Design of Wall-Destructive but Membrane-Compatible Solvents

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Supporting Information Placeholder

ABSTRACT: We report an extremely biocompatible solvent for plant cell walls based on a polar liquid zwitterion that dissolves cellulose, the most recalcitrant component of the plant cell walls. The polar liquid zwitterion does not affect the viability and activity of *Escherichia coli*, even at high concentrations. We demonstrate conversion of cell walls to ethanol *via* a starch-like process, namely successive dissolution, hydrolysis, and fermentation in the same reaction pot.

Cell walls exist on the surface of plant cells for the protection of fragile cell membranes and act like skeletons. Hence, they are one of the most chemically and physically robust and recalcitrant natural structures.[1] The extreme recalcitrance clearly points out that utilization of the cell walls as a renewable resource for next-generation energy and value-added materials[2] requires great breakthrough. In detail, the recalcitrance critically hinders hydrolysis and fermentation.[1a] To achieve efficient conversion of the cell walls to ethanol, starch-like process, namely successive dissolution, hydrolysis, and fermentation, is one of the promising routes because conversion of starch to first-generation biofuels has been industrialized.[3] On the other hand, cell membranes—the protective barriers for animal and microbial cells—are far more fragile than plant cell walls. Therefore, fermentative microorganisms are easily destroyed by surfactants and physical treatments. The dilemma is that the harsh conditions required to break down plant cell walls also destroy the cell membranes of the required microorganisms. For example, harsh molecular solvents required for the destruction of plant cell walls are known to destroy fermentative microorganisms.[4] Harsh thermochemical processes also tend to produce enzyme/microbial inhibitors and condense lignin, preventing rapid and complete conversion. Therefore, low toxicity solvating chemicals are in need for an integrated process.

Ionic liquids (ILs), which are not molecular solvents but salts that melt below 100 °C, are efficient solvents for cell walls and cellulose, the most recalcitrant component of these structures.[5] The characteristics of ILs can be controlled by designing the structures of the component ions. For example, ILs containing chloride, carboxylate, or phosphonate anions can easily dissolve cell walls and cellulose.[5d, 6] Although ILs totally differ from organic solvents, their toxicity has recently been found to be similar to or worse than these solvents.[7] Recently relatively low-toxic ILs, which have choline cation and acetate or amino acid anion, have been reported because they are composed of bio-derived ions, although they do not dissolve cellulose.[8] Nevertheless, they critically inhibit fermentation only at 5–10 wt%. IL toxicity is mostly a function of the cation structure, especially its alkyl chain length. A mechanism of the toxicity of ILs[9] is schematically illustrated on the left-hand side of Figure S1. Cations are electrostatically attracted to the phosphate groups of phospholipids, and the alkyl chains of the cations insert into the cell membranes *via* hydrophobic interactions with the lipid components. The cations accumulate in the membrane and eventually induce rupture.

To solve this problem, we designed new IL structures. Specifically, we introduced the polar anion onto the end of the cationic alkyl chain, i.e. zwitterions (ZIs) (see Figure 1), to suppress the hydrophobic interaction between the alkyl chains and the phospholipids (an image is shown in Figure S1). In detail, we challenged to satisfy both biomembrane-compatibility (or low toxicity to microorganisms) and cellulose dissolution with the ZIs which are an analogue of cellulose-dissolving ILs. However, it is not clear whether these ZIs have the same characteristics as ILs, for example cellulose dissolution

ability, because they are solids below 100 °C[10] and have not been utilized as solvents, except by Yoshizawa-Fujita *et al.*[11] Here, we synthesized a novel carboxylate-type ZI that is a liquid at room temperature because ILs with carboxylate anions are effective for dissolution of cellulose. The characteristics of the carboxylate-type ZIs were investigated: the cellulose dissolution ability and the toxicity towards a recombinant *Escherichia coli* (*E. coli*) which can ferment glucose to ethanol.

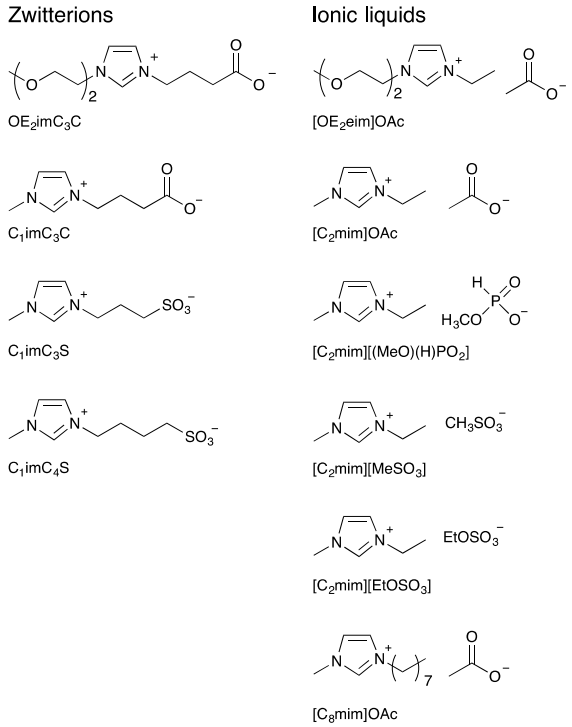

Figure 1. Structures and the abbreviations of ILs and ZIs used in this study.

A carboxylate-type ZI with an oligoether chain (OE_2imC_3C) was synthesized. OE_2imC_3C was liquid at room temperature. The melting point was not detected above -100 °C and the glass transition temperature was -62 °C. The oligoether chain is key to the liquid state of the ZI because when the chain was replaced with a methyl group (C_1imC_3C), the melting point was over 150 °C.

Figure 2. Cellulose dissolution in OE_2imC_3C.

OE_2imC_3C dissolved up to 10 wt% cellulose at 120 °C (Figure 2). Here, it is not sure that OE_2imC_3C dissolves more than 11 wt% of cellulose because the 11 wt% solution was not able to be stirred due to the high viscosity of OE_2imC_3C (1500 cP at 70 °C). OE_2imC_3C also dissolved hemicellulose and lignin (6 and over 10 wt%); OE_2imC_3C can dissolve all components of biomass and completely disrupt plant cell walls. To understand the dissolution mechanism especially for cellulose, the polarity of OE_2imC_3C was measured. The extremely high hydrogen bond basicity of ILs is known to play a key role in dissolving cellulose because the disruption of hydrogen bonds between cellulose molecules is necessary. The Kamlet-Taft parameters α, β and π^* (hydrogen bond acidity, hydrogen bond basicity and dipolarity/polarizability, respectively) of OE_2imC_3C have respective values of 0.46, 1.12 and 1.10. It is known that imidazolium-based ILs with β>0.8 can dissolve cellulose.[12] Thus, OE_2imC_3C has a sufficiently high β value to dissolve cellulose via a mechanism involving disruption of the hydrogen bonds in cellulose.

The toxicity of OE_2imC_3C to *E. coli* was characterized (Figure 3) by defining half maximal effective concentration (EC_{50}) as the concentration of ZIs or ILs when the *E. coli* growth ratio reduced by 50%. LiCl/*N*,*N*-dimethylacetamide (DMAc), a typical cellulose solvent, has an EC_{50} of 28 g/L. This is a relatively high toxicity because ethanol, a known sterilizer, has an EC_{50} of 17 g/L. A popular IL for dissolving cellulose, $[C_2mim]OAc$, has an EC_{50} of 9 g/L. The EC_{50} of OE_2imC_3C was 158 g/L, which indicates an extremely low toxicity; dimethylsulfoxide, a biocompatible organic solvent, has an EC_{50} of 91 g/L. Even in the case of simple salts, NaCl of around 60 g/L critically inhibits growth of *E. coli*[13], suggesting OE_2imC_3C is a very interesting liquid salt. An IL with an analogous oligoether chain, $[OE_2eim]OAc$, exhibits an EC_{50} of 7 g/L. Therefore, the oligoether chain does not affect the toxicity. In summary, the zwitterionic structure is a key factor in the development of a low-toxicity, extremely biocompatible solvent for cellulose; it is again stressed that oligoether side chain of OE_2imC_3C does not significantly affect its toxicity because C_1imC_3C has low toxicity (EC_{50}: 141 g/L), in addition to high toxicity of $[OE_2eim]OAc$.

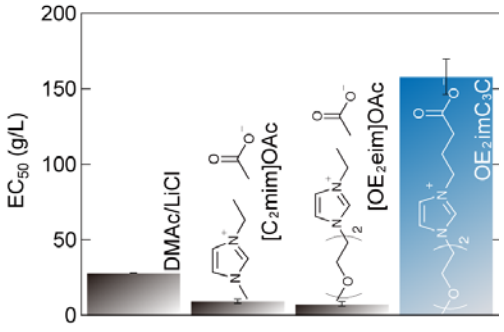

Figure 3. EC_{50} values of LiCl/DMAc, $[C_2mim]OAc$, $[OE_2eim]OAc$ and OE_2imC_3C toward to growth of a recombinant *E. coli* (KO11).

We confirmed the trend that ILs are toxic and ZIs are less toxic, by also investigating the toxicity of various ILs and ZIs regardless of their cellulose dissolution ability (see structures and the toxicity in Figure 1 and Table S1, respectively). All imidazolium-type ILs are highly toxic, regardless of the anion species, with EC_{50} values below 20 g/L. As mentioned above, the alkyl chain length of the cation significantly affected the toxicity. For example, the EC_{50} of [C_8mim]OAc was below 0.01 g/L. In contrast, a relatively biocompatible IL has also been reported as we mentioned above.[8] The imidazolium cation is effective for cellulose dissolution, but is relatively toxic. Choline acetate and choline amino acids are often suggested to be low toxic ILs, although they do not dissolve cellulose. Nevertheless, choline acetate and choline glutamate have EC_{50} of 70 and around 110[8c] g/L (although the definition of EC_{50} of choline glutamate is slightly different), less than OE_2imC_3C. All the ZIs have a high EC_{50}, regardless of their anion component and cation tail group (oligoether or alkyl chain). These data thus confirm that ILs are toxic and ZIs are less toxic.

We also examined the effect of OE_2imC_3C on ethanol production from glucose by a recombinant *E. coli*. In a pure medium without ZIs and ILs, 20.3 g/L of ethanol was obtained after 48 h of fermentation (Figure 4). A small amount of ethanol (3.8 g/L) was obtained in a medium containing 0.5 mol/L LiCl/DMAc. [C_2mim]OAc and [OE_2eim]OAc exhibited stronger fermentation inhibition relative to LiCl/DMAc; only small amounts of ethanol were obtained. In contrast, 19.4 g/L of ethanol was obtained in the medium containing 0.5 mol/L OE_2imC_3C. Because this value is 95% of that obtained in the pure medium, the OE_2imC_3C did not effectively inhibit fermentation.

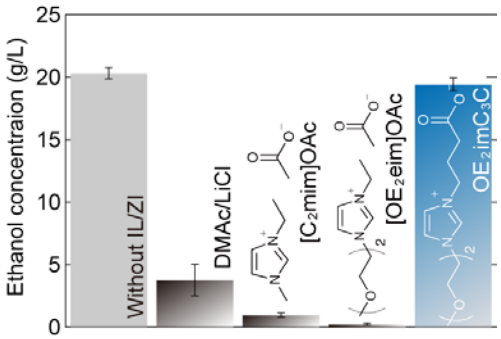

Figure 4. Concentration of ethanol produced by the recombinant *E. coli* in 0.5 mol/L LiCl/DMAc, [C_2mim]OAc, [OE_2eim]OAc and OE_2imC_3C solutions.

The effects of other ZIs and ILs on fermentation were investigated in detail (Table S1). Not only the inhibition of fermentation by ZIs, but also that by ILs has not been investigated until now. Unlike toxicity to cell growth, fermentation in ILs did not depend on the cation component but the anion. The inhibition strength was methylphosphonate > acetate > methanesulfonate > ethyl sulfate. Choline acetate, which has been recognized as a biocompatible IL, significantly inhibited fermentation at this high concentration. To discuss fermentation inhibition, we focused on the Kamlet-Taft β parameter. Figure 5 plots the relationship between the β values of ILs and ethanol concentration after 48 h of fermentation. The figure indicates that ILs with higher β values inhibit fermentation more strongly; indeed, only small amount of ethanol was produced for β values over 0.75. We then focused on glucose consumption by *E. coli* during the 48 h fermentation (Figure S2) to discuss the inhibition because other major metabolites were not observed as by-products (Table S2). Glucose consumption also decreased with increasing β values. This may suggest inhibition of protein activities associated with glucose uptake or metabolism because ILs with higher β values are denaturants that disrupt hydrogen bonds in proteins.[14] Here, only high β value ILs (>0.8) can dissolve cellulose as we mentioned above. Therefore, achieving both cellulose dissolution and low fermentation inhibition is not possible when using ILs.

However, we have demonstrated that OE_2imC_3C did not inhibit fermentation despite its high β value (1.12, see Figure 5); thus it satisfies both cellulose dissolution and low fermentation inhibition. This may be explained by the high protein compatibility of ZIs. Ohno *et al.* reported that hydrated ZIs form a specific structure, similar to that in hydrated biocompatible polymers, which is key to biocompatibility. Thus, hydrated ZIs did not work as denaturants[16] and consequently OE_2imC_3C did not inhibit proteins involved in glucose uptake and metabolism. The results that none of the other ZIs inhibited fermentation (Table S1) also support the hypothesis.

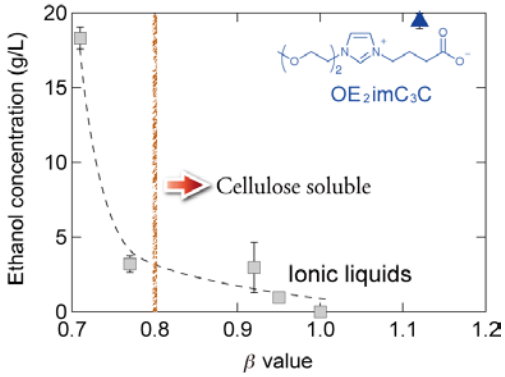

Figure 5. Relationship between β values of ILs and OE_2imC_3C and concentration of ethanol after fermentation in 0.5 mol/L ILs and OE_2imC_3C solutions. β values of [C_2mim]OAc and [C_2mim][(MeO)(H)PO_2] are from the literature.[15]

OE_2imC_3C enables a starch-like process for ethanol production from plant biomass. After treatment of bagasse in OE_2imC_3C at 120 °C for 8 h, acetate buffer was added to form a 0.5 mol/L OE_2imC_3C solution, and the

cellulose in bagasse was hydrolyzed with cellulase at 50 °C for 48h. The resulting glucose solution was directly fermented by *E. coli* at 37 °C for 48 h without addition of extra water. For comparison, we performed the same experiments with [C_2mim]OAc, without addition of ILs or ZIs. With OE_2imC_3C, 1.4 g/L of ethanol was obtained (Figure 6). No ethanol was obtained when we used [C_2mim]OAc because the [C_2mim]OAc strongly inhibited the fermentation by the *E. coli*. In the absence of ILs and ZIs, only 0.2 g/L of ethanol was produced because the high cellulose crystallinity prevented hydrolysis with cellulase. In summary, we have demonstrated successive cellulose dissolution, hydrolysis and fermentation in one-pot by exploiting OE_2imC_3C.

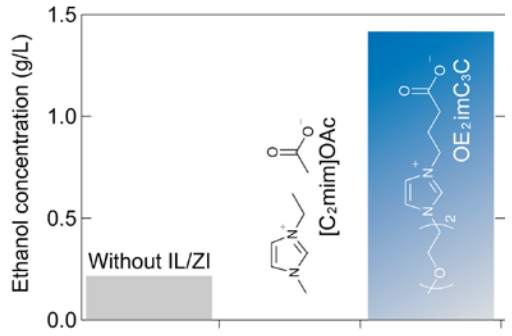

Figure 6. Concentration of ethanol produced via a starch-like process with or without [C_2mim]OAc/OE_2imC_3C.

We found that the *E. coli* produced ethanol without inhibition at concentrations of OE_2imC_3C up to 2 mol/L (over half of the solution, 516 g/L), by increasing the inoculated cell density. Ethanol production via the starch-like process was nevertheless performed at 0.5 mol/L in this study because native cellulase was used and denatured in 2 mol/L OE_2imC_3C (of course, it was also denatured in 2 mol/L [C_2mim]OAc solution). However, it is expected that modification of the enzyme will improve the IL-tolerance and that this will be possible in the near future because modification of enzymes is easier than that of microorganisms. In this study, we have removed the most critical bottleneck for starch-like ethanol production; the applicable concentration of solvents for cell walls and cellulose was significantly increased from several grams to several hundreds of grams per liter. Various value-added compounds such as terpene-based advanced biofuels[17] and building blocks[2] will be applied because *E. coli* can produce them with or without gene modification.

We anticipate that use of OE_2imC_3C will also enable medical applications of cellulose. Other polysaccharides, such as chitosan and alginic acid, have been used for wound healing, tissue engineering and drug delivery[18], but plant-derived cellulose has not been used because of solvent toxicity. Solvent use in medical applications is severely limited owing to their cytotoxicity, and all solvents for cellulose should be avoided (even dimethylacetamide without LiCl is limited).[19] The mechanism of cytotoxicity of ILs is the same as that toward microorganisms, and OE_2imC_3C is therefore a potential solution that will allow the use of plant-derived cellulose in medical applications.

In conclusion, we developed the cell-wall-destructive but cell-membrane-compatible solvent by modification of IL to liquid ZI. OE_2imC_3C dissolved cellulose up to 10 wt% as well as hemicellulose and lignin. Unlike other cellulose solvents, OE_2imC_3C was quite low toxicity in both *E. coli* growth and fermentation. Regarding growth, the toxicity of OE_2imC_3C was lower than dimethylsulfoxide, a biocompatible organic solvent, indicating OE_2imC_3C is an extremely biocompatible solvent among various solvents except for water. Regarding fermentation, OE_2imC_3C did not inhibit fermentation despite high β value, although none of ILs satisfies them. OE_2imC_3C must be a breakthrough to achieve starch-like process in biorefinery.

ASSOCIATED CONTENT

Supporting Information.
The Supporting Information is available free of charge on the ACS Publications website.
Experimental details, Figure S1 and S2, and Table S1 and S2 (PDF)

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Notes

The authors declare no competing financial interest.

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