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Effect of Microwave Heating on the Crystallization of Glutathione Tripeptide on Silver Nanoparticle Films

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Abstract

Effect of microwave heating on the crystallization of glutathione (GSH) tripeptide using the metal-assisted and microwave-accelerated evaporative crystallization (MA-MAEC) technique is reported. GSH crystals were grown from supersaturated solutions of GSH (300–500 mg/mL) on the iCrystal plates with silver nanoparticle films (SNFs) and without SNFs in three different microwave systems operating at 2.45 GHz: conventional (multimode, fixed power at 900W), industrial (monomode, variable power up to 1200 W), and the iCrystal system (monomode, variable power up to 100 W). The efficacy of the MA-MAEC technique, in terms of improvement in the crystallization time, crystal size and quality of GSH, was compared between the three microwave systems and the crystallization at room temperature (no microwave heating, a control experiment). Optical microscopy was used to visualize and quantify the growth of GSH crystals during and after microwave heating. Powder X-ray diffraction (XRD) and Fourier transform infrared (FTIR) spectroscopy data showed that GSH crystals had identical crystal structure to those grown at room temperature and microwave heating did not alter the chemical structure of GSH molecules during microwave heating, respectively. Using the MA-MAEC technique, the iCrystal system yielded high quality GSH crystals in a rapid manner.

Graphical Abstract

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The authors declare no competing financial interest.

Supporting Information

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Additional information related to temperature measurements during microwave heating, SEM, optical microscope images, and FTIR of GSH crystals under experimental conditions not shown in the main text (PDF)

INTRODUCTION

Crystallization of peptides affords for the structural analysis of important biological molecules and development of biological composite materials.^{1,2} Furthermore, crystallization of small peptides facilitates new pharmaceutical advances in drug design and delivery for subsequent treatment in medicine.³ One promising candidate for the study of medicine and drug development is glutathione (GSH). GSH, a low-molecular-weight tripeptide molecule (γ -glutamyl-cysteinyl-glycine), is one of the most abundant thiol compounds present in animal cells and is the primary antioxidant involved in maintaining redox status in cellular microenvironments.⁴ GSH also serves as a cofactor for many cytoplasmic enzymes and plays an important role in post-translational modification. GSH metabolism has been linked to HIV treatment, cancer, chronic stress, cardiovascular disease, and neurological disorders, such as, Alzheimer's disease and Parkinson's disease.^{5–8} Thus, the role of GSH supplementation as an effective treatment has been growing rapidly. Several crystallization methods that offer systematic control over the crystallization of GSH have been described in the literature. The most prominent of these methods deals with the recovery of GSH from natural sources in high yield and in uncontaminated form.⁹ However, these methods do not consider the rapidity of GSH crystallization and control over crystal size.

Previously, our research group demonstrated the crystallization of biomolecules (i.e., amino acids and proteins) using the MA-MAEC technique.^{10,11} The MA-MAEC technique offers the advantage of rapid crystallization and improved crystal size and quality for biomolecules of interest.¹⁰ The crux of the MA-MAEC technique is the generation of microwave-induced thermal gradients and the subsequent rapid mass transfer of biomolecules directed toward the metal nanostructures on planar surfaces that act as nucleation sites and microwave-transparent medium. Metal nanostructures of silver, gold, copper, nickel, and indium tin oxide are deposited onto poly(methyl methacrylate) (PMMA) platforms combined with silicon isolators (i.e., the iCrystal plates).^{12–16} The unique design of the iCrystal plates ensures that microwave radiation is distributed homogeneously across the wells formed by the silicon isolator.^{17,18} In studies based on the MA-MAEC technique by our research group to date, two different microwave systems operating at 2.45 GHz was employed: a multimode conventional microwave system (fixed power, 7001000 W)^{10,11} and the iCrystal system (monomode, variable power up to 100 W).¹⁹ Conventional microwave system is a commercially available microwave oven that was initially used to demonstrate the proof-of-principle demonstration and applications of the MA-MAEC technique for the crystallization of biological compounds.^{11–16} The iCrystal microwave system is an instrument developed by our research group and was also employed for the crystallization of a variety of biomolecules.¹⁹

Our research group seeks to improve the laboratory scale and rapid crystallization of biomolecules and pharmaceutical compounds using the MA-MAEC technique. Although the conventional and the industrial microwave systems were shown to facilitate rapid crystallization of biomolecules to date, a detailed comparative investigation of their capabilities for crystallization of simple amino acids and peptides was never carried out. Subsequently, there is still a need for the selection of a suitable microwave system that can

be used with the MA-MAEC technique for the rapid and on-demand crystallization of molecules of interest. In addition, there are several commercially available monomode industrial and research grade microwave systems that offer variable power up to several kilowatts, which were never used for crystallization of biomolecules.

This paper has two main aims: first, we demonstrate the effectiveness of the MA-MAEC technique for the rapid crystallization of a tripeptide (GSH) in all three different microwave systems, and second, we establish which of the three microwave systems is most effective for the rapid crystallization of GSH using the MA-MAEC technique. With respect to the latter, we compared several experimental factors, such as, crystallization speeds, crystal size, rate of crystal growth and crystal quality. Images of GSH crystals taken via optical microscopy are used to compare the aspects of crystal growth during microwave heating in all three microwave systems. Additionally, X-ray diffraction and FTIR analysis are used to confirm the preservation of GSH crystal structure. Moreover, computer simulations were carried out to predict and verify GSH crystal morphology based on crystallographic geometrical considerations. Our findings indicate that the use of the MA-MAEC technique improves crystallization rate of GSH in all three microwave systems and that the iCrystal system is the best choice for efficient crystallization in terms of crystal quality and size.

EXPERIMENTAL METHODS

Materials

Circular PMMA disks (5 cm in diameter) were purchased from McMaster-Carr (IL, USA), the L-glutathione, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS) and sodium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid, sodium hydroxide and ethanol was purchased from Pharmco-AAPER (Brookfield, CT). The silicon isolator with 21 wells was designed by The Aslan Research Group and manufactured by Grace BioLabs (Bend, OR). Each well had a capacity of 30 μ L of solution. A silver target (57 mm in diameter) was obtained from Electron Microscopy Sciences (Hatfield, PA).

Methods

Selection Solvent for GSH Crystallization Studies—Solubility of GSH in several solvents was investigated in the temperature range was between room temperature and 50 °C to obtain supersaturated solutions of GSH. The solvents used were DMF, DMSO, ethanol, water, PBS (pH = 7.4), sodium acetate (pH = 4.6), and sodium phosphate dibasic (pH = 9.1). Using a micropipette, a milliliter of each of the above solvents was pipetted into a 20 mL glass vial. The premeasured (<10 mg) of the solute was added into the solvent and the solution shaken thoroughly for 5 min and left on the corning magnetic stirrer to attain the room temperature. GSH in DMF, ethanol, and DMSO did not dissolve by the end of the 60 min, while water and buffer solution dissolved GSH completely. The above process was repeated at controlled temperature of 50 °C in a water bath.

Preparation of Sodium Acetate Buffer—A 8.2 g sample of the salt was dissolved in deionized water to make a 0.20 M and 4.6 pH solution of sodium acetate. pH of the buffer solution was adjusted by adding an acid (HCL) or a base (NaOH) to achieve the desired pH.

Preparation of GSH Solution—GSH of varying concentrations (final concentration: 100, 200, 300, 400, and 500 mg/ mL) were prepared by dissolving the appropriate amount of tripeptide in sodium acetate at 50 °C. The solutions were then checked for any undissolved substances using an optical microscope (Swift Digital M10). GSH solutions used in the crystallization experiments did not have any observable undissolved substances.

Preparation of Silver Nanoparticle Films—Circular PMMA disks were rinsed with ethanol and with deionized water, and wiped using a kimwipe and air-dried using air supply in the laboratory. Circular PMMA disks were coated with a silver thin film of 1 nm in thickness using a sputter coater (EMS 150R S, Electron Microscopy Sciences, Hatfield, PA). The SNFs-coated PMMA disks were modified with a silicon isolator with 21-well capacity (to form the iCrystal plates) were used within 1 day of preparation and those without SNFs were used as controls in the experiments.

Crystallization of GSH at Room Temperature—Twenty microliters of GSH solutions were placed in each well of a 21-well setup of the iCrystal plates with and without SNFs coating. Crystallization of GSH in all iCrystal plates was observed in one well using an optical microscope (Motic DV Camera). Optical images of crystals were captured every 5 min. GSH crystals in the other wells were imaged after the completion of the experiment and were collected in 5 mL polyethylene centrifuge tubes.

Crystallization of GSH using the MA-MAEC technique—The iCrystal plates and GSH solutions described above was employed. To accelerate the crystallization process, GSH solutions on the iCrystal plates were heated in three types of microwave systems: conventional microwave, industrial microwave, and the iCrystal microwave system. The first two of these systems facilitate crystallization by evaporation of the solutions on the iCrystal plates. The third microwave facilitates crystallization in a similar manner but without complete evaporation.

The conventional microwave oven (Rival, EM 720 CWA-PMB, 900 W) was operated at power level 1 (equivalent to 900 W in 10% of the microwave heating time or duty cycle of 3 s). The iCrystal plates were covered with thin polyethylene film and placed in the center of the microwave to ensure homogeneous heating throughout the experiment. Microwave heating of the iCrystal plates was continued for 5 min followed by observation under the optical microscope, and capturing the optical images of the grown crystals. This process was repeated every 5 min until complete crystallization (i.e., solvent was completely evaporated) was observed. The crystals from the remaining 20 wells were imaged first and were collected for FTIR and X-ray diffraction studies.

The industrial microwave system (GAE Variable Attenuator 1.2 kW CPR284) was operated at different power levels (200, 400, 600, 800, 1000 W). Each GSH solution was exposed to

each power level and again images were captured every 5 min until complete evaporation of the solvent was achieved.

The iCrystal microwave system was operated at 80 W. Microwave heating was performed for 15 min after which the iCrystal plates were removed from the microwave chamber and images of the target well were captured under the microscope. After 90 and 150 min, crystal size was measured and averaged for the given well. The 15 min microwave heating cycle was repeated for 3 h by which time the size of the GSH crystals were comparable to those grown with the other microwave systems.

Characterization of GSH Crystals—Size of GSH crystals was determined by (Motic images plus 2.0) vendor-provided software from the digital images captured using an 8-megapixel digital camera. Infrared spectrum of GSH crystals was obtained using an FTIR spectrometer (Agilent Cary 630 FTIR Technologies) in attenuated total reflectance (ATR) mode. GSH crystals were placed on the sample compartment and analyzed for spectra in the range of 4000 to 500 cm^{-1} . X-ray diffraction data was collected using Rigaku MiniFlex spectrometer equipped with Cu K α radiation.

RESULTS AND DISCUSSION

Solvent Selection for GSH Crystallization

The solubility of GSH was low in DMF and ethanol at both room temperature and 50 °C (Table S1, Supporting Information). Low solubility was also observed for GSH in DMSO at room temperature, but increased to <10 mg/mL at 50 °C. Solubility of <10 mg/mL for GSH was also observed with water, PBS, sodium acetate and sodium phosphate dibasic. DMF, DMSO, and ethanol were not chosen for the crystallization study because the solubility of GSH is lower in these solvents and initial screen for crystallization did not yield observable crystals. In addition, no or insignificant amount of GSH crystals were observed in deionized, PBS and sodium phosphate dibasic buffer both at room temperature and at 50 °C. Use of sodium acetate as solvent yielded large GSH crystals in 10 min at room temperature, and in 5 min at 50 °C. Subsequently, due to sufficient solubility and growth of GSH crystals in the initial screening, sodium acetate was the preferred solvent for the crystallization of GSH in this study.

Crystallization of GSH Using a Conventional Microwave

Table 1 shows the crystallization time (when all solvent was evaporated) for all GSH concentrations observed for the iCrystal plates with SNFs and without SNFs (i.e., PMMA) at room temperature, and using the MA-MAEC technique. The room temperature samples were used as controls for all microwave systems studied. No GSH crystals were observed at 100 and 200 mg/mL under any experimental conditions studied and will not be discussed further. For GSH solutions of 300, 400, and 500 mg/mL concentrations, the initial crystallization times were faster on the iCrystal plates with SNFs under both room temperature and microwave heating conditions (ascertained from Figures 1–3). The initial crystallization time was 25% to 30% faster for the iCrystal plates with SNFs when compared

to the iCrystal plates without SNFs. The fastest initial crystallization time occurred on the iCrystal plates with SNFs using microwave heating (the MA-MAEC technique).

Complete crystallization times decreased as the concentration of the initial GSH solution increased for all three microwave systems. For the initial GSH concentration of 300 mg/mL at room temperature, complete crystallization was observed at an average time of 628 ± 31.4 min and 415 ± 38 min for the iCrystal plates without SNFs (labeled as PMMA) and with SNFs, respectively. For the initial concentration of GSH 300 mg/mL, crystallization times using the MA-MAEC technique were 130 ± 20 min and 108 ± 12 min for the iCrystal plates without SNFs and with SNFs, respectively. These observations show that, when compared to crystallization at room temperature, the use of microwave heating yields GSH crystals ~5-fold and 4-fold faster crystallization times on the iCrystal plates without SNFs and with SNFs, respectively. In terms of platform type (i.e., iCrystal plates with and without SNFs), the presence of SNFs on the iCrystal plates reduced the crystallization times by <200 min at room temperature conditions and 22 min using microwave heating. At the range concentration of GSH studied here (300–500 mg/mL), the fastest crystallization times were observed when using the iCrystal plates with SNFs and microwave heating (i.e., the MA-MAEC technique), which yielded ~5.8-fold faster crystallization times as compared to the control experiments (iCrystal plates without SNFs at room temperature).

For initial GSH concentration of 400 mg/mL, complete crystallization of GSH at room temperature was observed at 242 ± 23 min and 227 ± 87 min for the iCrystal plates without SNFs (labeled as PMMA) and with SNFs, respectively. With the MA-MAEC technique, complete crystallization of GSH (400 mg/mL) occurred at 87 ± 8 min and 65 ± 8.7 min for the iCrystal plates without SNFs and with SNFs, respectively. On iCrystal plates with SNFs, crystallization times were shortened by 15 min at room temperature and 22 min under microwave heating. The fastest crystallization times for 400 mg/mL of GSH resulted using the MA-MAEC technique, which improved crystallization speeds by ~4-fold over the control experiments.

For initial GSH concentration of 500 mg/mL, complete crystallization of GSH at room temperature was observed at 257 ± 98 min and 170 ± 56 min using the iCrystal plates without SNFs and with SNFs, respectively. Crystallization times with the MA-MAEC technique were 63 ± 3 min and 47 ± 8 min for the iCrystal plates without SNFs and with SNFs, respectively. Using the MA-MAEC technique, complete crystallization of GSH (500 mg/mL) was ~4 times faster on the iCrystal plates without SNFs and with SNFs. At room temperature, crystallization time for GSH from an initial solution of 500 mg/mL was 87 and 16 min shorter on iCrystal plates without SNFs and with SNFs, respectively. Subsequently, the use of the MA-MAEC technique for the crystallization of GSH initial solution of 500 mg/mL resulted in the reduction of crystallization times ~5.5-fold faster than the control experiments.

Figure 1 and S1 (Supporting Information) shows the time- dependent growth of GSH crystals on iCrystal plates with and without SNFs using a conventional microwave and room temperature, respectively. Initial GSH crystals grown on both iCrystal plates in a conventional microwave observable by our optical microscope appeared in 5 to 10 min of

microwave heating. Size of the initial GSH crystals that were grown at both room temperature and by using the MA-MAEC technique was determined to be $\sim 20\text{--}30\text{ }\mu\text{m}$ (we refer to the length of GSH crystals throughout the text). As the microwave heating or the room temperature incubation continues, size of the GSH crystals reach $<100\text{ }\mu\text{m}$, where largest GSH crystals can be grown on the iCrystal plates using microwave heating. The observed difference in the size of GSH crystals can be attributed to the fact that rapid heating/cooling can lead to faster nucleation rates, which could result in larger crystals on the iCrystal plates with SNFs.

To ascertain the differences in the growth of GSH crystals on iCrystal plates with and without SNFs using the conventional microwave system, the rate of growth for GSH crystals from initial GSH solution of $300\text{--}500\text{ mg/mL}$ on both surfaces were calculated from the experimental crystal size measurements during microwave heating (Figure S2, Supporting Information). The rate of crystal growth on the iCrystal plates with SNFs (range of $20\text{--}40\text{ }\mu\text{m/min}$) were typically 2-fold larger than those observed on the iCrystal plates without SNFs (range of $7.0\text{--}33\text{ }\mu\text{m/min}$). For the initial concentration of GSH solution of 300 mg/mL , the initial rate of crystal growth on SNFs were significantly larger on surfaces without SNFs, which implies that at lower GSH concentration (300 mg/mL) rate of crystal growth is influenced by the presence of SNFs (surface- dependent growth). As the initial concentration of GSH solution was increased to 500 mg/mL , the gap between the initial rate of crystal growth on both surfaces decreases, that is the effect of SNFs becomes less significant (i.e., concentration- dependent growth). These observations demonstrate the advantage of combined use of SNFs and conventional microwave heating.

Industrial Microwave

We note that the current study is the first time such an industrial microwave was used with the MA-MAEC technique, subsequently, it is important to further describe the experimental conditions. The industrial microwave used in this study operates 2.45 GHz and has a variable power microwave source with true power control up to 1200 W (Figure 2). Our research laboratory has designed and custom built a microwave cavity to operate at 2.45 GHz , like the one that is currently in use with the iCrystal system. Our microwave cavity has nine open ports (3×3 , $D = 1\text{ cm}$, 2 cm distance between the ports) on top and bottom of the cavity, which affords for the in situ observations of the iCrystal plates (Figure 2).

Since the industrial microwave has a variable source, we investigated the effect of microwave power level on the crystallization of GSH. Table 1 displays the summary data for growth of GSH crystals as a function of microwave power level ($200, 400, 600, 800$, and 1000 W) using the MA-MAEC technique for the iCrystal plates with and without SNFs. The times for initial observation of GSH crystal formation was consistent at 5 min for all conditions of microwave power ($200\text{--}1000\text{ W}$, Figure 2, we note that data for all experimental conditions not shown for the sake of brevity), substrate type (iCrystal plates with and without SNFs), and GSH concentration ($300\text{--}500\text{ mg/mL}$). On the other hand, marked differences were observed for the complete crystallization of GSH based on the crystallization conditions as shown in Table 1. At GSH concentration of 300 mg/mL with microwave power level of 200 W , complete crystallization was observed at an average time

of 377 ± 5.77 and 230 ± 5.00 min using the iCrystal plates without SNFs and with SNFs, respectively. In contrast, at 500 mg/mL GSH concentration with 1000 W of microwave power, complete crystallization of GSH was observed 2-fold faster on the iCrystal plates.

As the microwave power increased from 200 to 1000 W, crystallization times were reduced by up to 2.4 times. Increasing GSH concentration from 300 mg/mL to 500 mg/mL reduced crystallization by ~ 2.7 times and similar trends were noted for all power levels. In the presence of SNFs on the iCrystal plates, crystallization times were improved across all conditions as shown in Table 1. On the iCrystal plates with SNFs, crystallization times were up to 2.3 times faster when compared to the iCrystal plates without SNFs at 200 W and 400 mg/mL. In addition, crystallization times were reduced by up to 6.4 times on iCrystal plates with SNFs at 1000 W and 500 mg/mL when compared to iCrystal plates without SNFs at room temperature (a control experiment) with the same GSH solution. The minimum average crystallization time observed was $\sim 40 \pm 13$ min and was observed at the highest microwave power level, the highest initial GSH concentration, and on the iCrystal plates with SNFs.

Figure 2 shows the time-dependent growth of GSH crystals on the iCrystal plates with and without SNFs using an industrial microwave operating at 1000 W. Initial GSH crystals grown on both iCrystal plates in the industrial microwave observable by our optical microscope appeared in 5 min of microwave heating. On the iCrystal plates with SNFs, the size of GSH crystals reach $<200 \mu\text{m}$ within 20 min of microwave heating and the GSH crystals appear to have similar size and quality. On the iCrystal plates without SNFs, GSH crystals vary in size and quality and took >40 min to reach a size of $<200 \mu\text{m}$. Crystal count was noticeably lower at 500 mg/mL GSH across all power levels. However, there was no evidence that microwave power level had a major impact on GSH crystal size or number. The largest GSH crystals, measured up to $\sim 400 \mu\text{m}$, was grown at 400 W using 300 mg/mL GSH on the iCrystal plates with SNFs.

The rate of growth for GSH crystals from initial GSH solution of 300–500 mg/mL on both surfaces were calculated from the experimental crystal size measurements during microwave heating using an industrial microwave system (Figure S3, Supporting Information). The rate of growth of GSH crystals on both iCrystal plates with SNFs and without SNFs (labeled as PMMA) was increased as the microwave power was increased from 200 to 1000 W. Although the rate of growth of GSH crystals was typically larger on SNFs as compared to PMMA using an industrial microwave system, an overall marked reduction in the rate values as compared to those calculated using conventional microwave system (and at similar microwave power) was observed. These observations can be attributed to the nature of the microwave source and the microwave cavity used, i.e., conventional microwave system offers heterogeneous heating in a larger cavity and the industrial system offers homogeneous heating in a smaller cavity. In addition, microwave heating of samples using an industrial microwave system is more consistent with higher degree of repeatability as compared to the heating of samples using a conventional microwave system.

The iCrystal System

The iCrystal microwave system utilizes variable power like the industrial microwave used in the previous section. However, it is important to note that due to the low power microwave heating (only up to 100 W at 2.45 GHz) complete evaporation does not occur in the iCrystal microwave system. Subsequently, crystallization times were not measured, but rather the crystal size was determined after a specific time which was used as an indicator of crystal growth (Figure 3). For all concentrations, GSH crystals grown via the MA-MAEC technique (iCrystal plates with SNFs and microwave heating) were larger than those grown on the iCrystal plates without SNFs, as shown in Table 1. After 90 min at 300 mg/mL, GSH crystals measured on average $\sim 90\ \mu\text{m}$ and $\sim 400\ \mu\text{m}$ for the iCrystal plates with SNFs and without SNFs, respectively. As the initial concentration of GSH solution was increased, the size of GSH crystals was gradually increased on both iCrystal plates. These observations imply that one can control the size of the GSH crystals and terminate the crystal growth when the desired size and number of GSH crystals are grown.

The rate of growth for GSH crystals from initial GSH solution of 300–500 mg/mL on both iCrystal plates were calculated from the experimental crystal size measurements during microwave heating using the iCrystal system (Figure S4, Supporting Information). The rate of growth of GSH crystals on the iCrystal plates with SNFs was ~ 3.3 -fold larger than the rate observed on the iCrystal plates without SNFs (labeled as PMMA) for GSH solutions of 300–400 mg/mL. As the concentration of initial GSH solutions was increased to 500 mg/mL, the rate of growth of GSH crystals on both surfaces were similar, that is, the effect of surface was negligible and the rate of crystal growth was dominated by the initial solution concentration. A comparison of rate of growth of GSH crystal using all three-microwave system reveals that the following order as listed from largest to lowest: conventional (up to $30\ \mu\text{m}/\text{min}$) > industrial (up to $15\ \mu\text{m}/\text{min}$) > the iCrystal microwave (up to $3.8\ \mu\text{m}/\text{min}$) systems.

Comparison of All Three Microwave Systems

A detailed comparison can be also made between the three microwave systems used in this study based on crystallization times, reproducibility, and crystal size. Using the MA-MAEC technique, initial crystallization times were fastest for the industrial microwave for all concentrations. For the industrial microwave system, initial crystal growth was observed within 5 min for all concentrations on iCrystal plates. In addition, for the iCrystal microwave system, initial crystallization occurred within the first 15 min for all concentrations. We note that 15 min microwave heating intervals were used in the iCrystal system and subsequently, observation of initial GSH crystals were not possible due to the experimental conditions. Initial crystallization times for the conventional microwave varied between 6–13 min across all initial GSH concentrations.

Complete crystallization times for GSH were observed to decrease by >50% for the industrial and conventional microwave systems as the initial concentration of GSH solution is increased from 300 to 500 mg/mL. The use of the industrial microwave resulted in slightly faster crystallization as compared to the conventional microwave at comparable power levels. For example, at initial concentration of 500 mg/mL of GSH, complete crystallization

was ~7 min faster in the industrial microwave when compared to conventional microwave system on the iCrystal plates with SNFs. At 400 mg/mL, complete crystallization was ~7 min slower in the industrial microwave. At 300 mg/mL, the industrial microwave was faster by only 2 min. No comparison for complete evaporation times was made between the iCrystal microwave system and the other microwave systems due to lack of complete evaporation of GSH solution. However, the crystal sizes measured in this microwave after 90 min gave indications of similar rates of crystallization.

Since the iCrystal plates have 21-well sample capacity, it is important to investigate the reproducibility of growth of GSH crystals within the iCrystal plate using the MA-MAEC technique with all three microwave systems. Figures 4–6 show the optical images of GSH grown in 21-wells of the iCrystal plates with SNFs using conventional microwave, industrial microwave system and the iCrystal microwave system, respectively. Figure 4 shows that the heterogeneous distribution of GSH crystals grown from 500 mg/mL GSH solution using the MA-MAEC technique in the conventional microwave, where only 5 of 21 wells of the iCrystal plates produces more than 10 GSH crystals of 100 μm in size. Furthermore, numerous crystal microaggregates (seen as dark spots in the optical microscope images) were observed indicating distorted and/or incomplete crystal growth. Figure 5 shows that the use of the industrial microwave results in the growth of more than 10 or more GSH crystals of 100 μm in size in 19 of 21 wells of the iCrystal plates, where the number of GSH crystals were uncontrollably higher than the rest of the wells (well number: 2 and 16). The use of the iCrystal microwave system resulted in the growth of GSH crystals in all 21 wells of the iCrystal plates in a homogeneous manner, as shown in Figure 6. Largest GSH crystals were obtained with the industrial and iCrystal microwave systems as compared with the conventional microwave. In addition, the use of the industrial and iCrystal microwave systems (Figure 5 and 6) afforded for improved crystal quality with well-defined crystals and better spatial distribution. However, the iCrystal microwave yielded the best crystal quality since there were fewer instances of distorted or dendritic crystal growth (Figure 6).

A qualitative comparison of the iCrystal plates (i.e., with or without SNFs) show that the growth of GSH crystals was better on SNFs-coated iCrystal plates than that without SNFs with respect to crystal size and size distribution. GSH crystals grown on the iCrystal plates without SNFs displayed significant variation in size and large numbers of very small crystals. In contrast, the use of the iCrystal plates with SNFs resulted in the growth of larger GSH crystals that were more uniform in length. These observations can be directly attributed to the presence of silver nanoparticles and the generation of microwave-induced thermal gradients between the SNFs and the solvent (due to selective heating of the solvent and not the SNFs). Subsequently, GSH molecules that are present in the initial supersaturated solution (warmer) are forced to move toward the cooler SNFs surface. In addition, SNFs also act as selective nucleation sites for the growth of GSH crystals due to the chemisorption of GSH molecules on to the silver surface through primary amine and thiol groups of the GSH molecules.

FTIR Analysis

FTIR spectra of GSH crystals grown at room temperature samples and using the MA-MAEC technique for all microwaves for each different concentration were measured to ascertain indirectly whether microwave heating affects the molecular structure of GSH molecules in solution. FTIR spectra under four different crystal growth conditions for 300, 400, and 500 mg/mL concentration were identical (Figure S2), which provides an indirect evidence that GSH molecules in solution retained their molecular structure during microwave heating in all microwave systems, and subsequently, were involved in the formation of GSH crystals on the iCrystal plates.

X-ray Diffraction Analysis

More definitive assessment and comparison of GSH crystal formation can be made using XRD analysis. XRD patterns for GSH crystals grown from an initial GSH solution of 500 mg/mL are shown in Figure 7, which reveals that the diffraction peaks were identical for GSH crystals for all conditions (on both iCrystal plates at room temperature and using the MA-MAEC technique) and were comparable to the XRD data reported in the literature (Figure S4).²⁰ XRD data provided additional evidence that the use of the MA-MAEC technique did not negatively affect the GSH crystal structure and resulted in the growth of GSH crystals comparable with other methods.²⁰ A comprehensive search of the literature yielded a small number of research reports on the XRD analysis of GSH.^{20,21}

On the basis of the observations described above, the iCrystal microwave system was proven to be the most cost-effective system for the growth of GSH crystals since better quality GSH crystals can be grown at a significantly lower power level over a similar period as compared to the other two microwave systems. In addition, the iCrystal system is smaller than either the industrial version or the conventional microwave.

Computer Simulations

We also carried out computer simulations (using Biovia Materials Studio, version 8) generate a model of the GSH crystals that resembles GSH crystals grown experimentally to identify the crystal faces of the GSH crystals. Since all GSH crystals grown experimentally have identical morphology as shown in optical microscopes images, a representative GSH crystal (Figure 8 and Figure S5) was used as a guide in the generation of model of the GSH crystal. Glutathione is known to crystallize in an orthorhombic unit cell with space group $F2_12_12_1$.^{21,22} The unit cell is composed of four molecules of the tripeptide. The SH group of the molecule is involved in hydrogen bonding and other short intermolecular contacts hold the unit cell together. Unit cell dimensions were obtained from the Cambridge Structural Database (CSD) and are given as follows: $a = 5.62 \text{ \AA}$, $b = 8.78 \text{ \AA}$, $c = 27.99 \text{ \AA}$, $\alpha = \beta = \gamma = 90^\circ$. The crystal morphology was calculated (per the procedure published previously¹²) and the morphologically similar surfaces, based on the observed experimental images for GSH crystal, were highlighted. These calculations were based on the Forcite module using the consistent valence force field method and the growth morphology method, which assumes that the growth rate of a crystal surface is directly proportional to its attachment energy.¹² The most significant crystal faces were found to be $\{002\}$, $\{012\}$ and $\{201\}$ as shown in Figure 8. Calculated attachment energies for the $\{201\}$ and $\{002\}$ crystal faces have the

lowest and highest attachment energy of -85.8 kcal/mol and -17.7 kcal/mol, respectively. The attachment energy values imply that GSH crystal is predicted to grow significantly faster on the (201) face and slower on the {012} and the slowest on the {002}. In addition, our calculations show that {002}, {012}, and {201} faces occupy ~28%, 61%, and 11% of the total surface area of the GSH crystals, respectively. We note that Materials Studio software does not account for all faces; however, virtual models generated using the Materials Studio software provide significant information related to morphology of the crystals and thermodynamic properties, otherwise extremely difficult to measure. Our research laboratory is currently working on the rapid crystallization of peptides with longer chains (up to 40 amino acids) and the results of these investigations will be reported in due course.

CONCLUSIONS

A comparative investigation for the rapid crystallization of a tripeptide, i.e., glutathione, using the MA-MAEC technique in three types of microwave systems (conventional, industrial, and iCrystal microwave) was presented. Our observations of GSH crystal growth via an optical microscope revealed that crystallization times in all microwave systems were most significantly improved on the iCrystal plates with SNFs as compared to the iCrystal plates without SNFs. Crystallization times were accelerated up to 5.8 times in the conventional microwave and 6.4 times in the industrial microwave as compared to room temperature experiments. In terms of GSH crystal size, the use of the both the industrial microwave and the iCrystal microwave system produced the largest crystals (>100 , μm) on the iCrystal plates with SNFs. Optical microscope images showed that the use of the iCrystal system yielded GSH crystals with the highest quality in all wells of iCrystal plates, where the growth of large GSH crystals with uniform size distribution and few instances of distorted growth were observed. Overall comparison of the three microwave systems implied that the most effective microwave system for the rapid crystallization of GSH using the MA-MAEC technique was determined to be the iCrystal microwave system since it affords both rapid crystallization and good crystal quality.

Supplementary Material

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Figure 1.

Optical images of GSH crystals grown from an initial GSH solution of 500 mg/mL using the conventional microwave (at 900 W, power level 1) on the iCrystal plates with (labeled as SNFs) and without SNFs (labeled as PMMA). A thin polymer cover placed in airtight manner over the top surface of the iCrystal plates to minimize or prevent the evaporation of the solvent. Real-color pictures of the iCrystal plates after the completion of the experiment was also provided.

Figure 2.

Optical images of GSH crystals grown from an initial GSH solution of 500 mg/mL using the industrial microwave (at 1000 W) on the iCrystal plates (A) without SNFs (blank PMMA) (B) with SNFs. Real-color pictures of the iCrystal plates after the completion of the experiment and the industrial microwave were provided.

Figure 3. Optical images of GSH crystals grown from an initial GSH solution of 500 mg/mL using the iCrystal system (at 80 W) on the iCrystal plates (A) without SNFs (blank PMMA) (B) with SNFs. Real-color pictures of the iCrystal plates after the completion of the experiment and the iCrystal system were provided.

Figure 4. Optical images of GSH crystals grown using a conventional microwave (at 900 W, power level 1) on all 21 wells of the iCrystal plates with SNFs.

Figure 5. Optical images of GSH crystals grown using the industrial microwave (at 1000 W) on all 21 wells of the iCrystal plates with SNFs.

Figure 6. Optical images of GSH crystals grown using the iCrystal system (at 80 W) on all 21 wells of the iCrystal plates with SNFs.

Figure 7.
X-ray diffraction analysis of GSH crystals grown in this study under all conditions.

Figure 8.

(Top) SEM image, simulated crystal habit and unit cell of GSH (Bottom) calculated properties of the morphologically important facets of GSH crystals.

Results of Conventional Microwave, Industrial Microwave and the iCrystal System, for Different Initial Concentrations of GSH Compared with Room Temperature (a Control Experiment)

power level 1 GSH concn. (mg/mL)	room temperature		conventional MW at 900 W	
	Crystallization time, ^a min		crystallization time, ^a min	
	PMMA	SNFs	PMMA	SNFs
300	628 ± 31.4	415 ± 37.8	130 ± 20.0	108 ± 11.6
400	242 ± 25.7	227 ± 87.4	86.7 ± 7.64	65.0 ± 8.66
500	257 ± 98.1	170 ± 56.4	63.3 ± 2.89	46.7 ± 7.64
industrial MW, variable power				
crystallization time, min				
microwave power, W	GSH concn = 300 mg/mL		GSH concn = 400 mg/mL	
	PMMA	SNFs	PMMA	SNFs
	377 ± 5.77	230 ± 5.00	235 ± 13.2	103 ± 18.9
	272 ± 2.88	210 ± 10.0	205 ± 5.00	135 ± 5.00
	207 ± 2.88	189 ± 7.63	115 ± 5.00	103 ± 2.88
800	190 ± 10.0	123 ± 2.88	145 ± 5.00	108 ± 17.5
1000	156 ± 15.3	106 ± 7.63	110 ± 5.00	71.6 ± 7.63
iCrystal system at 80 W				
GSH concn (mg/mL)	length ^b after 90min (µm)		length ^b after 150 min (µm)	
	PMMA	SNFs	PMMA	SNFs
	300	86.8	464	696
	400	160	456	714
	500	271	481	790

^a Crystallization time refers to the time when the solvent is completely evaporated.

^b Since GSH solution does not evaporate, length of GSH crystals are used as a parameter to assess the efficiency of the iCrystal system.