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RAPID COMMUNICATION

Effect of an ionic liquid on vancomycin crystallization

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Abstract−We first developed a vancomycin crystallization process using an ionic liquid (IL) and improved the crystallization efficiency by optimization of crystallization conditions (pH, conductivity, solution of distilled water and IL/acetone ratio, crystallization temperature, IL concentration). We also investigated the effect of major process parameters on crystallization, using an electron microscope, and identified morphology by XRD analysis. Using ILs (1-butyl-3-methylimidazolium tetrafluoroborate ([BMIm][BF_4]), 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIm][PF_6])), vancomycin crystals were successfully formed under the optimal crystallization conditions: pH 4.5; conductivity, 10 mS/cm; solution of distilled water and IL/acetone ratio, 1 : 3.5 (v/v); crystallization temperature, 10 °C; IL concentration, 20% (v/v). When using an IL ([BMIm][BF_4]), the time required for crystallization in the existing crystallization methods (~24 hr) was dramatically decreased (~9 hr) and high-quality vancomycin crystals were successfully formed.

Keywords: Vancomycin, Crystallization, Ionic Liquid, Process Parameter, Investigation

INTRODUCTION

Vancomycin is the first glycopeptide antibiotic that inhibits the cell wall synthesis of Gram-positive bacteria [1,2]. It is widely used to treat *Staphylococcus aureus* tolerant to methicillin and endocarditis allergic to penicillin or cephalosporin. It is also used for preventive treatment during heart surgery, orthopedic surgery or brain surgery [3].

The vancomycin content, total impurity content and individual impurity content of vancomycin registered in the United States Pharmacopeia and European Pharmacopoeia are strictly regulated [4]. To meet the regulations, several steps of the isolation and purification process are required. In the production process of high-purity drugs, such as antibiotics, a crystallization process is generally used as the final purification step. Crystallization, which can improve the purity of products and also control physical properties and morphology of products, has been used widely because it consumes less energy, is eco-friendly and has a simple process [5,6]. The crystal particles initially formed from the solution are called nuclei, and nucleation is classified into homogeneous nucleation, where nuclei are generated in the liquid state due to supersaturation, and heterogeneous nucleation, where nuclei are generated with the assistance of external surfaces (external impurity particles, reactor walls, agitators, etc.) [7]. In the case of solutes with high solubility, such as bioproducts including vancomycin, supersaturation of the solution is so high that nuclei formation occurs by heterogeneous nucleation. Therefore, vancomycin requires more time for nucleation, which is heterogeneous nucleation, improving the nucleation velocity through the surface area increased in the crystallizer [8].

In our previous study [9], most of the major process parameters (temperature, pH, conductivity, agitation, and initial vancomycin concentration) for the crystallization of vancomycin were optimized to obtain a highly pure (>97%) product with high yield (>95%). However, crystal formation required a long time (~24 hr), resulting in low productivity in the mass-production process. To overcome these drawbacks, a crystallization process with increased surface area per volume of reaction solution using glass beads or ion exchange resins was developed in 2011 and 2012, which decreased the time required for vancomycin crystallization (24 hr→12-18 hr) [10,11]. However, vancomycin crystallization still takes a long time and, therefore, additional reduction of crystallization time and improvement in the crystal quality are required.

Interest in ionic liquids (ILs) has continually increased due to their useful properties, such as negligible vapor pressure, high thermal stability, excellent solvation and high ion conductivity. They are eco-friendly solvents spotlighted as an alternative to the existing volatile organic solvents [12,13]. ILs are not only organic salts but also show ionic properties similar to the inorganic salts. As a result, many inorganic or organic materials are soluble in them [14]. They are commonly used for crystallization of protein or organic compounds [12,14-16]. In particular, it was reported that an IL had a great effect on crystal behaviors (nucleation, crystal growth, morphology/state, stability) in the process of protein crystallization [17]. However, there has been surprisingly little research in their use in protein crystallization. In fact, to date only the crystallization of some model soluble proteins from ILs has been reported [14,16,18]. The data do not indicate a single, clear mechanism for how ILs affect the crystallization process. Thus, this study was first conducted to develop a vancomycin crystallization process using an IL and to dramatically improve the crystallization efficiency (particularly, reduction of crystallization time and improvement in the crystal quality) by optimization of crystallization conditions (IL concentration, pH, conductivity, crystallization temperature, solution of distilled water and IL/acetone ratio).

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MATERIALS AND METHODS

1. Preparation of Vancomycin Sample

Vancomycin was here obtained through the fermentation of the microorganism *Norcardia orientalis* isolated from soil. Bacterial cells were removed from the fermentation solution containing vancomycin, which was then purified [19]. The solution was consecutively passed through cation exchange, anion exchange and porous cation exchange resins and eluted with ammonia to obtain 88% pure vancomycin in the form of hydrochlorate. Impurities such as pigment and protein were removed using alumina and a weak acidic cation exchange resin. The resulting product was used for the crystallization process.

2. Vancomycin Analysis

An HPLC system (SCL-10AVP, Shimadzu, Japan) and Cadenza CW-C18 column (4.6×100 mm, 3 μm, Imtakt, Japan) were used for analysis of vancomycin at 260 nm by using a UV detector. Mobile phase A was prepared by mixing 1,000 mL of distilled water and 1 mL of formic acid. Mobile phase B was prepared by mixing 1,000 mL of acetonitrile and 1 mL of formic acid. The analysis was performed in gradient and isocratic mode for 20 min. Elution was performed in a gradient using mobile phase A and mobile phase B mixture varying from 95 : 5 to 30 : 70 for the first 10 min. After that, the mixture of mobile phase A and mobile phase B (95 : 5) was isocratic from 10 min to 20 min. The flow rate was 0.8 mL/min and the injection volume was 20 μL. The concentration was calculated by using the peak area acquired with the standard materials. Each sample was analyzed in triplicate.

3. Crystallization Method

The crystallization process is influenced by many factors that directly affect the purity and yield as well as structure, size and distribution of crystal particles. A study was conducted to improve the vancomycin crystallization efficiency by optimization of major process parameters (pH, conductivity, solution of distilled water and IL/acetone ratio, storing temperature/time, vancomycin concentration) in the crystallization process using ILs. The diagram of the vancomycin crystallization process using ILs is shown in Fig. 1. First, the sample (vancomycin purity: 88%) was dissolved in distilled water and ionic liquid solution (vancomycin concentration: 0.1 g/mL) wherein the pH (2.0, 2.5, 3.0, 3.5) and conductivity (4, 9, 12, 15 mS/cm) were adjusted with 1 N hydrochloric acid and sodium chloride, respectively. Since sodium chloride has no influence on H^+, the pH was adjusted first. To efficiently induce crystal formation by a reduction in solubility, acetone was slowly dropped into the vancomycin solution while stirring. After addition of acetone, the pH and conductivity of crystallization solution were 4.0, 4.5, 5.0, 5.5, and 9, 10, 11, 12 mS/cm, respectively. Then, a crystallization experiment was performed by varying the storage temperature (5, 10, 15 °C) and time (9, 12, 15, 18 hr). 1-Butyl-3-methylimidazolium tetrafluoroborate ([BMIm][BF_4]) and 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIm][PF_6]), which are commonly used, typical ILs [14], were used at different concentrations (IL/distilled water: 10, 15, 20, 25%, v/v). After crystallization, the parent solution including the solvent adhered to crystal surfaces, so it was removed. The vancomycin was then washed with the organic solvent acetone used for the crystallization process to obtain a clear, final crystal product. Impurities were removed from crystal surfaces by filtration through filter paper (150 mm, Whatman); then the filtrate was dried under vacuum at 35 °C for 24 hr and analyzed by HPLC.

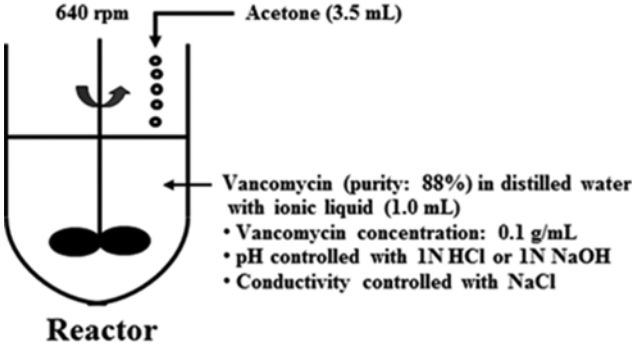

Fig. 1. Schematic diagram of crystallization process with IL for purification of vancomycin.

4. Analysis of Vancomycin Morphology and Size

The morphology and size of vancomycin particles formed during crystallization were determined using a video microscope (SV-35 Video Microscope System; Sometech, Korea) [9]. Precipitates were observed under high magnification (100×). Crystal morphology and size were determined from video images using the IT-Plus System (Sometech).

5. XRD Analysis

The morphology of vancomycin was analyzed by means of an X-ray diffractometer (MiniFlex 600, Rigaku) operated by the WINHRD 3000 program. XRD measurements were performed in the 5 to 25° 2θ range using CuKα radiation (40 kV, 15 mA) as the X-ray source. The amount of each sample was about 50 mg.

RESULTS AND DISCUSSION

1. Effect of pH and Conductivity of Crystallization Solution

pH is an important parameter in the crystallization process, having a direct influence on the solubility of vancomycin. A report from Food and Drug Administration about vancomycin solubility at various pH values shows that solubility is high at acidic pH (pH 3) and decreases near pH 4, then remains nearly constant between pH 4 and 8 [20]. To maximize crystallization efficiency, it is favorable to perform crystallization under the pH condition where the vancomycin solubility is lowest [7,9]. In addition, the pH stability of vancomycin has been known to be 2-5.5 [21]. Therefore, crystallization (18 hr) was performed with adjusting the pH of crystallization solution to 4.0, 4.5, 5.0 and 5.5, to investigate the effect of the pH on the vancomycin crystallization process using ILs ([BMIm][BF_4], [BMIm][PF_6]). As shown in Fig. 2, the most clear and uniform vancomycin crystals were obtained at pH 4.5 and, therefore, the optimal pH for vancomycin crystallization using ILs was confirmed. Vancomycin crystals were successfully formed at the specific pH (4.4-4.7). If it deviated from the specific pH, it was difficult to conduct crystallization because of gelation (a conglomeration phenomenon). This result was similar to the optimal pH (~4.5) of solution for vancomycin crystallization when not adding ILs [9]. Therefore, it was determined that the optimal pH of solution for vancomy-

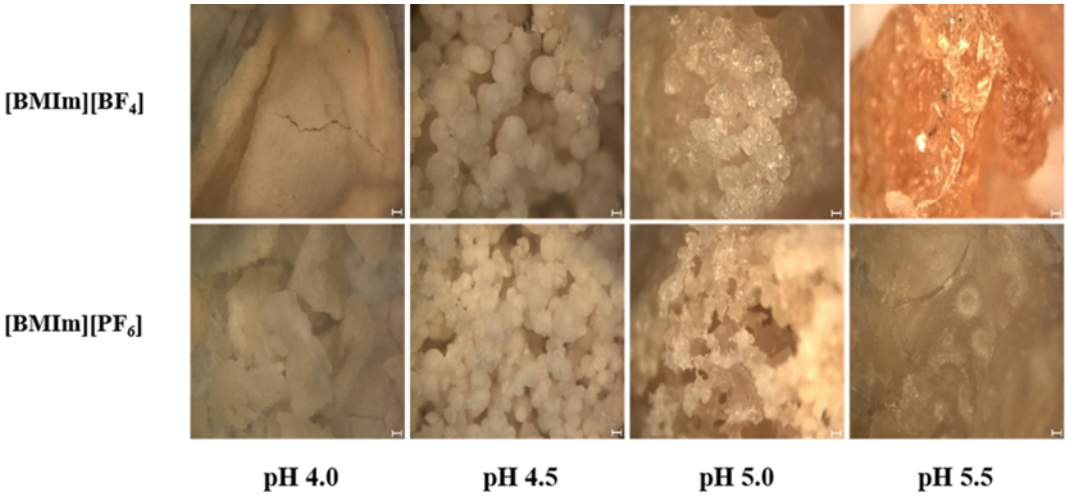

Fig. 2. Electron microscope images of vancomycin crystals formed by crystallization (18 hr) in ILs at various pHs. The crystallization temperature, conductivity, IL concentration, and distilled water and IL/acetone ratio were 10 °C, 10 mS/cm, 20% (v/v), and 1 : 3.5 (v/v), respectively. Scale bar indicates 100 μm.

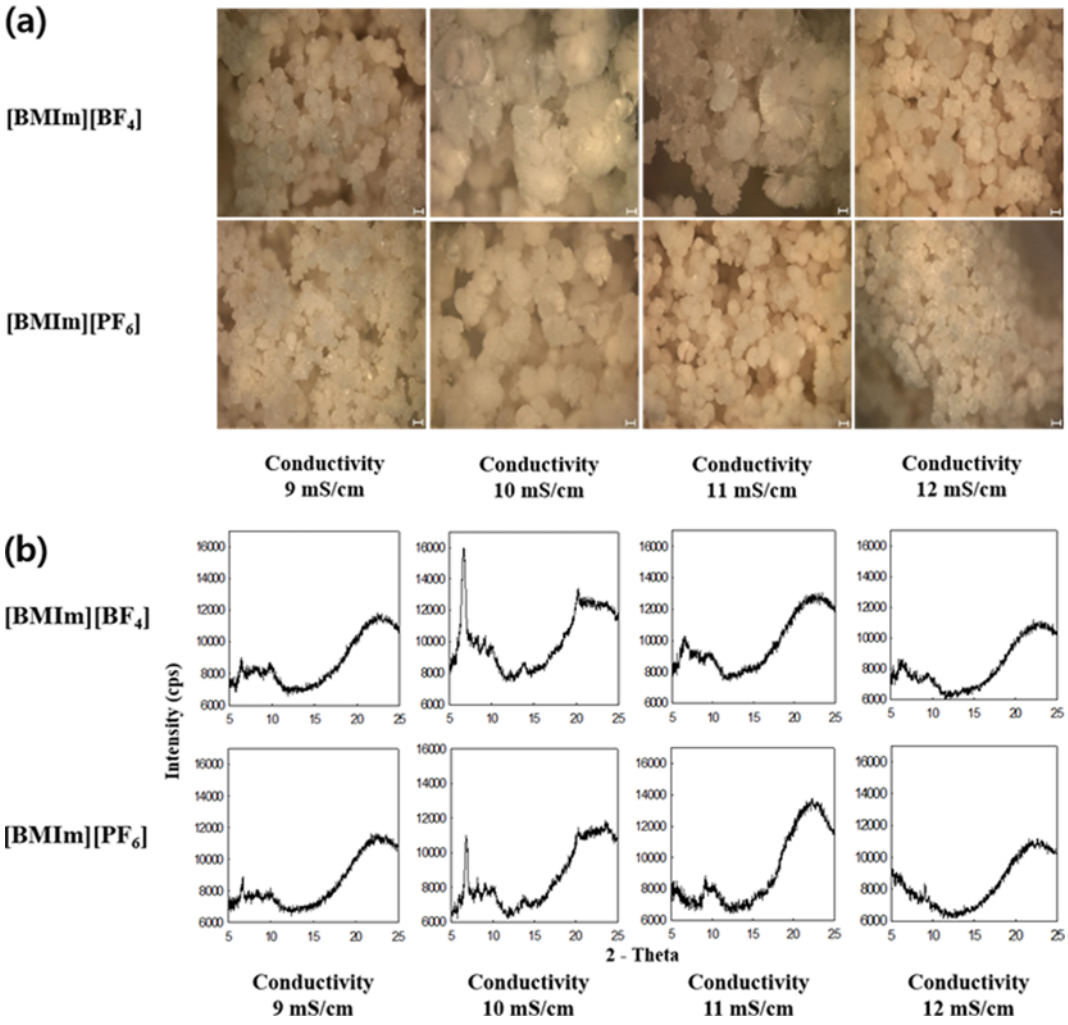

Fig. 3. Electron microscope images (a) and XRD patterns (b) of vancomycin crystals formed by crystallization (18 hr) in ILs at various conductivities. The crystallization temperature, pH, IL concentration, and distilled water and IL/acetone ratio were 10 °C, 4.5, 20% (v/v), and 1 : 3.5 (v/v), respectively. Scale bar indicates 100 μm.

cin crystallization using an IL is 4.5.

The effect of conductivity (9, 10, 11, 12 mS/cm) on vancomycin crystallization was investigated at a fixed pH (4.5) of crystallization solution. As shown in the electron microscope images, when using [BMIm][BF_4] and [BMIm][PF_6] at 18 hr of crystallization, vancomycin crystals were formed in all sections (Fig. 3(a)). Upon XRD analysis performed to confirm crystallization, meaningful peaks were observed at 10 mS/cm of conductivity (Fig. 3(b)). These peaks are in agreement with the previous study [22]. Therefore, it was verified that crystals were most successfully formed. In addition, a more meaningful peak at 10 mS/cm of conductivity was observed in [BMIm][BF_4] compared to [BMIm][PF_6], and so more clear and uniform (excellent degree of crystallinity) vancomycin crystals were obtained. The experimental results show that [BMIm][BF_4] is more suitable for vancomycin crystallization than [BMIm][PF_6]. The probable reason might be its high solubility in water. [BMIm][BF_4], the solubility in water of which is much higher than that of [BMIm][PF_6] [23], could compete with vancomycin for water molecules more strongly. Consequently, supersaturation of the solution was so low that the quality of crystals was very good. As a result, the most clear and uniform crystals were formed at a conductivity of 10 mS/cm and, therefore, it was most effective for vancomycin crystallization.

2. Effect of Solution of Distilled Water and IL/Acetone Ratio and Storage Temperature

In crystallization, supersaturation is one of the important parameters affecting the crystallization efficiency [10,11]. Supersaturation is a state of a solution that contains more of the dissolved solute than could be dissolved by the solvent and an organic solvent is added to reduce the solubility for supersaturation. In addition, a water-soluble organic solvent has a great effect on the crystal size and morphology. In this study, acetone, a common water-soluble organic solvent, was used for crystallization [9]. To identify the effect of the solution of distilled water and IL/acetone ratio, at 0.1 g/mL of the initial vancomycin concentration, vancomycin was dissolved in distilled water (IL 20%, v/v added) and the solution of distilled water and IL/solvent ratio (v/v) was changed to 1 : 2.5, 1 : 3.0, 1 : 3.5 and 1 : 4.0. Then, crystallization (18 hr) was conducted. As shown

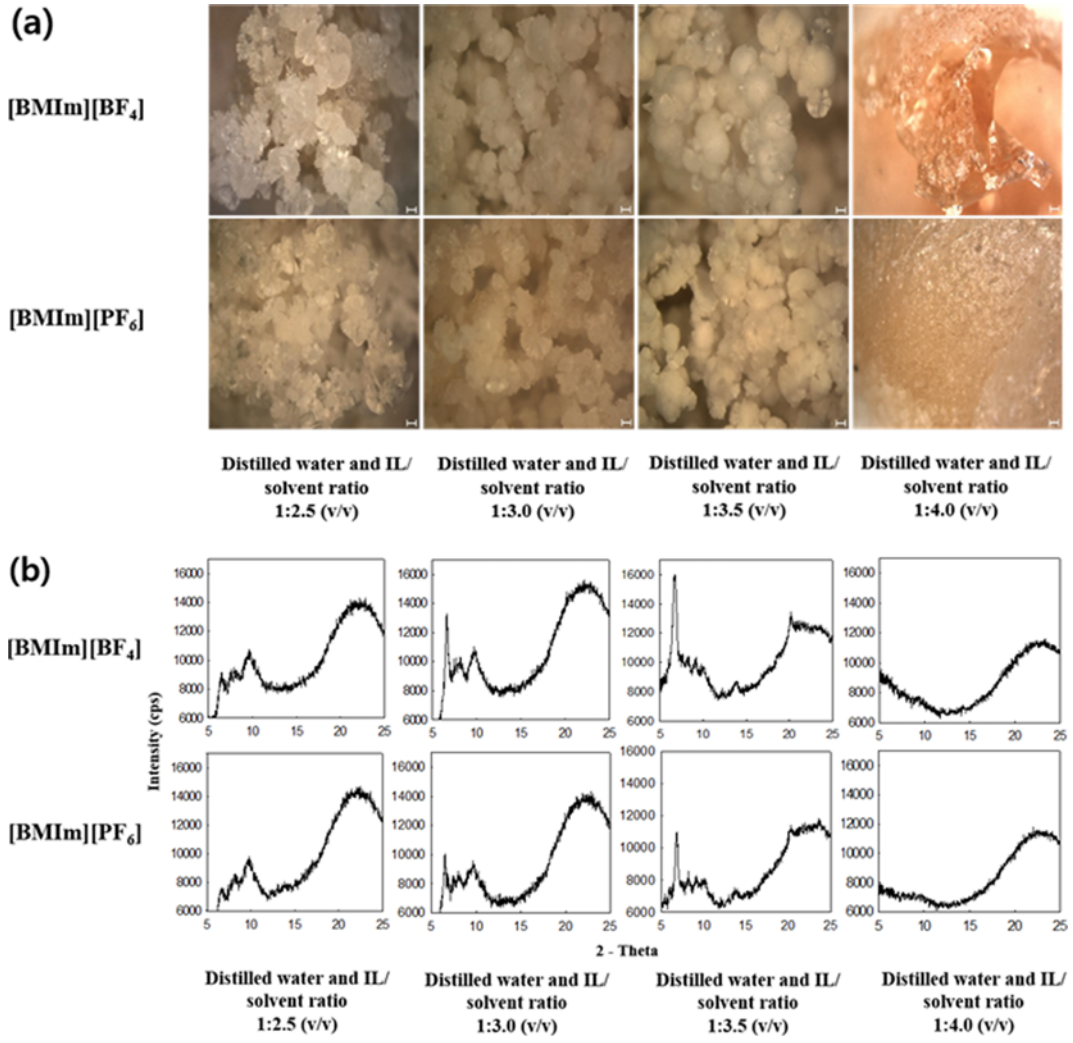

Fig. 4. Electron microscope images (a) and XRD patterns (b) of vancomycin crystals formed by crystallization (18 hr) in ILs at various distilled water and IL/solvent ratios. The crystallization temperature, pH, conductivity, and IL concentration were 10 °C, 4.5, 10 mS/cm, and 20% (v/v), respectively. Scale bar indicates 100 μm.

in Fig. 4(a), the crystal condition was different depending on the solution of distilled water and IL/acetone ratio (v/v). At a ratio of 1 : 2.5 and 1 : 3.0 (v/v), crystals were needle-shaped. At a ratio of 1 : 3.5 (v/v), the most clear and uniform morphology was shown and, at a ratio of 1 : 4.0 (v/v), no crystal was formed. Upon XRD analysis to confirm crystallization, a meaningful peak was observed at a ratio of 1 : 2.5, 1 : 3.0 and 1 : 3.5, as shown in Fig. 4(b). Therefore, it was confirmed that crystals were successfully formed. In particular, the most meaningful peak was observed and the most clear and uniform crystals were formed at a ratio of 1 : 3.5, and it was most effective for vancomycin crystallization. This trend was similar to the optimal range of the solution of distilled water and IL/solvent ratio (v/v) for vancomycin crystallization not using an IL [9]. As a result, vancomycin crystallization was most effective at a solution of distilled water and IL/acetone ratio of 1 : 3.5 (v/v). Therefore, we chose a ratio of 1 : 3.5 (v/v) to perform the next crystallization experiments.

For most bioproducts, solubility is sensitive to changes in temperature. In this case, temperature has a direct influence on the crystallization process and yield [7] and is thus an important parameter. To identify the morphology of vancomycin depending on changes in the storage temperature for crystallization, a sample was prepared under the following conditions: pH 4.5, conductivity 10 mS/cm, IL concentration 20% (v/v), solution of distilled water and IL/acetone ratio 1 : 3.5 (v/v). Then, it was stored at 0, 10 and 15 °C. The morphology of vancomycin was observed by an electron microscope in 18 hr of crystallization (data not shown). At 10 °C, the most clear and uniform crystals were formed. Crystal nuclei, the minimum units of crystal particles formed first in solution, were stably produced and grew at a storage temperature of 10 °C. Such nuclei are stable only at a certain temperature (less than the melting temperature); the presence of heat energy at a temperature exceeding the above is problematic [9]. At 15 °C, crystals were broken in the shape of a needle, or broken crystals were condensed. Also, at 5 °C,

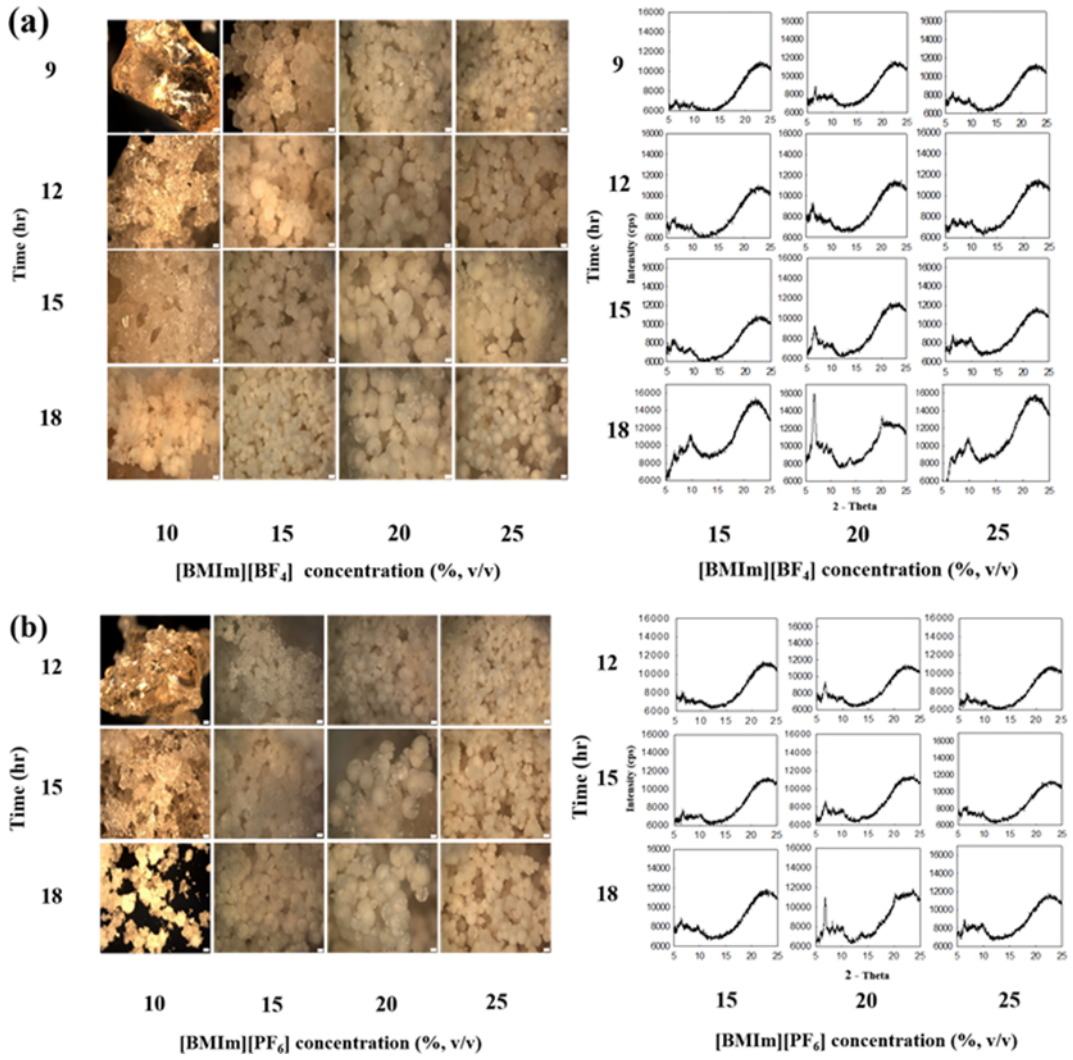

Fig. 5. Electron microscope images and XRD patterns of vancomycin crystals formed by crystallization at various IL concentrations over time. The ILs were 1-butyl-3-methylimidazolium tetrafluoroborate (a) and 1-butyl-3-methylimidazolium hexafluorophosphate (b). The crystallization pH, temperature, conductivity, and distilled water and IL/acetone ratio were 4.5, 10 °C, 10 mS/cm, and 1 : 3.5 (v/v), respectively. Scale bar indicates 100 μm.

gelation occurred in which the crystal particles adhered to each other because the density of the crystals in solution (quantity/volume) increased. This result is similar to the optimal range of temperature for vancomycin crystallization not using an IL [9]. Therefore, the optimal crystallization temperature was set at 10 °C and then the next experiments were performed.

3. Effect of IL Treatment Conditions

The most effective IL treatment conditions were investigated for vancomycin crystallization. [BMIm][BF_4] and [BMIm][PF_6] were used for crystallization at different crystallization times (9, 12, 15, 18 hr) and concentrations (10, 15, 20, 25%, v/v). The vancomycin crystal pattern depending on changes in the IL type, IL concentration and crystallization time is shown in Fig. 5. In 10% (v/v) [BMIm][BF_4], the first crystal formed 18 hr after crystallization setup. On the contrary, in 15-25% (v/v), the first crystal formed 9 hr after crystallization setup and the degree of crystallinity of vancomycin increased over crystallization time. In particular, in 20% (v/v) [BMIm][BF_4], the most clear and uniform vancomycin crystals were formed. However, at all concentrations (15, 20, 25%, v/v) except 10% (v/v) [BMIm][PF_6], the first crystal formed 12 hr after crystallization setup, and the degree of crystallinity of vancomycin increased over crystallization time. As with [BMIm][BF_4], the most clear and uniform vancomycin crystals were formed at 20% (v/v), and so high-quality vancomycin crystals were obtained. The degree of crystallinity in [BMIm][BF_4] was higher than that in [BMIm][PF_6] under the same conditions, and the time required for crystallization was more reduced in [BMIm][BF_4]. Therefore, it was confirmed that [BMIm][BF_4] was more effective. This result can be attributed to differences in water solubility of ILs. [BMIm][BF_4], the solubility in water of which is much higher than that of [BMIm][PF_6] [23], could compete with vancomycin for water molecules more strongly. Supersaturation of the solution was so low that the quality of crystals was very good. As a result, in 20% (v/v) [BMIm][BF_4], the time required for crystallization could be dramatically decreased (24 hr→9 hr) and high-quality vancomycin crystals were also obtained. At up to 20% (v/v) IL, the shape of vancomycin crystals became clear and uniform and the size increased as the amount of the IL added increased. At concentrations higher than 20% (v/v), however, the crystal quality decreased and the size also decreased. The reason might be that the diffusion velocity was highly decreased due to inhibition of the growth of vancomycin crystals by certain concentrations (~20%, v/v) of the IL and, therefore, it contributed to the formation of clear and uniform crystals [14,18,24]. In addition, the low diffusion velocity caused by an IL also inhibited crystal nucleation, and so the number of crystals formed was small but the size was large. The quality, shape and number of vancomycin crystals over crystallization time were observed after adding a certain amount of an IL, and it was confirmed that the IL contributed to the effective formation of uniform and high-quality vancomycin crystals. In addition, the purity (~96%) and yield (>95%) of vancomycin over crystallization time were not significantly different (data not shown).

CONCLUSIONS

We developed a crystallization process using an IL to efficiently promote vancomycin crystallization, and optimized major process parameters (pH, conductivity, solution of distilled water and IL/acetone ratio, storage temperature, IL concentration). In particular, the effect of parameters on crystallization was observed by electron microscope during crystallization and morphology was identified by XRD analysis. Using ILs ([BMIm][BF_4], [BMIm][PF_6]), the first vancomycin crystal was formed 9 hr after crystallization setup in [BMIm][BF_4] and, therefore, the time required for crystallization was dramatically reduced (24 hr→9 hr). In particular, using [BMIm][BF_4] (20%, v/v), the most clear, uniform and high-quality vancomycin crystals were obtained under the optimal crystallization conditions (pH 4.5, conductivity 10 mS/cm, crystallization temperature 10 °C, distilled water/acetone ratio 1 : 3.5 (v/v)). The quality, shape and number of vancomycin crystals over crystallization time were observed after adding a certain amount of an IL, and it was confirmed that the IL contributed to the effective formation of uniform and high-quality vancomycin crystals. Therefore, this result could be effectively applied to the mass production of vancomycin.

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