Bacteria in Nonaqueous Solution

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Bacteria Incorporation in Deep-eutectic Solvents through Freeze-Drying**

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Biocatalysis (based on either enzymes or whole microorganisms) has matured to a standard technology in the fine-chemicals industry, as reflected in the number of biotransformation processes run on a commercial scale.[1] Interestingly, the use of whole microorganisms (mostly bacteria and fungi) prevails owing to the difficulties in isolating and purifying certain enzymes.[2] Biocatalytic processes are typically performed in aqueous solutions, but the use of ionic liquids (ILs) as solvents has received increased attention lately since they may offer advantages over normal organic solvents. For example, ILs do not react with water, they are nonvolatile and biodegradable, and they can be designed for specific reaction conditions, for example, in order to modify the enzyme selectivity or to tailor the reaction rate.[3] In biocatalytic processes carried out in ILs, the use of enzymes has prevailed over whole microorganisms since some difficulties still remain in incorporating microorganisms in pure ILs.[4] Thus, microorganisms are first cultured in buffered aqueous solutions and then added to the ILs, resulting in monophasic (for water-miscible ILs) and biphasic (for non-water-miscible ILs) systems which have been used successfully for whole-cell-catalyzed synthesis of fine chemicals.[5]

Deep-eutectic solvents (DESs) have been recently described as a new class of ILs. DESs are obtained by complexion of quaternary ammonium salts with hydrogen-bond donors.[6,7] The charge delocalization occurring through hydrogen bonding between the halide anion with the hydrogen-donor moiety is responsible for the decrease of the freezing point of the mixture relative to the melting points of the individual components. DESs share many characteristics of conventional ILs (e.g. they are nonreactive with water, nonvolatile, and biodegradable), but their low cost makes them particularly desirable (more than conventional ILs) for large-scale synthetic applications. DESs have also been the solvent of choice for a number of enzyme-based biotransformations because of their excellent properties for a wide variety of solutes, including enzymes and substrates.[8] However, biotransformations based on whole microorganisms are strongly limited in DESs since the incorporation of microorganisms by means of aqueous solutions is not possible. Hydration of the individual components of the DES would result in the rupture of the hydrogen-bonded supramolecular complexes, the DES would become a simple solution of the individual components, and the special features of the DES in its pure state would vanish. Thus, suitable strategies should be designed for incorporation of whole microorganisms in a DES in its pure state. A plausible strategy for this would require transitioning from aqueous chemistry to DES chemistry. We have recently reported on the incorporation of liposomes in a DES in its pure state by freeze-drying aqueous solutions of the DES that also contained liposomes.[9] Liposomes, vesicles consisting of a lipid bilayer membrane, can be considered as models of living cells. Nonetheless, the membrane structure is significantly more complex in microorganisms (Scheme 1).

Herein, we describe freeze-drying methods developed to suspend microorganisms in DES in its pure state. The DES of choice was a mixture of glycerol and choline chloride in a 2:1 molar ratio (e.g. GCCl-DES) while the microorganisms of choice were bacteria, in particular, the bacterial strain *Escherichia coli* (*E. coli*) TG1/pPBG11.[10] This strain is derived from *E. coli* TG1[10] by introduction of plasmid pPBG11. This multicopy plasmid contains two relevant elements: 1) the *gfp* gene encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* cloned immediately downstream from the inducible promoter *PalkB*, from which it is expressed, and 2) the *xylS* gene, which encodes a transcriptional regulator that activates the *PalkB* promoter when an inducer (e.g. dicyclopropyl ketone, DCPK), present in the medium, permeates the bacterial membrane (Scheme 1).[11] The GFP protein is extensively used as a reporter to monitor gene expression[12] and hence, bacteria viability. Besides, it provides information about membrane integrity. Intact cells retain the GFP inside (i.e. in the cytoplasm). However, if the cell is damaged, the GFP is released into the medium. Another interesting feature of GFP is its intrinsic fluorescence; no additional cofactors or exogenous substrates are needed to yield a signal. Several GFP variants exist; the one used in this work showed maximum emission at 510 nm when excited at 485 nm.[13]

Bacteria were immobilized in GCCl-DES in its pure state as described in the Experimental Section. Because of the noticeable antibacterial activity exhibited by choline chloride at concentrations above 0.5 M (typical of trimethylammonium salts,[14] see Figure S1 in the Supporting Information), freeze-drying was achieved immediately after incorporation of the

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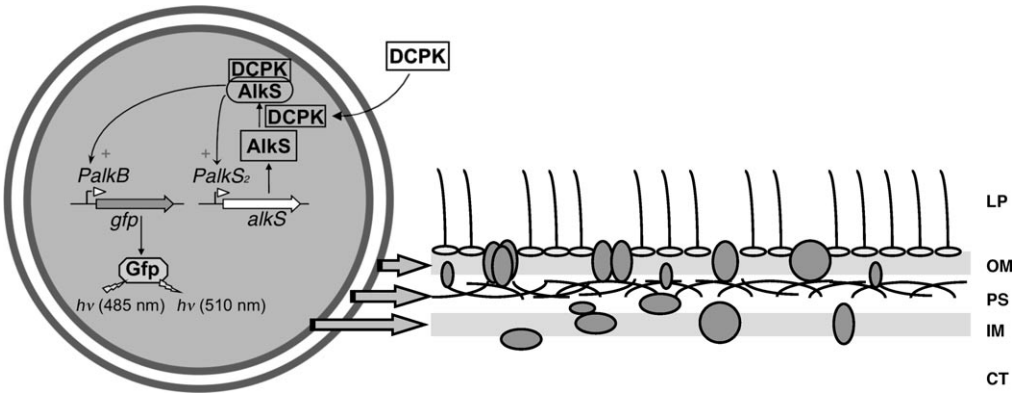

Scheme 1. Left: Representation of GFP expression by genetically modified *E. coli* induced by DCPK (see text for details). Right: Cell wall of typical Gram-negative bacteria such as *E. coli*. The lipid bilayers of the outer membrane (OM) and inner membrane (IM) are represented in light gray. The peptidoglycan polymer, which provides rigidity to the cell wall, is depicted within the periplasmic space (PS). Proteins of the inner and outer membranes (porins, transporters, enzymes) are indicated as dark gray ovals. LP: lipopolysaccharide, CT: cytoplasm.

bacteria into the aqueous solution of DES in order to ensure bacterial integrity. Freeze-drying was used for the transition from aqueous to DES environments, which are characterized by the formation of supramolecular complexes consisting of the halide ion and the hydrogen-bond donor. The ^{1}H NMR spectra and thermogravimetric (TG) data measured for GCCl-DES prepared by freeze-drying were identical to those obtained from GCCl-DES prepared by regular thermal procedures, which indicates that the freeze-drying process is highly efficient at removing water (Figure 1). As mentioned above, water elimination is crucial if one desires to obtain DES in its pure state. Otherwise, choline chloride and glycerol are solvated by water molecules and do not form ion pairs. ^{1}H NMR spectroscopy is a suitable tool for studying the threshold water concentration for the formation of glycerol/choline chloride ion pairs, as some chemical shifts are strongly influenced by this event. Thus, the chemical shifts of HO-C*H*$_2$-CH$_2$-N(CH$_3$)$_3$ and (HO-CH$_2$)$_2$-C*H*-OH in samples diluted to 86 wt% revealed a major presence of supramolecular complexes, which decrease and even vanish at dilutions of 43 wt% and below, respectively (see downfield shifts of up to 0.12 and 0.15 ppm in Table S1 and Figure S2 in the Supporting Information). Further insight provided by the ^{1}H NMR spectra on the rupture of ion pairs upon dilution can be found in the Supporting Information.

The feasibility of freeze-drying processes to obtain DES in its pure state was also studied by cryo-etch scanning electron microscopy (cryo-etch-SEM).[15] In cryo-etch-SEM experiments, the aqueous solution is first plunge-frozen by immersion in subcooled liquid nitrogen, that is, liquid nitrogen at vacuum pressure. The sample temperature is subsequently raised to −90 °C, which allows exposed ice to sublimate (etching). For low solute contents, this temperature (ca. 47 °C above the glass-transition temperature (T_g) of water) favors the formation of crystalline ice, which readily frees itself of any dissolved solute. Meanwhile, for high solute contents, ice formation is not favored and cryo-etch-SEM can provide a map of the water distribution within the sample. Cryo-etch-SEM images of aqueous solutions of GCCl-DES with low solute contents (ranging from 5 to 20 wt%, see Figure 2a–c) revealed the formation of "fencelike" structures that consisted of solutes (in this case, GCCl-DES) surrounded by empty areas where ice originally resided. It is worth noting that highly dilute aqueous solutions of different ice-avoiding substances (e.g. DESs based on urea/thiourea and choline

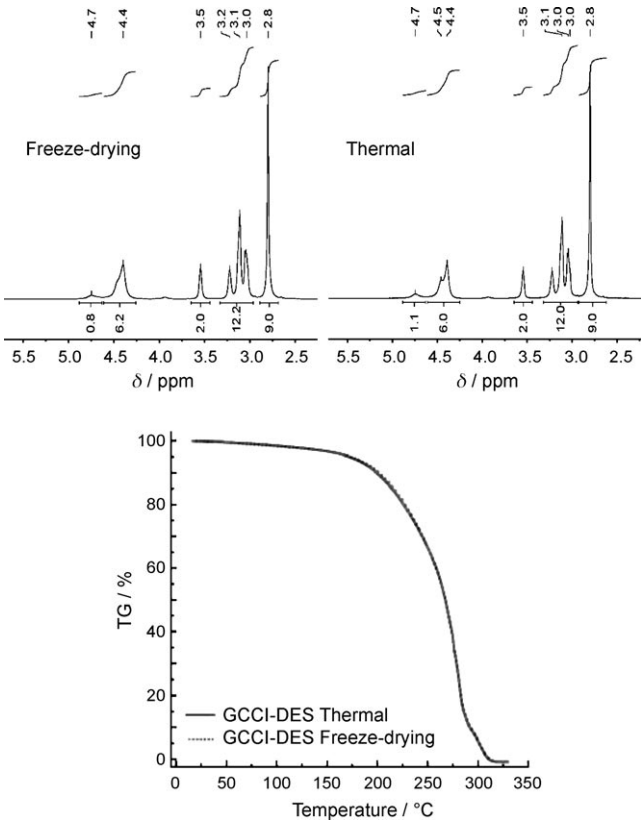

Figure 1. ^{1}H NMR spectra (top) and TG analysis (bottom) of GCCl-DES prepared by thermal and freeze-drying procedures. The baseline signal at ca. $\delta = 3.9$ ppm in the ^{1}H NMR spectra as well as the weight lost at 100–150 °C in the TG analysis indicate that the water content in both GCCl-DES samples is ca. 1.5 wt%.

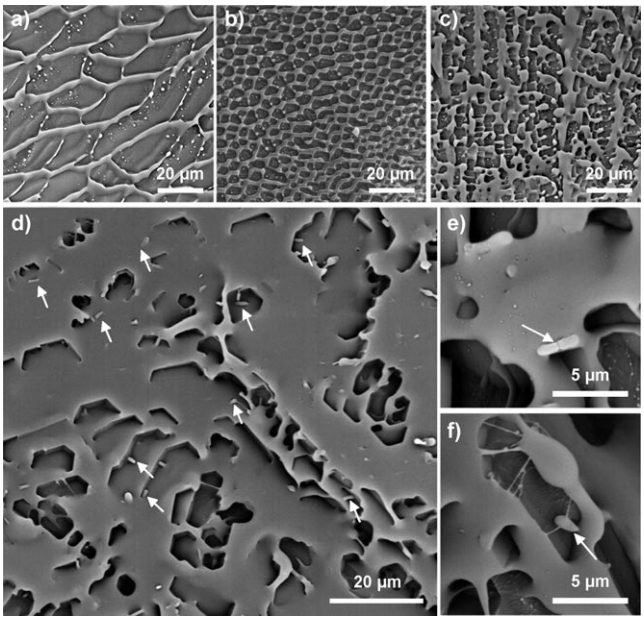

Figure 2. Cryo-etch-SEM images of aqueous solutions of GCCl-DES having a solute content of a) 5, b) 10, and c) 20 wt%, and of bacteria (marked with arrows) incorporated in a buffered (minimum medium) solution of GCCl-DES having 20 wt% solute (glycerol and choline chloride in a 2:1 molar ratio) content (d–f).

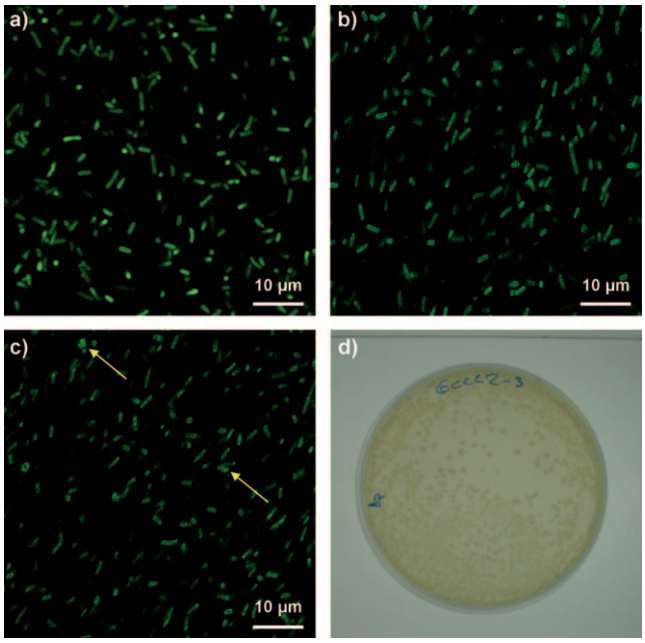

Figure 3. Confocal fluorescence micrographs of bacteria exposed to DCPK a) before freeze-drying (e.g. suspended in M9 minimal salts medium) and b,c) after freeze-drying (e.g. suspended in GCCl-DES). In this latter case, DCPK was added 3 h (b) and 24 h (c) after freeze-drying. Arrows in (c) point to some damaged bacteria. d) Bacteria colonies grown in agar plates cultured from bacteria in GCCl-DES resuspended in LB media (dilution #3 in experimental).

chloride[8] or even ionic salts like NaCl[16]) exhibit quite similar features as a consequence of ice segregation.[17]

Cryo-etch-SEM was also a suitable tool for obtaining first insights on the suitability of GCCl-DES as a cryoprotecting agent. The bacteria visualized in Figure 2 d–f display well-preserved cell envelope integrity (e.g. neither lysis nor even collapsed membranes of irregular shape are evident). In previous work a clear correlation was reported between the loss of viability and the presence of structural damage at the bacterial membrane.[18] Further corroboration of the viability of bacteria incorporated in GCCl-DES was the occurrence of cell division (see Figure 2 e).

The viability of cells incorporated in GCCl-DES was also investigated by confocal fluorescence microscopy. As mentioned above, the *E. coli* strain used in this work was genetically engineered to express green fluorescent protein (GFP) in response to the presence of DCPK. GFP expression occurs only when the bacteria are viable. Thus, the following procedure was applied: The bacteria were 1) grown in LB medium, 2) collected by centrifugation at the end of the exponential-growth phase, 3) resuspended in M9 minimal salts medium, 4) incorporated in GCCl-DES by freeze-drying, and 5) exposed to DCPK for GFP expression. Freeze-drying processes typically damage bacteria membranes. For this reason, non-freeze-dried bacteria (that is, bacteria resuspended in M9 minimal salts medium) were also exposed to DCPK and studied by confocal fluorescence microscopy for comparison. The confocal fluorescence images show a noticeable presence of fluorescent bacteria in all cases, even when the bacteria were stored in GCCl-DES for 24 h before induction (Figure 3 and Figure S3 in the Supporting Information). It is worth noting that, in this latter case, a few collapsed, irregularly shaped cells were observed (see arrows in Figure 3 c). The ability of bacteria suspended in GCCl-DES to express GFP in response to DCPK indicated remarkable bacteria viability even after freeze-drying and storage for up to 24 h. The loss of metabolic activity of bacteria due to freeze-drying and storage can be estimated from the fluorescence intensity emitted by bacteria which ultimately depends on how capable the bacteria are to express GFP. The overall fluorescence intensity was collected from confocal fluorescence images recorded at low magnifications (see Figure S3 in the Supporting Information). The fluorescence intensity of bacteria exposed to DCPK before freeze-drying was considered as 100% given that bacteria are in optimum conditions for GFP expression; that is, they were not submitted to any deleterious process (e.g. freeze-drying[19]) previous to DCPK induction. The fluorescence of bacteria suspended in GCCl-DES exhibited roughly 30% loss of intensity relative to that of bacteria induced in buffered solutions. In fact, the fluorescence intensity of bacteria freeze-dried in the presence of an efficient cryo-protectant such as glycerol was just 1.2-fold that of bacteria in incorporated into GCCl-DES (Figure S3 in the Supporting Information). Also corroborating the excellent cryo-protecting function of GCCl-DES for the preservation of membrane integrity was the absence of collapsed membranes of irregular shape (Figure 3).[18] Meanwhile, storage over 24 h resulted in only a further 2% loss of intensity as a consequence of the appearance of some few damaged bacteria (Figure 3 c and Figure S3d in the Supporting Information). Bacteria stored

over 24 h in GCCl-DES were diluted in buffered solutions containing LB and cultured on LB–agar plates to further corroborate their viability (Figure 3d and Figure S4 in the Supporting Information).

In summary, we have reported on the first use of freeze-drying processes for the incorporation of bacteria in DES in its pure state with outstanding preservation of bacteria integrity and viability. Our findings open interesting perspectives for the use of whole microorganisms in biocatalytic processes carried out in nonaqueous solvents. It is worth noting that in our case substrate concentration should not exceed 16 wt% to preserve the eutectic mixture (see the Supporting Information). Preliminary work aiming to extend this procedure to conventional ILs (e.g. 1-butyl-3-methylimidazolium tetrafluoroborate) seems to indicate that cryoprotection is crucial for preservation of bacteria integrity and viability.

Experimental Section

GCCl-DES in its pure state was prepared by dissolution of glycerol and choline chloride in water, followed by freeze-drying. The molar ratio of glycerol and choline chloride was 2:1, and the solute content ranged from 5 to 20 wt%. The viscous liquids resulting from freeze-drying were studied by ^{1}H NMR spectroscopy (using a Bruker spectrometer DRX-500), TG analysis (using a SEIKO TG/ATD 320 U SSC 5200, from room temperature to 350 °C at a heating rate of 10 °C min^{-1} and under nitrogen flow of 100 mL min^{-1}) and cryo-etch-SEM (using a Zeiss DSM-950 scanning electron microscope). Cryo-etch-SEM experiments were conducted as described elsewhere.[8,15b,20]

Bacteria were grown at 37 °C in complete LB medium[9] with aeration, collected by gentle centrifugation at the end of the exponential-growth phase (A600 of 0.8–1), and resuspended in fresh M9 minimal media (1:100 of the original volume).[21] The incorporation of bacteria in GCCl-DES in its pure state was accomplished by dispersing the concentrated suspension of bacteria in minimal media (ca. 10^6 bacteria per mL) in an aqueous solution of glycerol and choline chloride (20 wt% solute content) with a 2:1 molar ratio. The resulting aqueous suspension was studied by cryo-etch-SEM as described above. Confocal fluorescence microscopy was performed after freeze-drying using a Radiance 2100 (Bio-Rad) Laser Scanning System on a Zeiss Axiovert 200 microscope and a SLM Aminco 4800. Fluorescence studies were conducted on bacteria suspended in minimal media and in GCCl-DES (before and after freeze-drying, respectively). Freeze-dried bacteria with glycerol as a cryo-protecting agent were also induced for comparison. Bacteria suspended in minimal media (40 μL in a 0.2 mL Tris buffered solution) were induced using DCPK previously dissolved in Tris-buffered solution (0.25% v/v, 50 μL) for a final DCPK concentration of 0.05% v/v. Bacteria suspended in either GCCl-DES or glycerol were induced right after freeze-drying, by direct addition of DCPK to the medium for a final DCPK concentration of 0.05% v/v. Induction was also performed on bacteria stored in GCCl-DES for 24 h. In every case, fluorescence was measured 3 h after induction. Bacteria viability was also studied by performing serial tenfold dilutions of the bacteria suspended in GCCl-DES over 24 h in LB medium (e.g. 100 μL of bacteria in GCCl-DES in 900 μL of LB for dilution #1, 100 μL of dilution #1 in 900 μL of LB for dilution #2 and so on). LB dilutions were plated into LB–agar plates (e.g. 0.1 mL of each dilution onto one LB-agar plate) and incubated overnight at 37 °C. The assay was performed in triplicate.

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