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Note

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Ionically-Tagged Water Soluble Artificial Enzyme Promotes Dephosphorylation Reaction with Nitroimidazole: Enhanced Ionic Liquid Effect and Mechanism

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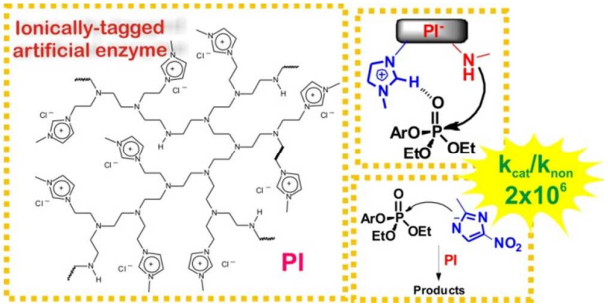


ABSTRACT

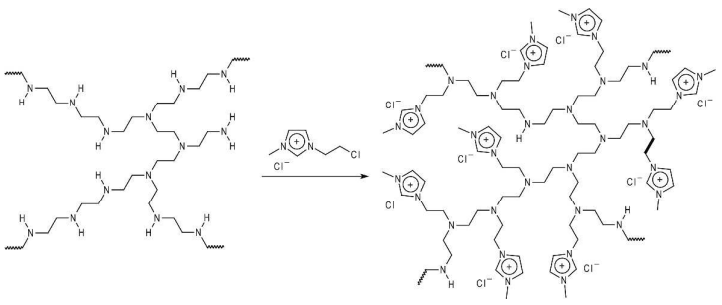
In this paper, we describe a novel synthesized ionically-tagged water-soluble artificial enzyme (PI) that can efficiently cleave phosphate esters, with enhanced ionic liquid effect through cooperative effects for the substrate activation and further nucleophilic reaction. Dephosphorylation reaction with PI was evaluated in the presence and absence of 2-methyl-4(5)-nitroimidazole, showing impressive rate enhancements up to $2x10^6$-fold, accounted for by the imidazolide species known as excellent nucleophiles, and formed favorably at lower pH values in the presence of PI.

Key-words: artificial enzyme, nitroimidazole, dephosphorylation, ionic liquid, catalysis

Dephosphorylation processes are a particular class of vital biological reactions, intrinsically related to phosphorylated structures such as DNA and RNA. These processes are indeed extremely unfavorable, therefore requiring enzymes.[1] There is also a great interest in developing novel bio-inspired catalytic systems that may help with both the understanding about these enzymatic processes and the development of artificial enzymes.[2] Macromolecules are in this sense ideal backbones for this purpose since they encompass active sites, as well as neighboring domains that can synergistically assist the reaction. They can also assemble, leading to nanoreactors that can concentrate reactants and thus accelerating the reactions. When designing new catalytic systems, optimum reactive groups are required. In this context, much emphasis is given to imidazole/imidazolium moieties that are present in many enzymatic active sites because of their versatility. They may efficiently act as general acid-base pairs and nucleophilic catalysts.[3,4] The presence of imidazolium moieties also favors the so-called ionic liquid effect with a typical enhancement in both yields and selectivities as a consequence of ion-pairing and the formation of supramolecular aggregates.[5] Although the origin of the ionic liquid effect is hotly debated in the scientific literature, some compelling evidences point to a cooperative cation-anion stabilizing effects.[6] In principle, ion pairs of imidazolium-based cations as neighboring polar domains could assist the reaction more efficiently as a consequence of solubility effects and the ionic liquid effect. Imidazolium-based derivatives are in this sense thought to be ideal due to both their known high thermal and chemical stabilities (plus their nearly universal solubility), which may certainly help to bring all reactants to the same phase, or to the artificial synthetic enzyme domains, therefore facilitating the reaction to take place.

Herein, we studied the dephosphorylation reaction promoted by a novel synthesized ionically-tagged (imidazolium-based) water-soluble artificial enzyme (PI,

Scheme 1), expected to act as a nanoreactor with enhanced ionic liquid effect. The model substrate evaluated was the triester 2,4-dinitrophenyl phosphate (DEDNPP). The incorporation of imidazolium cations should potentiate the ionic liquid effect in the artificial enzyme toward dephosphorylation reactions and, to the best of our knowledge, no ionically-tagged synthetic enzyme has been described before.



Scheme 1. Synthesis of the water-soluble ionically-tagged artificial enzyme (PI).

We also studied the reaction of 2-methyl-4(5)-nitroimidazole (MNI) with DEDNPP in the presence of PI in an aqueous medium, which is not possible to study without PI since MNI is a water insoluble nucleophile. These nitrated imidazole derivatives are of great interest since they constitute many pharmaceuticals[7,8] and little is known about their action, especially against phosphate esters, that could point to possible carcinogenic activities. In fact, dephosphorylation processes are known to be related to many dangerous diseases.

The PI structure was firstly characterized by ^{1}H-^{15}N HMBC experiments (see Supporting Information, SI), typically used for the characterization of artificial enzyme synthesized from commercially available polyethylene imines,[9-11] and spectra show typical hydrogens from the imidazolium-based ionic liquid structures (9.30 and 9.11 ppm for C2-H; 8-7 ppm C4-H and C5-H; 5.01 C7-H; 4.05 and 4.08 for methyl C6-H) correlated with different types of nitrogen atoms at 167.0 ppm and 174.1 ppm (^{15}N)

from the imidazolium ring. As expected, the presence of varied signals for similar ^{1}H and ^{15}N atoms are the net result from the differences in chemical environments of the polymeric structure of PI. In addition, the signal at 25 ppm observed in the ^{15}N analysis, which is correlated with ^{1}H at 2.80 ppm, reveals that tertiary amines are predominant in the PI structure.

Potentiometric titration were carried out to characterize PI as well as the system PI/MNI, regarding the existing pK_a´s. These provide important information in order to infer the catalytic activity of specific species, since the pH effect in the reactions studied (*vide infra*) will be evaluated. Fitting the titration profiles (see SI, Figure S2) with the program BEST7, for PI, 4 values of pK_a´s were determined: pK_{a1} =5.41, pK_{a2} =7.50, pK_{a3} =8.99 and pK_{a4} =9.69 (error for all pK_a´s ±0.03), accounted to the amine sites (NH_2^+). Previous reports[12] agree with the assumption of multiple pK_a´s for a macromolecule with several units of similar acid-basic moieties, attributed to the neighboring groups that affect the equilibrium, *e.g.*, stabilizing the deprotonated species. The pK_a of an amine group can therefore be shifted depending on whether it bears a neutral or protonated amine group, just as observed in natural enzymes. The values are additionally consistent with other complex amine-based macromolecules[13] such as lysozyme.[14] In the case of MNI/PI, the same four pK_a´s for PI were determined with an additional pK_a =10.48±0.01, attributed to MNI (formation of imidazolide species), which agrees with other nitroimidazole derivatives.[15]

The reaction of DEDNPP with PI in the presence and absence of MNI was followed at different pH; and the pH rate profiles obtained are shown in Figure 1 along with data for the spontaneous reaction of DEDNPP in water.[3] Results clearly show that the dephosphorylation of DEDNPP is accelerated with PI and PI/MNI when compared to the spontaneous reactions. It is also noted that k_{obs} increases with pH for the reactions

studied, suggesting that reactive species are formed at higher pH values. In the case of solely PI, this can be attributed to the amine groups, potential nucleophilic sites, which are knowingly reactive at higher pH, *i.e.*, neutral. For PI/MNI, the imidazole group from MNI can additionally act as nucleophilic sites, with a reactivity that depends on the pH, evidencing the importance of the previous titration study. The data in Figure 1 were fitted with Equation 1, which in all cases considers the reaction of DEDNPP with water (k_0) and hydroxide (k_{OH}). With only PI, the last terms of the equation were not considered, related to the MNI species. Although PI has many possible pK_a´s for the amine moieties (*vide supra*), only one pK_a was required to fit the data, and two species were relevant in this reaction: a partially protonated species (*e.g.* bipolar) with some neutral amine groups with neighboring protonated groups (molar fraction χ_{PI+}; k_{PI+}), and totally deprotonated species with neutral amine groups (χ_{PI-}, k_{PI-}). In the presence of MNI, an additional species was considered, regarding the deprotonated imidazolide group (χ_{MNI-}, k_{MNI-}), known to form at ca. pH 12 for nitroimidazoles.[15] All these possible pathways are illustrated in Scheme 2.

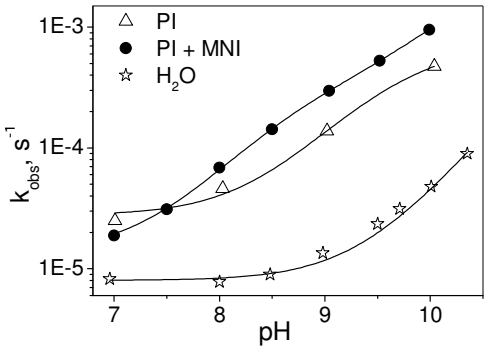
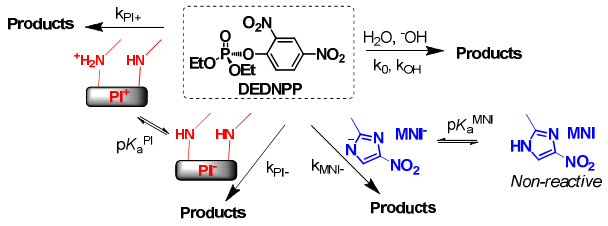


Figure 1. pH-rate profile for the reaction of DEDNPP with PI (3.14 mg/mL) in the absence (Δ) and presence of MNI 0.02 M (●). Spontaneous hydrolysis of DEDNPP is shown for comparison (✯) at 25 °C.



Scheme 2. Pathways for the possible reactions of DEDNPP with PI and PI/MNI.

$$k_{obs} = k_0 + k_{OH}[OH] + k_{PI+}\chi_{PI+} + k_{PI-}\chi_{PI-} + k_{MNI}\chi_{MNI} \quad (1)$$

Table 1 presents the kinetic parameters obtained from the fitting data in Figure 1, where values of k_0 and k_{OH} are accordingly to previous studies.[3] For the reaction of DEDNPP with only PI, results show that the neutral species (PI^-) is the most reactive, as expected, leading to rate enhancements ~80-fold when compared to the spontaneous reaction. Considering that PI has many of its nucleophilic sites hindered, acting more as an aggregated ionic liquid, this reactivity is impressive. The kinetic pK_a determined for PI is consistent with the titration data, showing that above this critical value, the amine moieties of PI are neutral, crucial for inferring nucleophilic reactivity. As mentioned, macromolecules with multiple protonation sites with the same functionality (*e.g.* amine) are known to have several pK_as, since neighboring groups affect deprotonation of subsequent sites.[12] Multiple equilibria are however not crucial in the kinetic evaluated, and an overall equilibrium can be reasonably considered (PI^+ to PI^-). The species PI^+ has indeed little contribution but is necessary for fitting the data, evidencing that at lower pH (<9.5) an overall partially neutral species of PI is reactive in the reaction evaluated. The concentration of PI was varied, showing a linear profile with k_{obs} (see SI, Figure S3), characteristic of a nucleophilic attack. The obtained second-order constant for PI was 0.11 g^{-1} mL^{-1} s^{-1}, extremely high for macromolecule-mediated dephosphorylation reactions. For example, the powerful α-nucleophilic polyhydroxamate is effective in cleaving DEDNPP[12] with k_N= 0.017 g^{-1} mL^{-1} s^{-1},

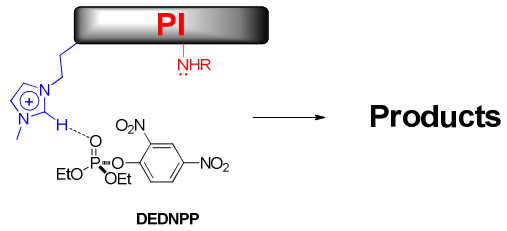
evidencing the high reactivity of PI. For the reaction with PI/MNI, similar constants for PI were found and for MNI, rate enhancements were even more impressive: ~ 700-fold, accounted for by the anionic imidazolide species (MNI^-). It should be noticed that the reaction with solely MNI cannot be evaluated since the substrate is water insoluble in the absence of PI (even at low concentration, varying pH, with different surfactants, etc.). Likewise, MNI is highly reactive at a considerably low concentration (0.02 M), and the most commonly compared parameter in nucleophilic reactions is the second order rate constant, which is 0.27 M^{-1} s^{-1} (among the highest reported with DEDNPP). Even imidazole, that lacks the nitro group which readily decrease reactivity, presents k_N=0.177 M^{-1} s^{-1}.[3] The kinetic pK_a determined for MNI is also consistent with the titration study. Finally, the neutral species of MNI did not show significant reactivity.

Table 1. Kinetic parameters obtained for the reactions of DEDNPP.

H_2O, ^-OH: k_0=8.0x10^{-6} s^{-1}; k_{OH}=0.42 M^{-1} s^{-1}		
Constant	**PI**	**PI / MNI**
k_{PI+} , s^{-1}	1.88x10^{-5}	3.14x10^{-5}
k_{PI-} , s^{-1}	5.97x10^{-4}	5.65x10^{-4}
k_{MNI-} , s^{-1}	-	5.4x10^{-3}
pK_a^{PI}	9.69	
pK_a^{MNI}	-	10.56

Regarding the mechanism, the presence of imidazolium rings in the PI structure is possibly helping in activating the substrate toward dephosphorylation reaction. A remarkable acceleration on the transesterification reaction of some phosphate ester derivatives in the presence of imidazolium-based ionic liquids has been demonstrated.[16] Dupont[17] and others[18] have also proposed C=O activation in the presence of imidazolium cations through the interaction of C=O---H-C2 between the acidic hydrogen at C2 position of the imidazolium ring and the basic oxygen of the C=O group. Based on the data obtained herein and on the literature evidences, an activation mode could be proposed for DEDNPP (Scheme 3). In this sense, both parts of the

synthetic enzyme play a role for the reaction acceleration. That is, the amine groups (basic sites) and the imidazolium rings (acidic sites) are displaying a cooperative effect for the dephosphorylation reaction.

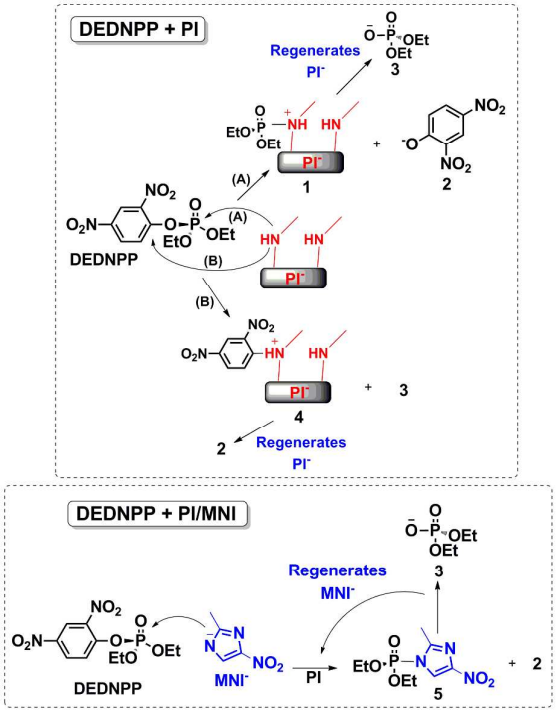


Scheme 3. Substrate activation by the imidazolium cation of PI.

Scheme 4 presents an overall mechanism proposed for the reactions studied here. Thus, DEDNPP can be attracted to the PI neighboring domains by the polar regions of the imidazolium cation, therefore being activated to react with the amine sites. Nucleophilic attack by the amine sites occur via the following two paths: (A) on the phosphoryl group leading to a phosphorylated intermediate (1); and path (B) on the aromatic carbon affording intermediate 4. These intermediates are formed by a concerted step[3,19] and can readily decompose, regenerating PI and thus comprising a catalytic nanoreactor with cooperative effects through the ionic liquid effect.

Analogous mechanisms of DEDNPP with amines have been reported[3,19-23] but without any ionic liquid effect exploited so far. In the present study, we propose the attack on the phosphorus atom is predominant since the initial formation of the phenolic product 2 should mostly come from path A, in accordance with the literature.[19] Thermodynamic parameters were also obtained (Eyring plot given in the SI, Figure S4) for the reaction of DEDNPP with PI, giving $\Delta S^{\ddagger}$ = -24.87 cal K^{-1} mol^{-1}, $\Delta H^{\ddagger}$=15.5 kcal mol^{-1}, $\Delta G^{\ddagger}$ = 22.91 kcal mol^{-1} (25°C), which agree with the proposed nucleophilic pathway.[3]

In the case of the reaction of DEDNPP with PI/MNI, which should also be attracted to the PI domains, an additional nucleophilic reaction should occur between the anionic nitrogen of imidazolide (MNI) and the phosphoryl group, leading to the intermediate 5, knowingly very unstable.[3] Imidazolide is not usually studied since its formation is associated with a very high pK_a, over 14, for solely imidazole.[15] Herein, the withdrawing nitro group lowers this pK_a (10.5, *vide supra*), enabling the formation of the highly reactive species MNI^-. This is also favored by the solubility and stabilizing effects due to the presence of imidazolium-based neighboring domains in the synthetic enzyme structure. The neutral species of MNI is not reactive, since the available nitrogen is known to be a weak nucleophile (with a pK_a < 3).[15] Finally, an attack on the aromatic carbon may be discarded, since previous studies showed that this path is highly unfavorable with imidazole derivatives.[3]



Scheme 4. Proposed mechanism for the studied reactions. Note the substrate activation by the imidazolium cation has been omitted for clarity.

Overall, results showed that PI may efficiently cleave phosphate esters, such as DEDNPP, behaving as a catalytic nanoreactor with an enhanced ionic liquid effect through cooperative effects for the substrate activation and further nucleophilic reaction. PI proved to be capable of dissolving MNI in an aqueous medium, and this system PI/MNI catalyzed the cleavage of DEDNPP with rate enhancements up to $2x10^6$-fold, comparing second order constant k_N with the spontaneous reaction. The high reactivity of MNI is mostly accounted for by the imidazolide species, known to act as excellent nucleophiles, and formed favorably at lower pH values in the presence of PI (*i.e.* milder conditions), in contrast to other imidazole-based systems (pH>13). As previously mentioned, nitroimidazoles comprise many pharmaceuticals and the present results evidence that precautions are necessary, since these compounds are potential nucleophiles that can attack our biological phosphate esters (*e.g.* DNA, RNA), causing defects, for example, and leading to tumoral processes. Lastly, these results have great potential in designing water-soluble artificial enzymes with enhanced ionic liquid effect and also detoxifying agents, since organophosphorus compounds constitute many chemical weapons and pesticides that need to be monitored and eliminated (*i.e.*, detoxification).[24]

EXPERIMENTAL SECTION

Materials. MNI was obtained commercially and DEDNPP was prepared as described previously.[3] PI synthesis: Commercially available polyethylene imine (branched, Mw ~800 by GPC, Mn ~600 by GPC) was dried by azeotropic water removal. The anhydrous PEI (5.00 g) was dissolved in anhydrous CH_2Cl_2 (10 mL) in a sealed Schlenk tube. 1.40 g of 1-(2-chloroethyl)-3-methylimidazolium chloride was added followed by 2,6-lutidine (10 g). The mixture was heated at 100 °C for 2 h. After this time period, the

Schlenk was unsealed and the mixture washed with CH_2Cl_2 followed by AcOEt affording PI in nearly quantitative yield.

NMR. All NMR measurements were carried out on a spectrometer (11.75 T) operating at 500.13 MHz for ^1H and at 50.68 MHz for ^{15}N. Typically, 2D ^1H-^{15}N HMBC pulse sequence from the Bruker User Library was used. All experiments were performed using 40 mg of PI in 600 μL of CD_3OD in a NMR tube containing a sealed capillary tube charged with nitromethane used as the external reference to set the scale (4.80 ppm for ^1H and 381.7 ppm for ^{15}N).[25]

Potentiometric titration. Potentiometric titration was carried out in a 100.0 mL thermostated cell at 25°C. The solutions were acidified with HCl (0.11 M) and titrated with small increments of KOH ($9.95x10^{-2}$ M, CO_2 free). The pH was monitored by a pHmeter. The program BEST7 was used to determine the equilibrium constants.[26]

Kinetics. Reactions that were followed spectrophotometrically were started by adding 10.0 μL from a stock solution of the substrate DEDNPP ($7.5x10^{-3}$ M in acetonitrile) in 3.0 mL of the reaction solution, under pseudo-first-order kinetics. Solutions were buffered with 0.01 M of $KHCO_3$ (pH 7.0) and K_2HPO_4 (7.5-10.0). Reactions were followed by the appearance of 2,4-dinitrophenol (**2**) at 400 nm with a thermostated cell holder maintained at 25 ºC. Observed first-order rate constants (k_{obs}) were calculated from non-linear plots against time by using the Levenberg algorithm using the program Origin8.

SUPPORTING INFORMATION

NMR spectra, potentiometric titration curves and kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org

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