

Microwave energy: a versatile tool for the biosciences

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As the range of techniques for microwave heating has expanded, so have the areas in which it can have a profound impact. Two emerging areas are the application of microwave heating for the synthesis of peptides, peptoids, oligopeptides and carbohydrates and in the field of proteomics.

Introduction

Microwave heating is a valuable tool for synthetic chemists. It is capable of improving product yields and enhancing the rate of reactions as well as being a safe and convenient method for heating reaction mixtures to elevated temperatures.^{1,2} The field has developed significantly since the first reports of microwave-promoted synthesis in 1986.^{3,4} Domestic microwave ovens are increasingly being replaced by scientific microwave apparatus for use in synthesis. As well as being safer, these new instruments allow for accurate control of key parameters such as initial microwave power, reaction temperature and, in the case of sealed vessel reactions, internal pressure.

Microwave heating occurs on a molecular level as opposed to relying on convection currents and thermal conductivity when using conventional heating methods. This offers an explanation as to why microwave reactions are so much faster. With microwave

irradiation, since the energy is interacting with the molecules at a very fast rate, the molecules do not have time to relax and the heat generated can be, for short times, much greater than the overall recorded temperature of the bulk reaction mixture. In essence, there will be sites of instantaneous localized superheating where reactions will take place much faster than in the bulk. This localized superheating can be especially marked when the reaction mixture contains highly polar reagents or catalysts.

Chemists often would use microwave irradiation as a last resort, heating reaction mixtures to high temperatures in sealed tubes in an attempt to make their reactions go. However, microwave heating has a much more valuable role in the preparative chemist's portfolio. It is possible to perform reactions at modest reaction temperatures and still see great improvements in rate and yield. While sealed reaction vessels are one option, standard reflux and open vessel chemistry can also benefit from microwave irradiation. When performing synthesis using microwave heating, the usual protocol is to heat the reaction mixture to a desired temperature and then hold it there for a period of time. This is known as temperature control. During the initial heating stage a significant amount of microwave power is directed at the sample, but once at temperature, the power drops such as to hold the reaction mixture at the desired temperature. By irradiating a reaction

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mixture with microwaves while simultaneously cooling the outer vessel walls with compressed air or cryogenic fluid, it is possible to irradiate the sample with significant microwave power during the whole period of the reaction. As well as potentially opening up avenues for new chemistry, it is possible to perform chemistry at lower bulk temperatures.⁵⁻⁷ As an example of this, in the Pd/C catalyzed Suzuki reaction of aryl chlorides with boronic acids in water as a solvent, significant decomposition of the aryl chloride substrates occurs at the elevated temperatures required to effect the coupling. By using microwave heating in conjunction with simultaneous cooling it is possible to perform the coupling at a lower bulk temperature but in good yield.⁸ Another way to introduce significant energy into a reaction mixture is to simply irradiate the sample with a constant microwave power, allowing the temperature to rise continually during the course of the reaction. This is known as power control.

As the range of techniques for microwave heating has expanded, so have the areas in which it can have a profound impact. One of the emerging areas is the application of microwave heating to biologically relevant processes. Examples of this are in the synthesis of peptides, peptoids, oligopeptides and carbohydrates and in the field of proteomics. Here, we offer a perspective of these two categories. Rather than provide an exhaustive survey, we have chosen to highlight the advantages of microwave heating in these areas as well as discuss the potential of the technique to impact and transform the field in the future.

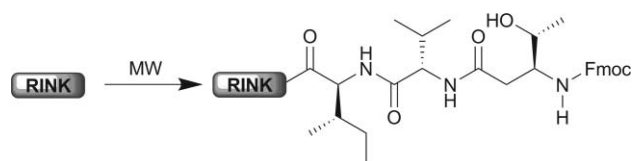
Microwave-promoted synthesis of peptides

Peptides are highly involved in many biochemical processes including cell-cell communication, metabolism, immune response, and reproduction. Additionally, peptides act as hormones and neurotransmitters in receptor-mediated signal transduction. As the role of peptides in many physiological and biochemical processes has become more understood, so has interest in their value as potential drug candidates. Peptides, when compared to small molecule drugs, have the advantage of higher potency and specificity with fewer toxicological problems.⁹ A recent article reported that there are more than 40 marketed peptides worldwide, around 270 peptides in clinical phase testing, and about 400 in advanced preclinical phases.^{10,11}

Obtaining peptides from natural sources can often be a very difficult task. In tissue samples, desired peptides are often at very low concentrations requiring highly sensitive assay methods. The availability and storage of natural tissue samples can also limit availability. While recombinant genetics has been the major production tool for synthesis of proteins, this can be difficult, time consuming and laborious. Chemical synthesis of peptides allows for site-specific control of backbone and side chain modifications with a specificity unavailable through recombinant strategies. Additionally, chemical synthesis allows for peptide sequences to be synthesized not only rapidly, but also free from DNA impurities or endotoxins that may be present during recombinant synthesis. The combination of different chemical synthesis strategies has been successful in producing small proteins of up to 200 amino acids. Although chemical synthesis of peptides and proteins has developed greatly in the last several decades, it can be very time consuming and frequently requires the use of significant quantities of expensive reagents. In addition, in many cases synthesis can

suffer from incomplete reactions that significantly reduce final product purity. Chemical synthesis can be performed either by solution phase, solid phase, or a combination of both. Solid-phase methods predominate however, due to ease of purification at each step.^{12,13}

The first example of microwave irradiation as a tool for peptide synthesis came in 1992. In their solid-phase synthesis of three test peptides, Yu and co-workers reported that microwave heating offered a 2-4 fold reduction in amino acid coupling time, especially when using side-chain hindered amino acids. In addition, no racemisation was observed.¹⁴ The reactions were performed using an un-modified domestic microwave apparatus and thus accurate temperature measurement was not possible and also reproducibility from microwave to microwave proved an issue. While the results appeared promising, lack of proper instrumentation and concerns regarding acceleration of potential side-reactions delayed further exploration. It was not until almost a decade later that the field began to develop in earnest. As modern microwave systems that generated a homogeneous microwave field and offered temperature control became available, new interest was generated in peptide synthesis applications. α -Aminoisobutyric acid (Aib), was coupled with sterically hindered natural or non-coded amino acids, using conventional and microwave heating methods with either PyBOP/HOBt or HBTU/HOBt as coupling agents.¹⁵ In the microwave heating experiments, the reaction mixture was heated to 55 °C for 15 min followed by a further 15 min at 60 °C. Common activators such as HBTU and PyBOP have been shown to be effective with microwave heating up to 110 °C. In the synthesis of a tripeptide (Fmoc-Thr-Val-Ile-NH₂) and two dipeptides (Fmoc-Ala-Ile-NH₂ and Fmoc-Thr-Ile-NH₂) (Scheme 1).¹⁶ The couplings were performed on a polystyrene resin using the Rink amide linker. Couplings of Fmoc-protected amino acids were performed using microwave heating while the Fmoc deprotection steps were conducted at room temperature. The microwave steps were performed in sealed vessels. The reaction mixtures were heated using a temperature control protocol to 110 °C and held at this temperature for 20 min. Significant pressure was generated during the course of the reactions due to the volatile nature of the coupling agents used. The efficiency of the solid-phase methodology was however limited by the need to transfer between different reaction vessels to perform the coupling and washing steps. The application of microwave heating can be expanded to both deprotection and coupling reactions. The ⁶⁵⁻⁷⁴ACP peptide, a standard peptide used to test synthetic methods, was prepared reproducibly and in high purity using 2 min deprotection and 3 min coupling steps and ending with a 10 min cleavage step.¹⁷



Scheme 1 Microwave-promoted solid-phase synthesis of a small tripeptide.

As attractive as the increased deprotection and coupling rates appeared, there was concern over possible enhancement

of sequence dependent side reactions that are well-documented conventionally. Aspartimide formation can occur during Fmoc removal with piperidine and can be a serious problem during chain assembly. Nucleophilic attack by the nitrogen atom on the α -carboxy group of aspartic acid or asparagine on the side chain ester or amide group, respectively results in generation of a 5-membered ring. This potential side reaction will accumulate during each successive deprotection step and can lead to substantially reduced purity. During the coupling reaction, formation of an activated ester and concomitant increased acidity of the α -carbon proton can lead to racemisation. Conventionally this has been problematic for cysteine and histidine derivatives. Racemisation can be difficult to detect because of difficulties with separation and identical mass identification. While microwave peptide synthesis had shown no evidence of racemisation by HPLC analysis with smaller peptides, there had not been a focus on cysteine and histidine containing peptides until recently when a group attempted to prepare a 20 amino acid peptide containing His and Cys residues using microwave heating.¹⁸ The first synthesis in the microwave at 70 °C showed significant racemisation (3–5%).¹⁹ This was suppressed by cooling the contents of the reaction vessel in an ice bath before microwaving for a short period and then repeating this whole procedure a number of times. This allows for significant input of microwave power while at the same time maintaining a low bulk temperature. The optimized conditions for making the 20-mer involved deprotection steps of 3 pulses of 30 s at 100 W with cooling in between ($T_{\max} = 40$ °C) and coupling steps of 5 pulses of 30 s at 50 W, again with cooling in between ($T_{\max} = 40$ °C).

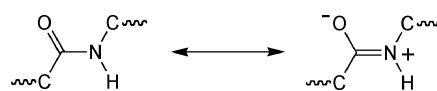
A nonapeptide has also been prepared using a similar “cooling before microwave irradiation” approach.²⁰ The deprotection and coupling reactions were performed in standard glass vials and the washing steps in polypropylene syringes equipped with a frit and PTFE valve. Using a power control protocol, the maximum temperature reached in the steps depended on whether the contents of the reaction mixture were cooled to room temperature or to 0 °C prior to microwave irradiation as did the purity of the final product. As with the previous synthesis of the 20- and 25-mers, cooling in ice prior to microwave irradiation proved optimal.

While performing pre-cooling of reaction mixtures prior to microwave irradiation leads to peptides in high purity with little racemisation, it is inconvenient and not practical either in an automated reactor or when considering performing couplings in parallel or on a large scale. Addressing this problem has been the focus of a recently published report.²¹ A model 20-mer peptide was synthesized both at room temperature and using microwave heating. The model peptide chosen for the study contained each of the natural 20 amino acids, but with a selectively placed C-terminal Asp-Gly segment to encourage maximum potential aspartimide formation (VYWTSPFMKLIHEQCNRADG-NH₂). At room temperature, although the racemisation levels were low, the crude product purity was only 68% due to a number of deletions. An initial microwave-promoted synthesis of the 20-mer indicated that the sequence is susceptible to racemisation. Using deprotection steps of 30 s at 50 W followed by 180 s at 50 W ($T_{\max} = 80$ °C) and a coupling step of 300 s at 40 W ($T_{\max} = 80$ °C) led to significant racemisation at His, Cys and Asp. Simply ensuring that the reaction mixture does not exceed 50 °C when using a power control microwave heating protocol proved effective in reducing racemisation of His and Cys residues and it is not necessary

to pre-cool the reaction mixtures. A coupling protocol of 120 s at 0 W followed by 240 s at 40 W ($T_{\max} = 50$ °C) was used. Additionally, once Cys or His are incorporated into the peptide they do not show any further increase in racemisation during subsequent chain extension steps, even when the reactions are performed using the original conditions of heating to a maximum of 80 °C. As an additional approach, couplings could be performed using microwave heating with the exception of those involving Cys and His which instead are done at room temperature. This combines the time savings of microwave irradiation for coupling of the majority of amino acids with the low racemisation possible by coupling Cys and His conventionally. To limit aspartimide formation both incorporation of HOBt and using piperazine rather than the traditional piperidine as a reagent in deprotection steps proved effective.

Solid-phase peptide synthesisers for use with conventional heating have been around for many years and it was clear that if microwave technology was to have a major impact on peptide synthesis, an analogous automated synthesiser would be required. This is because although the deprotection and coupling steps could be accelerated using microwave heating, if manual transfer between vessels for washing was required, any time savings would be lost. While dedicated microwave synthesisers for organic synthesis have been around for a few years, it was in 2003 that the first automated equipment for peptide synthesis using microwave heating became available.²² The apparatus can perform all steps necessary for the completely automated synthesis of a peptide including all deprotection, coupling, wash and peptide-resin cleavage steps. As an example, a 41 amino acid peptide has been prepared using the apparatus in a total time of 31 h.²³

The question arises as to why the microwave heating has such a profound effect on the deprotection and coupling reactions to make peptides, even at the relatively low microwave powers and bulk temperatures used. Optimal coupling conditions require a fully solvated peptide-polymer matrix that allows for efficient reagent penetration. During the synthesis of difficult peptides the reaction matrix becomes partially inaccessible, typically 6–12 residues into chain assembly.^{24,25} This could be attributed to the formation of secondary structures that result in poor solvation of the peptide-polymer matrix. As a peptide is built stepwise on a resin bead, it can form aggregates with itself or neighbouring chains as a result of hydrogen bonding between peptide backbones. One hypothesis is that irradiation with microwave energy leads to de-aggregation of the peptide backbones thus allowing for reagents to reach the reaction sites at the end of growing chains more easily (Fig. 1). The amide group of amino acids in a peptides has two resonance forms (Scheme 2) thus leading to several important properties, one of which is an unusually high dipole moment of roughly 3.5 debye. When molecules that possess such a dipole moment are exposed to microwave irradiation, the dipoles try to align with the applied electric field. Since the electric field is oscillating, the dipoles constantly try to realign to follow this. At 2.45 GHz, molecules have time to align with the electric field



Scheme 2 Resonance forms of an amide group in an amino acid.

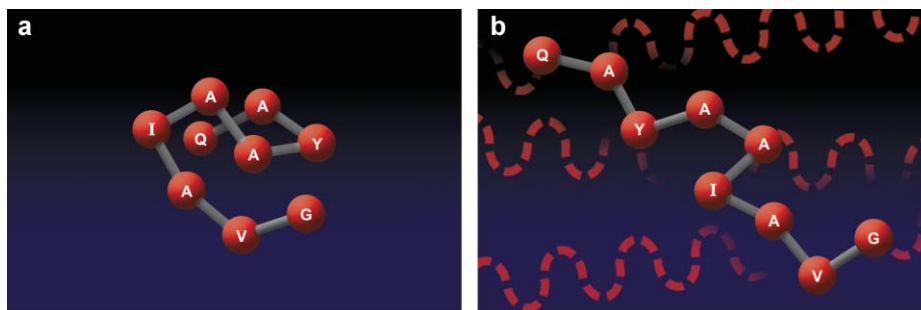


Fig. 1 A peptide backbone (a) before and (b) during microwave irradiation.

but not to follow the oscillating field exactly. This continual re-orientation of the molecules is one of the accepted mechanisms by which microwaves lead to localised heating of reaction mixtures.²⁶ In a polypeptide, this movement could quite feasibly lead to de-aggregation of the peptide backbone as well as to localised superheating which can have an acceleratory effect on the reaction rate for deprotection and coupling steps.

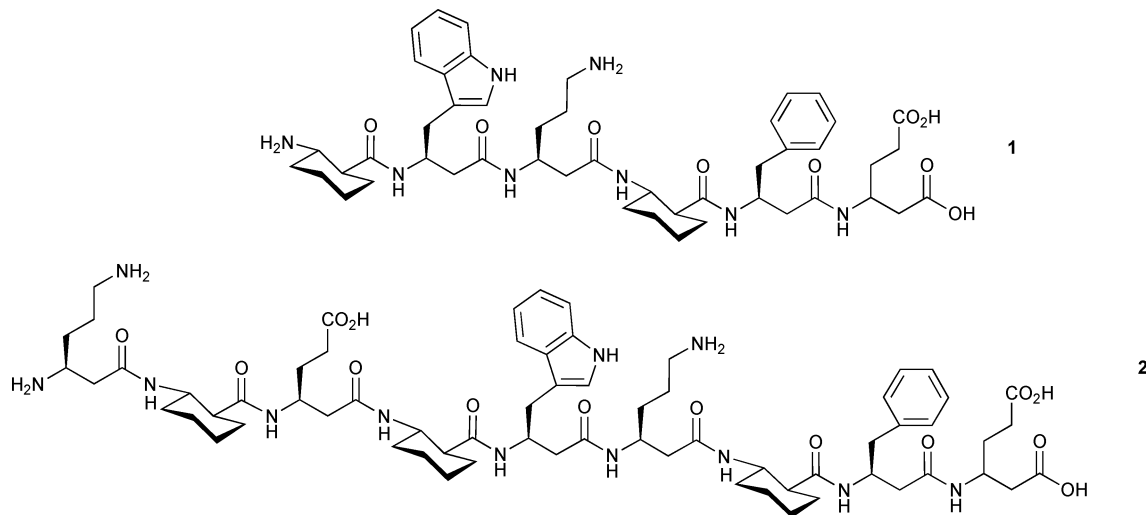
To show the effectiveness of microwave heating in the preparation of longer peptide sequences a β -amyloid, the main constituent of the fibrillar aggregate responsible for Alzheimer's disease, has been prepared.²⁷ The 42 amino acid peptide could be prepared in 69% purity in 19 h using the automated microwave peptide synthesiser.

Microwave heating has been used to prepare 14-helical β -peptides.²⁸ Initial studies focused on the optimisation of the conditions required to prepare single peptides.²⁹ Hexamer **1** was prepared manually both conventionally and also in a monomode microwave apparatus. When using conventional methodology, although the penta- β -peptide precursor was >95% pure, hexamer **1** was only 55% pure with significant quantities (33%) of the unreacted pentamer as well as some of the Fmoc-protected hexamer being present. Using controlled microwave heating, **1** was obtained in 80% purity with only 5% of the unreacted pentamer and none of the Fmoc-protected hexamer being observed. Studies were extended to the deca- β -peptide **2**. However this was obtained in only 57% purity, indicating the challenge of coupling extra residues to **1**. A solution to the problem was to use a solution of

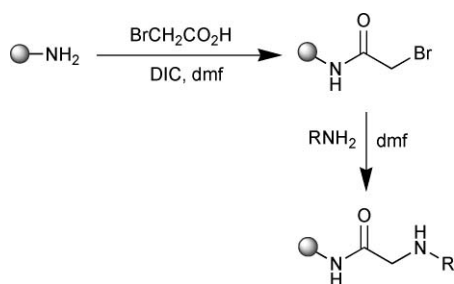
LiCl in DMF in the coupling protocol. Salt additives are known to alleviate, at least in part, the problems of aggregation and/or folding of resin-bound α -peptides as they increase in length.³⁰ Thus, in this case, the effects of microwave heating alone may not be enough to disaggregate the β -peptide backbone. With the modified reaction conditions, **2** could be prepared in 88% purity and 81% yield. Returning to the synthesis of **1** and using the modified conditions, the already high purity could be increased even more (94%). As well as de-aggregating the peptide, the salt additive could also enhance the ionic conduction mechanism for conversion of microwave energy into heat in the reaction mixture.

Moving to a multimode microwave apparatus, the same group have prepared a library of 14-helical β -peptides.³¹ First, conditions for the preparation of **1** in the multimode microwave apparatus were re-optimised. Then, to confirm the homogeneity of heating and to optimise stirring in a 96-well plate, **1** was prepared in 26 different wells scattered across the plate. A library of peptides was then prepared. Some variation in product purity was observed. While this could be attributed to inhomogeneity of heating across all the wells, since the differences in purity were no more than 10%, it could be as much dependent on the amino acid sequence as the location in the 96-well plate.

Peptoids can be prepared efficiently using microwave heating. Peptoids differ from peptides in that the side chain is connected to the amide nitrogen rather than the α carbon atom and are known as oligo(*N*-alkyl) glycines. Peptoids exhibit enhanced stability towards proteolysis relative to α -peptides.³² They also



find applications as biological probes and in drug discovery.^{33,34} In preparing peptoids, each building block is added in a two-step strategy known as the “sub-monomer” route (Scheme 3).³⁵ Firstly the amine function of the existing chain is acylated by the addition of bromoacetic acid and *N,N*-diisopropyl carbodiimide (DIC) and then nucleophilic replacement of bromide with a primary amine. The commercial availability of a wide range of structurally diverse primary amines means that large, diverse peptoid libraries can be made easily and cheaply, often without the need for protecting groups. However, the procedure is slow. Starting from Rink MBHA amine resin, the solid-phase synthesis of a 9-mer could take 20–32 h. Using a domestic microwave oven, this has been reduced to about 3 h.³⁶ The reactions were performed in a 1000 W microwave oven with the power set at 10%. Solutions were irradiated for 15 s, gently agitated and then irradiated for a further 15 s. Under these conditions, the bulk temperature of the reaction mixture was found not to exceed 35 °C as determined using a thermometer after the second 15 s irradiation. This 2 × 15 s irradiation protocol was used for both the acylation and bromide substitution reactions. Yields and purities were comparable to those obtained when performing the chemistry conventionally at 37 °C (45 min for acylation, 1 h for bromide substitution).

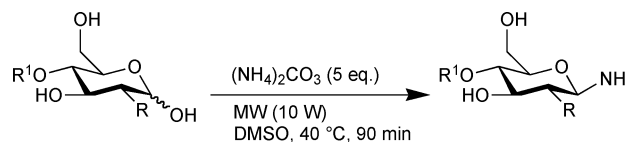


Scheme 3 Synthesis of peptoids using the sub-monomer route.

Following this work, a protocol has been developed for the preparation of peptoids in a scientific microwave apparatus.³⁷ As well as offering a more reproducible methodology, some interesting observations were made. In moving from domestic to scientific microwave equipment, simply repeating the reactions using the same conditions (100 W for 30 s) gave peptoids that were some 10–50% lower in purity. In attempts to determine the origins of this difference, it was discovered that in the case of unhindered primary amines no microwave irradiation was required in order to obtain the corresponding peptoids in high purity. Thus neither microwave heating nor the lengthy conventional protocol is necessary, the reactions being complete within approximately 1 min at room temperature. With electronically deactivated benzylamines, microwave heating was found to have a positive effect. The optimal conditions for the acylation step involved irradiation for 30 s and heating to 35 °C using temperature control, and for the bromide displacement, irradiation for 90 s and heating to 95 °C again using temperature control.

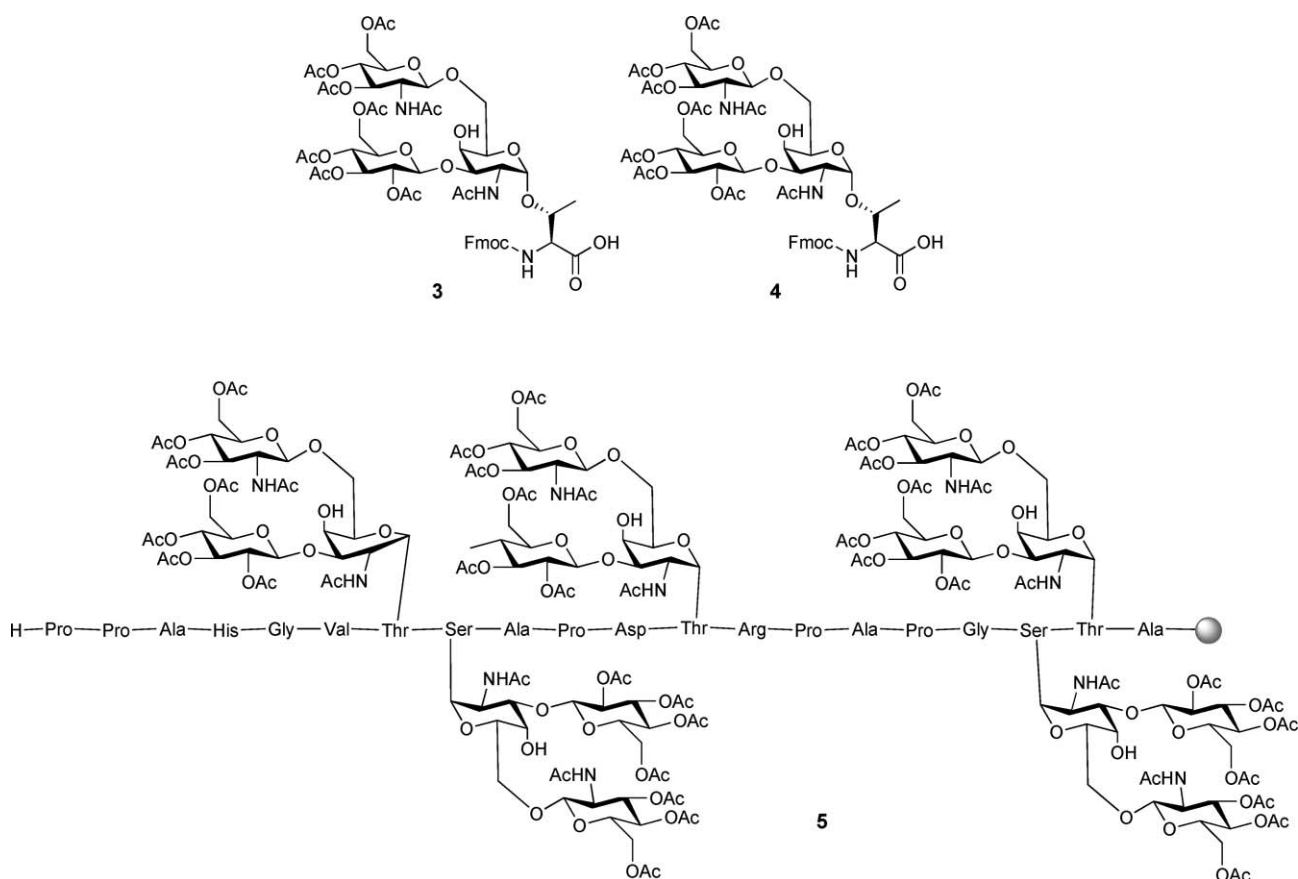
More recently, microwave heating has been found to be useful for generating a poly-cationic peptid and its conjugation to a 13-mer peptide. In an extension to the work, microwave heating has also been used to label the peptides with a variety of fluorophores and quenchers.³⁸

Since more than half of all proteins carry carbohydrate side-chains, the synthesis and study of glycopeptides has become an important area of research.^{39,40} The chemical synthesis of glycopeptides has particular advantages. Glycoprotein samples traditionally obtained from biological sources are very complex and, as a result, little is known about how the glycan chains specifically modulate stability and activity. One synthetic route to glycopeptides involves the use of β -glycosylamines as intermediates, these then being reacted with a suitably protected amino acid or polypeptide side chain.⁴¹ The β -glycosylamines can be prepared directly from a fully deprotected sugar by treatment with 40–50 equivalents of ammonium bicarbonate at room temperature for 6 days, this being known as the Kochetkov reaction.⁴² By using microwave heating, this reaction can be performed in 90 min at 40 °C (Scheme 4).⁴³ Key to the success of the reaction is use of DMSO as a solvent. It is also possible to reduce the quantity of ammonium bicarbonate required to 5 equivalents. A microwave power of 10 W is used and a temperature control protocol used. If the reaction temperature is increased to 60 °C, significant dimerisation is observed. The crude glycosylamines obtained were subsequently used for the preparation of glycoamino acid building blocks.



Scheme 4 Microwave-promoted synthesis of β -glycosylamines.

Microwave heating has also been used for the coupling of sterically hindered, glycosylated amino acid building blocks on solid supports.^{44,45} Amino acid building blocks **3** and **4** were used in the building of a 20 amino acid protein, **5**, known as MUC1. This five *O*-glycan containing glycopeptide has an antigenic structure and is found on the surfaces of epithelial cells in a variety of tissues.⁴⁶ The solid-phase synthesis was performed on both Tentagel and poly(ethylene glycol) poly(dimethylacrylamide) copolymer (PEGA) supports functionalised with Rink amide linker. Reactions were performed at 50 °C in a monomode microwave apparatus using a temperature control protocol as well as conventionally at 50 °C and room temperature. Starting with the Tentagel support, when coupling regular Fmoc-amino acids a reaction time of 10 min was used whereas for couplings involving **3** and **4**, the time was extended to 20 min. Deprotection steps were performed in 3 min. The whole procedure for building the 20-mer was undertaken manually, taking 7 h in the case of the microwave reactions and the conventional control at 50 °C. For the couplings at room temperature, reaction times were significantly extended (2 h for Fmoc amino acid couplings, 20 h in the case of **3** and **4** and 20 min for Fmoc deprotection steps). As a result, the whole process took approximately 99 h. The overall yield of the desired 20-mer in the microwave protocol was significantly higher than when using conventional heating but comparable to that obtained at room temperature. Changing to the PEGA resin improved the yield of the final glycopeptide. The PEGA resin may permit the permeation both of reagents and steric building blocks **3** and **4** into the porous surfaces of the polymer particles. The 20-mer was



elaborated further by enzymatic sugar elongation to give a range of more complicated glycopeptides.

Microwave heating as a tool for proteomics

Proteomics is broadly defined as the large-scale study of proteins, particularly their structures and functions.^{47,48} To obtain detailed structural information, proteins are selectively cleaved into smaller polypeptide fragments by controlled chemical reactions or enzymatic digestion. The resulting mixtures can then be analysed by various mass spectroscopic techniques and, from this, the structure of the original protein determined.

Identification of the amino acid at the C-terminus of proteins is possible using the Akabori reaction, devised over half a century ago.⁴⁹ It involves heating the protein with anhydrous hydrazine under reflux at 125 °C for several hours. The amino acid at the C-terminus of the peptide is liberated and can be distinguished from the remaining amino acid residues that have been converted to hydrazides. In addition, hydrazinolysis provides quantitative information for the presence of amino acid residues containing guanidino, β -mercapto, carboxy, or carboamido groups. The reaction has been performed in a domestic microwave, the time to reach completion being reduced to 3–5 min.⁵⁰ The dipeptide Trp-Phe, tripeptide Tyr-Gly-Gly, tetrapeptide Pro-Phe-Gly-Lys, heptapeptide Ala-Pro-Arg-Leu-Arg-Phe-Tyr, and an N-terminal blocked tripeptide (*N*-acetyl-Met-Leu-Phe) have all been used as test substrates.

When using enzymic methodologies, digestion times depend on the nature of the proteins and can vary from hours to days. It is

necessary to ensure that sufficient quantities of the peptides are generated such that the detection limit of the analytical techniques is surpassed. Trypsin is the most commonly used enzyme because it specifically hydrolyses peptide bonds at the carboxyl side of lysine and arginine residues, except when either is followed by proline. Unmodified trypsin is subject to proteolysis, generating fragments that can interfere with protein sequencing or peptide analysis. As a result, trypsin modified by reductive methylation is often used, this treatment rendering it resistant to proteolytic digestion. The combination of trypsin digestion with controlled microwave heating has attracted attention.

Trypsin digestion of bovine cytochrome c, bovine ubiquitin, horse heart myoglobin, modified chicken egg lysozyme and recombinant human interferon α -2b (rh-IFN α -2b) has been performed in a scientific monomode microwave apparatus.⁵¹ The reactions were performed on a 100–350 μ L scale. The digestion was optimised at 60 °C for 10 min. Additionally, no non-specific cleavage was detected using the microwave approach. A comparison of conventional and microwave heating in the digestion of cytochrome c showed that microwave irradiation for 10 min produced similar results to the classic method of 6 h of digestion at 37 °C. The beneficial effect of microwave irradiation was confirmed by performing the digestion of cytochrome c in 12 min under microwave and conventional heating. In the case of microwave irradiation, after this time approximately 90% sequence coverage was possible whereas there were no hydrolysis products in the conventional experiment. In the absence of the protease, cytochrome c remains intact after 20 min irradiation, showing that the microwave energy only enhances the enzymatic digestion of the

protein and does not itself induce degradation. Similar shortening of the digestion time has been found when using a domestic microwave and bovine serum albumin and human urinary protein as substrates.⁵²

The digestion of rh-IFN α -2b was repeated at different temperatures and rapid elevation of the solution to above 60 °C was found to enhance the process.⁵¹ In both cases the percentage of digestion increased rapidly over the first 5 min before levelling off at approximately 70% at 10 min. In an attempt to mimic conventionally the temperature profile seen in the microwave experiments, the same digestion was performed using a metal block pre-heated to 60 °C. The rate of enzymic cleavage was found to be essentially identical suggesting that, at least in part, the rapid increase in temperature is responsible for the rate of acceleration seen upon microwave irradiation.

Using more accurate temperature measurements and control, the microwave-mediated digestion of glycosylated haemoglobin (HbA1c) has been studied.⁵³ Using trypsin modified by reductive methylation as the proteolytic enzyme for digestion, the optimum conditions involved controlled microwave heating to 50 °C and holding at this temperature until a total time of 20 min had elapsed with an enzyme-to-protein ratio of 1 : 100. Under these conditions, the digestion efficiency is about 20% greater than that observed using conventional conditions for 18 h. Changing the enzyme from trypsin to Glu-C has a significantly negative effect on digestion efficiency. The optimum temperature for Glu-C was found to be 40 °C but, even at this zenith, the efficiency was significantly lower than under conventional conditions at room temperature. The difference in enzyme activity could be attributed to several factors. Firstly, Glu-C was used at temperatures significantly higher than the manufacturer's recommendation of 25 °C. Also, while it is possible to modify trypsin to make it less susceptible to proteolytic digestion, the same is not true of Glu-C. However, since analysis of the Glu-C digest showed no evidence for proteins originating from the enzyme, it is more likely that thermal deactivation is the major cause of the loss of activity as opposed to autolysis of the enzyme.

Digestions are traditionally performed in standard buffer solutions. The effect of solvent on the efficiency of microwave-promoted trypsin digestion has been studied using myoglobin, cytochrome c, lysozyme and ubiquitin as substrates.⁵² The efficiencies and sequence coverages increased when acetonitrile was added to the reaction mixture. If methanol is used as an additive, as the quantity increases so the enzyme activity decreases indicating that it is being deactivated. This is not surprising given the fact that methanol inhibits many enzyme-catalysed processes.

It is also possible to use microwave heating to perform digests on substrates separated on gels and results suggest that this significantly improves peptide recovery compared to standard in-gel digestion protocols.^{51,52,54} The ability to be able to perform in-gel digests means that the technology can be in conjunction with separation techniques for analysis of protein mixtures. Five proteins, including lysozyme, chicken egg albumin, bovine albumin, conalbumin, and ribonuclease have been separated by gel electrophoresis, stained, cut out and digested with trypsin solution either in a microwave for 5 min or incubated for 5 min or 16 h at 37 °C. Using microwave irradiation resulted in more matched fragments than either of the traditional methods for all the proteins except conalbumin; in this case the number of matched fragments is slightly lower but close to the 16 h traditional method. Other

Table 1 Comparative dipole moments⁵⁵

Molecule	Dipole moment (debye)
Water	1.8
Peptide bond	3.5
Myoglobin	170
Horse serum albumin	380
Horse carboxy haemoglobin	480

studies show similar results. For example, with both bovine serum albumin and yeast lysate the number of proteins identified with an in-gel microwave methodology was either the same or better than that from conventional studies.⁵²

Again the question arises as to why the microwave heating has such a profound effect on the rate of enzymatic digestion. Together, α -helices and β -conformations compose a major portion of the secondary structure of large proteins. In the α -helix, the polypeptide backbone is tightly wound around the molecule axis with each amino acid side-chain pointing outwards and downwards from the backbone. The α -helix shows highly optimised use of internal hydrogen bonding and creates a stacking of peptide bond dipoles that are added across the hydrogen bonds in the helix, this leading to a large net dipole from one end of the helix to the other. The magnitude of these dipoles is illustrated in Table 1. The presence of a large net dipole moment across α -helices may be responsible, at least in part, for increased digestion rates of certain proteins upon microwave irradiation. If the microwave energy interacts with the dipole of the α -helix, perturbation of the three-dimensional structure of the protein may result (Fig. 2). This could facilitate digestion of previously enclosed areas of the protein.

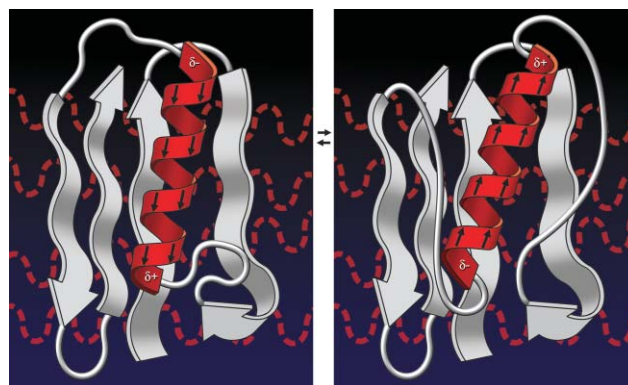


Fig. 2 Dipole moment across an α -helix and interaction with microwave radiation.

Microwave heating can also be used for determination of amino acid content (but not structural information) by means of vapour-phase protein hydrolysis using 6 M HCl in a sealed tube.⁵⁶ A particular advantage of the microwave-promoted hydrolysis is that it reduces the time required for cleavage of difficult to hydrolyse hydrophobic peptide linkages without excessive degradation of the labile amino acids, serine and threonine. In a recent development, apparatus specifically designed for microwave-promoted hydrolysis has become available. The 45 mL vapour-phase hydrolysis vessel allows processing of up to ten 100–300 μ L HPLC autosampler vials at one time and is connected to vacuum and nitrogen sources. The sealed hydrolysis vessel is alternately evacuated and purged

with nitrogen. The hydrolysis is then performed under inert, anaerobic conditions to prevent oxidative degradation of amino acids. Using this, hydrolyses on the picomole to nanomole scale are possible.

Microwave heating can be used as a tool for deglycosylation of antibodies.⁵⁷ Many biotechnological products consist of antibodies raised against an oncological, auto-immunological or other antigen of interest. Characterisation of these antibodies is important, but the fact that they are heavily glycosylated means that the task of measuring the molecular weight of the intact protein is complicated. By deglycosylation, it is possible to facilitate accurate molecular weight verification. A common method for achieving this is to use an enzymic cleavage. Peptide-N-glycosidase F (PNGase F) is one of the most widely used enzymes for the deglycosylation of glycoproteins. The enzyme releases asparagine-linked (N-linked) oligosaccharides from glycoproteins and glycopeptides. With controlled microwave heating it is possible to perform deglycosylation reactions using PNGase F in between 10 min–1 h depending on the substrate. This compares to a time of up to 2 days when performing the same transformations conventionally. The reactions were performed in a monomode apparatus using just 1 W microwave power. The optimum reaction temperature was 37–45 °C above which recovery of the protein at the end of the cycle drops significantly.

Microwave heating as a tool for other biochemical applications

Microwave heating has found uses in a number of other biochemical processes. To show the scope, we have selected some examples.

Microwave-promoted catalysis of organic transformations using enzymes has been the subject of a number of studies, mainly using domestic microwave ovens.⁵⁸ While an apparent rate enhancement has been reported, more control experiments are required before a definite conclusion can be drawn. Controlled microwave irradiation has been used to accelerate by at least 15-fold metal-catalyzed oxidation reactions that site-specifically oxidize the amino acids bound to copper in Cu/Zn superoxide dismutase.⁵⁹ When combined with mass spectrometry, these reactions provide a sensitive method for determining Cu-protein binding sites. The maximum microwave power suitable for maintaining the structural integrity of the protein can be determined readily by measuring the oxidation extent of different peptide fragments as a function of power.

DNA amplification by polymerase chain reactions (PCR) is a very powerful process, finding applications in medical and biological research labs for a variety of tasks such as the detection of hereditary diseases, the identification of genetic fingerprints, the diagnosis of infectious diseases, the cloning of genes and DNA computing.^{60–62} The PCR process is conventionally carried out in a thermal cycler using a DNA polymerase. The most common is Taq polymerase. The application of microwave heating to PCR has been studied both to see how the process can be facilitated and also probe the effects of heating Taq polymerase in many cycles.^{63,64} Using controlled microwave heating, it is possible to reduce the cycle time by approximately half. This is due mainly to the fact that it is possible to reach the target temperatures very rapidly and then hold it there easily. As a result, incubation times

can be shortened over the conventional counterparts since, in the case of the latter, time for equalisation of the temperature is required. Also, because the heating is on a localized level when using microwave irradiation, it is possible to perform PCR on the mL scale. Conventionally, the slow distribution of heat together with the importance of short process times and reproducibility limits the volume for most reactions to 0.2 mL. Using microwave heating, PCR on the 2.5 mL and 15 mL scales has been performed rapidly with very high efficiency. The sequence to be amplified was a 53 bp fragment from human chromosome 13. A total of 33 cycles was performed.

By combining the use of metal-enhanced fluorescence (a near field effect that can significantly enhance fluorescence signatures) with low power microwave heating, the sensitivity of surface assays in a model protein avidin–biotin assay could be greatly increased as well as being essentially completed within a few seconds.⁶⁵ A greater than 5-fold fluorescence enhancement coupled with an approximate 90-fold increase in assay kinetics was observed. This technology has the potential to impact high throughput fluorescence-based processes, such as in biology, drug discovery and general compound screening. In another enabling technology, the power of combining cleavable isotope-coded affinity tags (ICAT) and microwave heating can offer an efficient, high throughput analysis of proteins.⁶⁶

Summary

Application of microwave energy for peptide synthesis has shown advantages in terms both of higher purity and shorter synthesis time. With two separate chemical reactions required for addition of each amino acid, the benefit of microwave is accumulated at each cycle. Synthesis of other types of polymers such as oligonucleotides and carbohydrates should offer similar benefits. Phosphoramidite chemistry has become the preferred synthesis technique of oligonucleotides. While DNA synthesis is fast and efficient conventionally (30 s coupling), RNA synthesis is more difficult. In comparison to deoxyribose phosphoramidites, the ribose phosphoramidites associated with RNA contain an extra 2'-hydroxyl group that requires additional protection during synthesis. The commonly used protecting groups 2'-*O*-(*tert*-butyl)-dimethylsilyl (TBDMS) and 2'-*O*-[(triisopropylsilyl)-oxy]-methyl (TOM) can interfere with the coupling reaction requiring routine 12–15 minute reaction times with standard tetrazole activation. Use of alternative activators such as 5-ethylthio-1*H*-tetrazole (ETT) and 5-benzylthio-1*H*-tetrazole (BTT) has shown decreased coupling times, but their increased acidity has been associated with premature deprotection of trityl groups leading to unwanted dimerisation. Microwave represents a strategy for potentially decreasing RNA coupling times to less than a minute even while using standard tetrazole activation. Removal of the 2'-protecting group of TBDMS or TOM is a slow process that is critical for high purity RNA. Conventionally, this process requires around 3 h and is a potential area where microwave synthesis could provide benefits.

Carbohydrates have many biological roles including energy storage, metabolism, and bioreceptors in cell-to-cell communication. However, research of carbohydrates has been limited due to difficulty of synthesis. Development of solid phase synthesis of oligosaccharides (SOS) represents an area of active research

to overcome this problem. However, generation of synthetic oligosaccharides represents a major challenge due to the fact that they are branched rather than linear, monosaccharide units can be connected by α or β linkages, and multiple selective protecting strategies are required. Attempts at coupling monosaccharide units are also difficult due to steric hindrance associated with the monosaccharide units. While development of an orthogonal protecting strategy for SOS represents a major current challenge, microwave represents a valuable tool that can be applied to assist the rapidly developing SOS methods.

A proteome is thought to be an order of magnitude more complex than the genome itself due to post-translational mechanisms, genes coding for multiple proteins, and proteins assuming multiple forms. For this reason throughput is a major focus in proteomics and enzymatic digestion is often conventionally performed in 96-well formats. While enzymatic digestion has shown benefit with microwave energy, its application has typically been limited to single samples. A high throughput format is currently an issue for microwave instrumentation as the individual wells do not heat evenly in larger multimode cavities and do not fit into smaller single mode cavities. Research focused on development of a new cavity to heat a 96-well plate uniformly has proved challenging due to the small sample sizes used and the need for accurate temperature monitoring. Development of a 96-well plate microwave system should make a significant impact in improving throughput and sequence coverage for proteomics.

In conclusion, microwave chemistry has shown its scope is not limited only to synthetic organic chemistry, but also includes many biological applications. In many cases, comparison of conventional and microwave methodologies has shown that peptides can be prepared in higher yield and purity using microwave irradiation.^{21,29,37,38} Due to the large net dipole moment associated with peptides, proteins, and other biomolecules, microwave energy offers a unique tool in the bioscience field. Through dipole rotation and ionic conduction, controlled microwave irradiation can be used to transfer significant amounts of energy into biosystems for enhancing a wide variety of processes. As the bioscience field continues to expand so the application of microwave will grow in parallel.

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