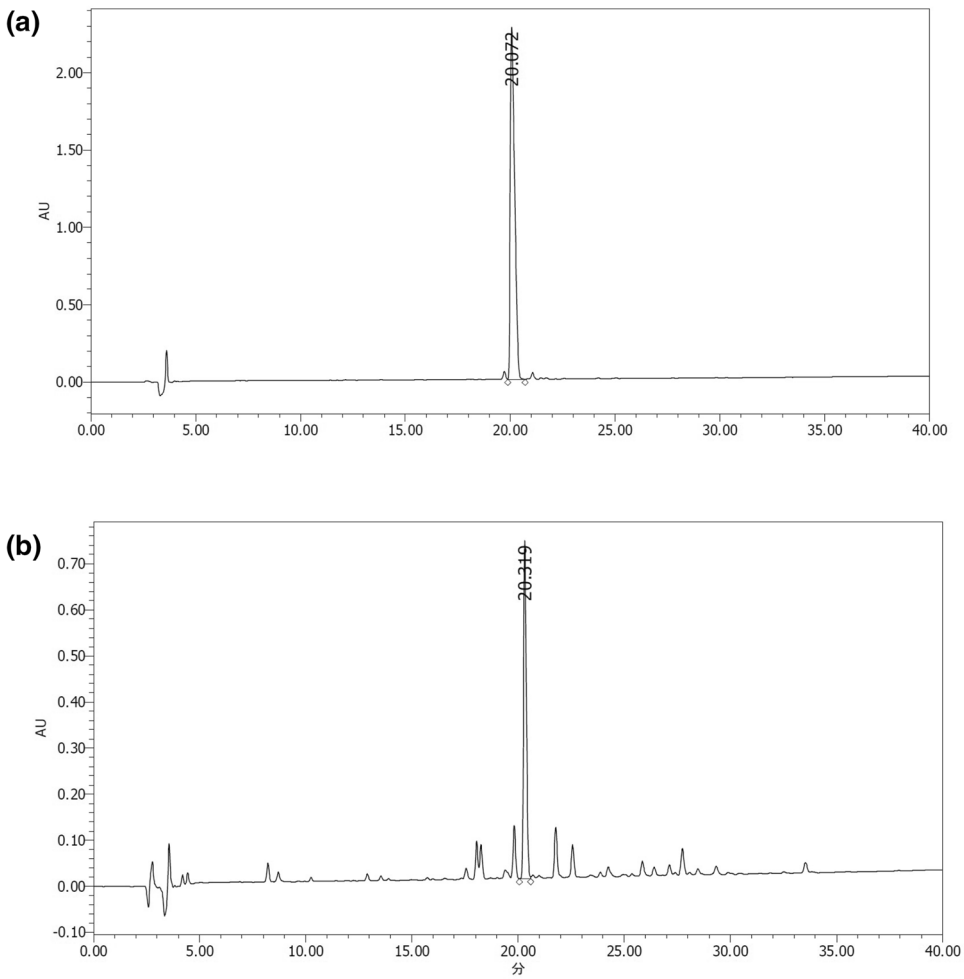

Fig. 5 **a** Analytical HPLC profiles; and **b** Mass spectra of crude Val-Ala-Val-Ala-Gly-OH obtained by aqueous MW assisted SPPS using water-dispersible Boc-amino acid nanoparticles. Elution was carried out over 20 min at a flow rate of 1 ml/min with a linear gradient from 9:1 to 5:5 mixture of 0.05% aqueous TFA and 0.1% TFA in acetonitrile. (Hojo et al. 2013)

DLS to have a mean diameter of 749 ± 272 nm. The nanomicelles solution looked clear; however, the Tyndall phenomenon was observed on laser irradiation (Fig. 6b). To evaluate the feasibility of nanomicelles as building blocks in water, we examined the solid-phase MW-assisted coupling reaction of Boc-Phe-OH nanomicelles with DMTMM. The coupling reaction using these nanomicelles proceeded quantitatively and was completed in as little as 5 min. We subsequently tested this MW-assisted method using nanomicelles for the synthesis of Leu-enkephalin on HMBA-PEG grafted resin according to the aqueous protocol (Table 3). Figure 7b showed analytical HPLC result of crude Leu-enkephalin obtained by aqueous SPPS using nanomicelles. Since there are some deletion peaks, it might be necessary to optimize aqueous coupling condition and deprotection condition of the aqueous protocol using nanomicelles. But the purity and yield of crude Leu-enkephalin was satisfactory (Fig. 7b), and comparable to those of crude peptide obtained by the conventional Fmoc strategy in organic solvent (Fig. 7a).

This technique using nanomicelles of Boc amino acids with DMTMM, which are easily prepared without special powder technology, might be not only suitable for aqueous peptide synthesis but also more environmentally friendly than the method using nanoparticles because it uses less energy due to a reduction of the wet-milling process. Furthermore, this

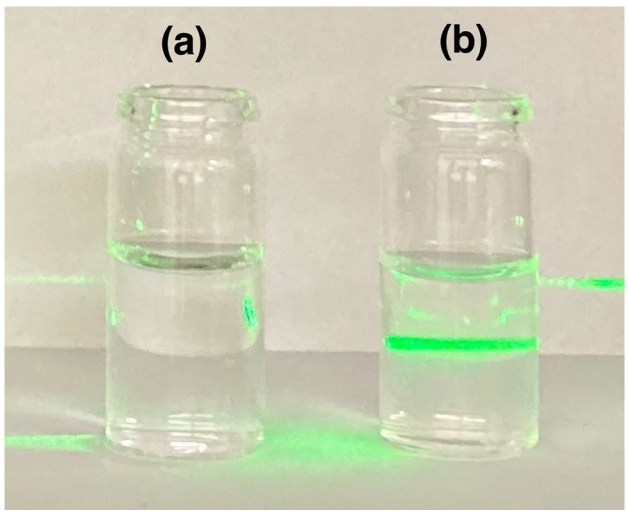

Fig. 6 Photo image of **a** water; and **b** Tyndall phenomenon with aqueous nanomicelles of Boc-Phe-OH with DMTMM

Table 3 Protocol for aqueous MW-assisted SPPS using aqueous nanomicelles

Program	Step	Reagent	Temp	Time
Wash	Wash	Water	rt	1 min × 4
Coupling	Coupling reaction	Aqueous nanomicelle of Boc-amino acids with DMTMM, NMM	75 ℃	5 min
	Wash	Water	rt	1 min × 4
Wash	Wash	DCM	rt	1 min × 2
Deprotection	Deprotection	50% TFA/DCM	rt	5 min × 2
	Wash	DCM	rt	1 min × 2
	neutralization	0.05 mol/L aq $NaHCO_3$ solution	rt	1 min × 2

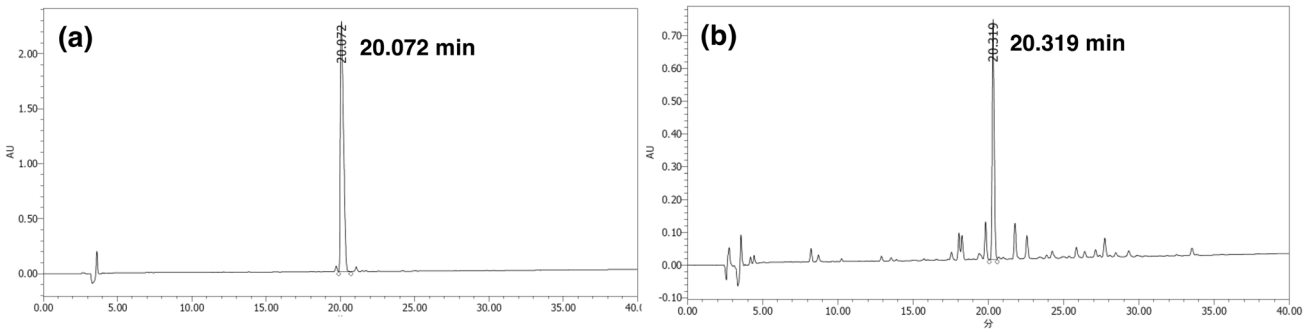

Fig. 7 Analytical HPLC profiles of crude peptides Leu-enkephalin obtained by **a** general Fmoc strategy in DMF; and **b** obtained by aqueous MW assisted SPPS using aqueous nanomicelles of Boc-amino acid with DMTMM. Elution was carried out over 40 min at a flow rate of 1 ml/min with a linear gradient from 9:1 to 5:5 mixture of 0.1% aqueous TFA and 0.1% TFA in acetonitrile

method is greener than an approach using micellar systems with additional surfactant because it produces less waste.

Conclusion

Currently, most industrial processes involving organic synthesis are carried out in organic solvent because it has been generally accepted that the reacting molecules must be dissolved in solvent for an efficient reaction. In recent years, several attempts to achieve environmentally conscious chemical synthesis have been made, including catalytic reactions in which the organic solvent is replaced by water. However, many of these novel reactions are applicable only to water-soluble raw materials, and they remain at the stage of laboratory research and development, yet to be industrialized.

Industrial manufactured materials including intermediates are sparingly soluble in water and thus not suitable for aqueous reactions. If a technique that allows for an efficient reaction in water can be established, then many chemical synthesis plants will be able to switch to industrial processes using water, even for compounds that have poor solubility in water. The present approach based on water-dispersed nanoparticles and nanomicelles is applicable to various organic reactions with insoluble materials. It is anticipated that further advances in this methodology will lead to a more sustainable chemical industry.

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Author Contributions KH supervised the study, reviews the literature, and prepared original draft. SF, HI, and YM managed the experiments. YT reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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