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## Smart Urea Ionic Co-crystals with Enhanced Urease Inhibition Activity for Improved Nitrogen Cycle Management

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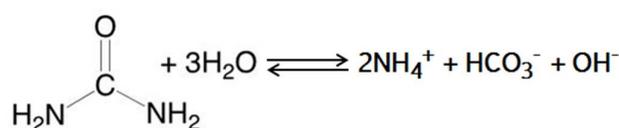
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A smart ionic co-crystal of urea with KCl and ZnCl<sub>2</sub> has been obtained in two polymorphic modifications by mechanochemical and solution methods and proven to be a very efficient urease inhibitor while, simultaneously, able to provide soil nutrients to complement N supply.

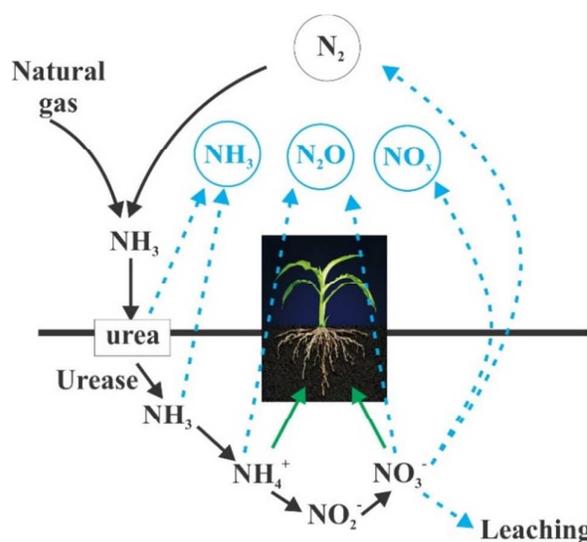
A reliable, cost-efficient and sustainable supply of nitrogen (N) and other plant nutrients is essential for crop growth and accelerated economic development, while maintaining population growth and high life quality.<sup>1</sup> Urea is a predominantly used N-containing fertilizer synthesized from ammonia (NH<sub>3</sub>) and carbon dioxide (CO<sub>2</sub>) with an annual production projected to reach 226 million tons in 2021.<sup>2</sup> NH<sub>3</sub> is synthesized via hydrogenation of N<sub>2</sub> using H<sub>2</sub> derived from natural gas, a process that consumes ~1% of the global energy and ~4% of the total natural gas supply.<sup>3</sup> Once deposited in soil, urea quickly undergoes fast enzymatic hydrolysis catalysed by urease, according to Scheme 1, generating a variety of N species that can quickly dissipate into the environment rather than being absorbed by the cultivated plants.

Urease is a nickel-dependent, non-redox key enzyme that operates within the global N cycle transformations; it is found in plants, fungi and bacteria, and accelerates urea hydrolysis to a rate 10<sup>15</sup> times higher than the non-catalysed reaction, making it the most efficient enzyme known to date.<sup>4</sup> However, this reaction causes a number of agronomic, environmental and economic problems.<sup>5</sup> Indeed, only ~47 % of the total N delivered to soil with fertilizers reaches and is utilized by plants.



Scheme 1. Overall urea hydrolysis catalyzed by urease.

The loss of N derived from urea is partly due to the rapid pH increase upon urea hydrolysis, catalysed by soil urease activity<sup>6</sup> (see Scheme 1), leading to the release of gaseous ammonia from soil<sup>7</sup> that can reach more than 90% of the applied fertilizer.<sup>8</sup> Beside N loss, ammonia is toxic to plants<sup>9</sup> and has several other consequences because of ammonia nitrification<sup>10</sup> and tropospheric pollution with N containing NO<sub>x</sub>, NH<sub>3</sub> and N<sub>2</sub>O (a greenhouse gas with 300 times the heat-trapping capacity of CO<sub>2</sub><sup>11</sup>), as shown in Scheme 2.



Scheme 2. Schematic representation of the N cycle via NH<sub>3</sub> synthesized using natural gas as H<sub>2</sub> source and the resulting urea fertilizer. Loss pathways of the reactive N shown in blue.

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N species available to plants, absorption of  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , in green. Urease enzyme is responsible for the decomposition of urea in soil and the strategy of inhibiting its action via urea ionic co-crystals design is presented in this work.

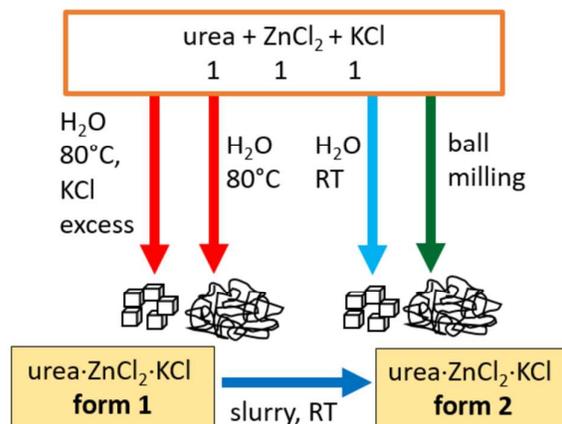
The control of urea stability in fertilised agricultural soils has been pursued via two different approaches. First, low rates of urea dissolution in moist soil can be achieved by coating<sup>12</sup> or encapsulation<sup>13</sup> of urea granules and/or copolymerization of urea with other reactive organic molecules, such as formaldehyde.<sup>14</sup> An alternative approach is the amendment of urea with urease inhibitors. Established reversible inhibitors that target the Ni(II) ions in the active site are phosphate, diamidophosphate, thiols, sulfite, fluoride, as well as hydroxamic, citric and boric acids, while Michael-type reagents such as catechols or quinones irreversibly target enzyme cysteine thiols essential for catalysis.<sup>15</sup> The most widely used urease inhibitor in agronomic practices is the organophosphorus compound N-(n-butyl)thiophosphoric triamide (NBPT).<sup>16</sup> Inhibitors are typically applied via liquid formulation by mixing with N containing liquid fertilizers, such as urea-ammonium nitrate (UAN) solution, or sprayed onto solid urea granules.

In this work we describe an innovative crystal engineering<sup>17</sup> approach to obtain urea-based solid fertilizers via the association of urea with inorganic salts that can act *both* as nutrients and urease inhibitors. Co-crystals, usually composed of two or more neutral components, are providing a wide range of objectives to crystal engineering research<sup>18-20</sup>, as they represent new ways to design or alter the properties of solid active ingredients (thermal stability, solubility, dissolution rate, compressibility, etc.). Recently, organic co-crystals of urea have been scrutinized in the search for less soluble urea carriers.<sup>21</sup>

An important subset of the co-crystal class of compounds is that of ionic co-crystals,<sup>22</sup> which are also being investigated in the quest for smart materials for a variety of applied areas (pharmaceuticals, food, agrochemicals, optoelectronics).<sup>23</sup> Very recent work shows that urea ionic co-crystals with Mg and Ca salts, mechanochemically synthesised, may be used to provide additional primary and secondary nutrients, such as P, Ca, Mg and S, which are necessary for a balanced N uptake.<sup>24, 25</sup> Since urea is a good complexing molecule, ionic co-crystals present new opportunities to provide nutrients and fertilizers while simultaneously tackling the urease inhibition issue.

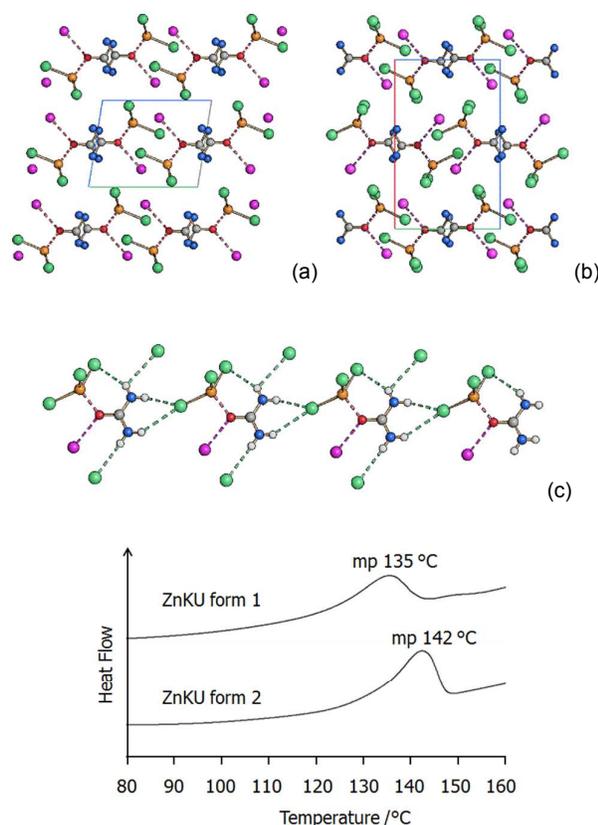
Here we demonstrate this approach by synthesizing, using both solution and mechanochemical methods,<sup>26</sup> a new ionic co-crystal of urea with KCl and  $\text{ZnCl}_2$ , namely urea· $\text{ZnCl}_2$ ·KCl (ZnKU). As potassium (K) is a major nutrient necessary to the plants, while Zn is both micronutrient<sup>27-30</sup> and urease inhibitor,<sup>31,32</sup> the synthesis of ZnKU permits the production of a substance in which known, non-organic and non-toxic, natural mineral based urease inhibitors can be supplied together with urea in one integral crystalline unit, concomitantly reducing urea solubility with respect to pure urea. This type of approach

would also contrast the zinc deficiency in soils, which represents a major issue in crop production.



**Scheme 3.** ZnKU forms 1 and 2 obtained by reacting urea,  $\text{ZnCl}_2$  and KCl in 1:1:1 stoichiometric ratio. An excess of KCl was necessary to grow single crystals of form 1.

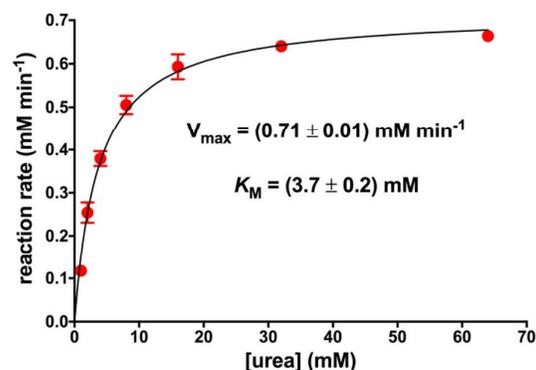
Depending on the preparation method (see Scheme 3) two polymorphic<sup>33, 34</sup> modifications of crystalline ZnKU, form 1 and form 2, have been obtained by reacting urea with KCl and  $\text{ZnCl}_2$  in 1:1:1 molar ratio (see Table ESI-1 and Fig.s ESI-9a-c). The crystal packings of forms 1 and 2 are shown in Fig. 1a-b, while the hydrogen bonding network involving urea N-H donors and the chloride acceptor ions is shown in Fig. 1c. The ionic co-crystals shown in Fig. 1 also possess bifurcated bonding of the urea O-atom to both Zn and K: this suggests that shared bonding of urea to Zn is instrumental to the inclusion of K, as confirmed by the presence of only two urea and K containing crystals (refcodes VIHNOT and XULGAM) in the CSD.<sup>35</sup> Pure ZnKU form 2 is obtained in aqueous solution at room temperature or via ball milling, while pure form 1 can only be obtained from solution if the reaction and the evaporation crystallization are conducted at 80 °C. The high temperature is required for the formation of the kinetic form 1, which is metastable and converts into form 2 upon slurry in water at RT (see Fig. ESI-9d). Form 1 also converts to the stable form 2 under pressure. The two forms of ZnKU constitute a dimorphic, monotropic system (see DSC traces in Fig. 1a-b and Fig.s ESI-6-8), with form 2 being the thermodynamic stable component at all temperatures, and form 2 melting at higher temperature than pure urea (142 vs 137 °C, see Fig. ESI-5). A knowledge of the mechanical response and of the relative thermodynamic stability of ZnKU polymorphs is essential for all practical applications of this crystalline material.<sup>36</sup> The solubility of pure urea versus urea co-crystallised in ZnKU has also been investigated in water (see ESI), revealing that urea solubility decreases by about 20% w/v (see Fig.s ESI-1 and ESI-9e).



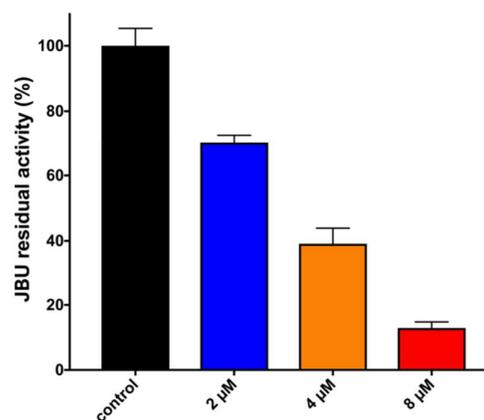
**Fig. 1.** Packings comparison for ZnKU form 1 (a) and form 2 (b), and chain motif (c) evidencing complexation of urea to potassium (pink) and zinc (orange) via the oxygen atom, and hydrogen bonds involving  $\text{NH}_2$  groups and chlorides (green) in form 2; DSC traces for ZnKU form 1 (d) and form 2 (e) show that form 2 melts at higher temperature with a higher associated  $\Delta H$  (81 vs 60 J/g for forms 1 and 2, respectively) indicating that the system is monotropic.<sup>34</sup>

In order to establish the urease inhibition properties of ZnKU, the kinetic parameters for urea hydrolysis by *Canavalia ensiformis* (Jack bean) urease (JBU) were first determined using the pH-STAT method for enzymatic assay<sup>36,37</sup> (see SI for the details of the experimental protocol). The activity of urease as a function of urea concentration is presented in Fig. 2. Data fitted by using the Michaelis-Menten equation yielded  $V_{\text{max}} = (0.71 \pm 0.2) \text{ mM min}^{-1}$  and  $K_M = (3.7 \pm 0.2) \text{ mM}$ , in full agreement with data previously reported for the same enzyme.<sup>37</sup> A representative enzyme saturating concentration of urea (64 mM) was used to determine the inhibition properties of ZnKU on urease activity as a function of increasing concentrations of ZnKU. The results, presented in Fig. 3, show the enzymatic residual activity in the absence (black bar) and in the presence of 2, 4, and 8  $\mu\text{M}$  ZnKU form 2 (blue, orange and red bars, respectively). A similar representation is given for ZnKU form 1 (in comparison with ZnKU form 2) in Fig. S10. Results show that both forms of ZnKU significantly inhibit urease in a concentration-dependent

manner. In particular, at the lowest concentration used, *ca.* 30% activity loss is observed, the latter increasing to *ca.* 90% at the highest concentration. In all cases, the N/Zn ratio was at or below the theoretical value of 0.05 weight %<sup>24</sup> strongly supporting a simultaneous function of ZnKU as both an environmentally compatible nutrient and a urease inhibitor.



**Fig. 2.** Michaelis-Menten curve describing urease reaction rate in the presence of urea as a substrate. The experimental data, showing the enzyme activity as a function of the substrate concentration, are shown as red dots. Data fitting, represented as a solid line, was carried out using the Michaelis-Menten equation.



**Fig. 3.** Residual percentage activity of jack bean urease (JBU), referred to 100% (control, black bar) in the presence of increasing concentrations of ZnKU form 2, at pH 7.5, at 64 mM urea. The blue, orange and red bars correspond to 2, 4 and 8  $\mu\text{M}$  of ZnKU form 2, respectively.

In this communication we have reported the preparation, characterization and evaluation of a multipurpose material able to supply both urea and important nutrients (such as K and Zn) to soil and to inhibit significantly urease activity for the purpose of improving the efficiency of soil N fertilisation. In practical applications, ZnKU might be supplied mixed with urea in order to optimize the inhibition/fertilizing dual action. In summary, we have shown that a combination of organic and inorganic precursors in ionic co-crystals, such as ZnKU,

achieves several objectives simultaneously: i) prepare a stable urease inhibitor that would reduce ammonia release in the atmosphere, ii) provide plant nutrients for crop production, and iii) use a simple, quantitative, scalable, and eco-friendly (solvent free) synthetic procedure. We have proven that crystal design strategies can also be used to devise materials for biotechnological and agricultural applications that are of top importance for food production, human nutrition, energy conservation and environment protection, areas of increasing global importance.

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### Conflicts of interest

There are no conflicts to declare.

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