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Identification and directed development of non-organic catalysts with apparent pan-enzymatic mimicry into nanozymes for efficient prodrug conversion

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Abstract: Nanozymes, nanoparticles that mimic the natural activity of enzymes, are intriguing academically and are important in the context of Origin of Life. However, current nanozymes offer mimicry to a narrow range of mammalian enzymes, near-exclusively performing redox reactions. In this work, we present an unexpected discovery of non-proteinaceous enzymes based on metals, metal oxides, 1D/2D-materials, and non-metallic nanomaterials. Specific novelty of our findings lies in the identification of nanozymes with apparent mimicry of diverse mammalian enzymes, including unique pan-glycosidases. Further novelty lies in the identification of the substrate scope for the lead candidates, specifically in the context of bioconversion of glucuronides, that is, human metabolites and privileged prodrugs in the field of enzyme-prodrug therapies. Lastly, nanozymes are employed for conversion of glucuronide prodrugs into marketed anti-inflammatory and antibacterial agents, as well as "nanozyme prodrug therapy" to mediate antibacterial measures.

Nanoparticles that mimic the natural activity of enzymes, termed nanozymes, comprise a unique set of materials at the interface between inorganic and bio-organic chemistry. [1] Engineering mammalian enzyme-like behavior into non-proteinaceous molecules is intriguing academically and is poised to open up immense opportunities in biotechnology and biomedicine. Specifically, advantages of nanozymes over the natural enzymes include stability, scalability, chemical diversity, and performance in non-aqueous solvents.[2] From a different perspective, enzymatic activity of inorganic materials (regardless of their dimension) is considered highly important in the context of Origin of Life. [3] However, successful in their own right, current nanozymes and mineral surfaces offer mimicry to a very narrow range of mammalian enzymes, almost exclusively performing redox reactions.[4] Compared to the magnitude of enzymes in the human body, this range is only humble at the very best.

In this work, we present a highly unexpected identification of nanozymes with apparent mimicry of diverse mammalian enzymes performed under physiological conditions, including non-proteinaceous pan-glycosidases. This was accomplished via a screen of ca. 100 enzyme-mimic candidate materials against ca. 20 fluoro/chromogenic substrates. Specific novelty lies in that we identified non-proteinaceous enzymes based on metals, metal oxides, 1D/2D-materials, and non-metallic nanomaterials (detailed in Supporting Information, Figures S10-S31). Further novelty lies in that we identified the substrate scope for the lead nanozymes, specifically in the context of bioconversion of glucuronides, that is, natural metabolites and privileged prodrugs in the field of enzyme-prodrug therapies.[5] Lead nanozymes performed efficient conversion of glucuronide prodrugs to produce anti-inflammatory and antibacterial agents. To our knowledge, we report the first example of pan-enzymatic mimicry evidenced in non-proteinaceous materials, as well as the first example of nanozymes as mimics of glucuronidase for enzyme-prodrug therapy.

With our broader interest in enzyme prodrug therapies (EPT), [6] the first screen of nanomaterials for potential enzymatic activity performed in this work aimed to identify mimics of glucuronidase. Glucuronides hold a privileged position in EPT[5] and are also natural metabolites that are formed during phase II metabolism in the human liver. Enzyme activity was evaluated with the use of a turn-on fluorescent probe, resorufin-β-D-glucuronide (Figure 1A). Over a 24 h incubation time, inorganic enzyme-mimics afforded conversion of the substrate to yield fluorescent product in content as high as 1 μM (Figure 1B). The catalytic activity of the particles was readily registered by fluorescence imaging which showed significant increase in the fluorescence of substrate solutions in the presence of nanozymes (Figure 1C). Strikingly, enzyme mimics were identified within each class of nanomaterials tested and leads included metals, metal oxides, 1D/2D materials and non-metallic nanoparticles.

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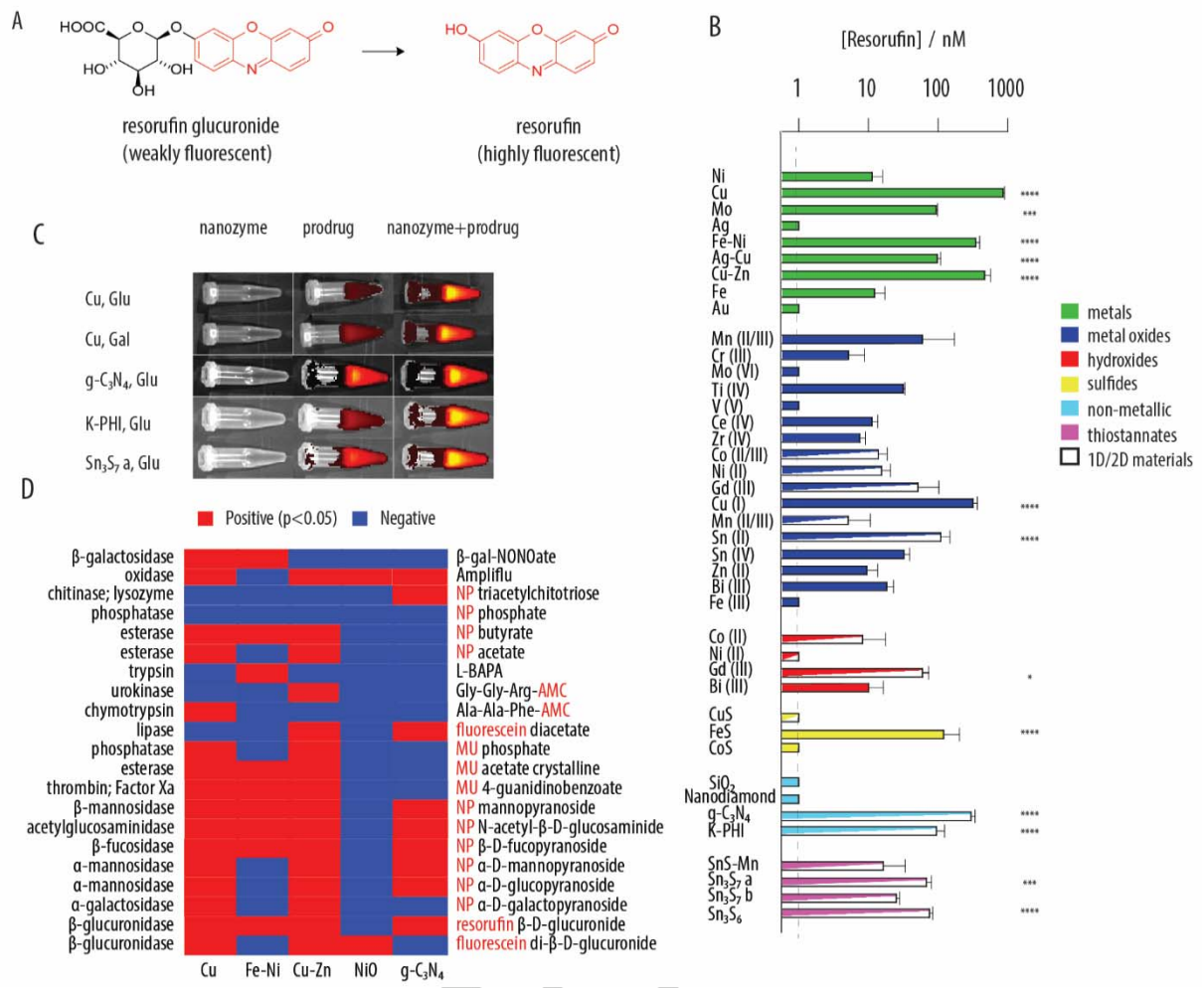

Figure 1. High throughput screen of nanozymes identifying nanoparticles with activity of β-Glucuronidase and pan-enzymatic activity. (A) Conversion of weakly fluorescent resorufin β-D-glucuronide into the corresponding fluorescent product, resorufin, allowing for a fluorescence based readout of nanozyme activity; (B) Glucuronidase mimicry screen for nanozymes based on metals, metal oxides and other nanomaterials using resorufin β-D-glucuronide as an enzyme-specific fluorogenic probe; (C) imaging of catalytic output for selected nanozymes using resorufin β-D -glucuronide (Glu) and - β-D -galactoside (Gal); (D) "Heat map" representation of the results of the screen for nanozyme activity (Cu, Fe/Ni, Cu/Zn, NiO, *g*-C_3N_4) using enzyme-specific substrates corresponding to diverse mammalian enzymes. NP = 4-nitrophenol, AMC = 7-amido-4-methylcoumarin; MU = 4-methylumbelliferone. "Positive" activity is defined herein as the one with statistically significant enhancement of the catalytic substrate conversion over non-catalyzed reaction ($p \leq 0.05$). Statistical evaluation was performed using one-way ANOVA with Dunnett's multiple comparison test or unpaired t-tests with or without Welch's correction.

Optimization of the catalytic output of the nanozymes (performed using commercial copper nanoparticles) was performed using the computer-assisted design of experiments. Solvent pH and temperature had a non-negligible influence on the catalytic output of the nanozyme particles. However, the highest level of influence was revealed by the choice of the buffer, phosphate buffer being a significantly better medium than HEPES for catalysis, and the presence of physiological concentration of sodium chloride (Figure S1). This observation indicates that nanozyme activity, at least for copper nanozymes, is strongly affected by the ion adsorption to the metal surface. [7] With regards to the kinetics of (bio)conversion of resorufin-β-D-glucuronide, nanozyme based on commercially available copper nanoparticles expectedly revealed *K*cat values that were significantly lower than for the natural catalyst, while *K*m values were surprisingly similar to β-glucuronidase (β-Glu) (Table 1, raw data presented in Figures S6-S9). We note that while contribution of the metal ions released from the particles in the overall catalytic performance [8] cannot be fully ruled out, we found that ion-mediated catalysis was significantly lower, e.g. for copper ions compared to the copper nanozyme (Figure S2); this consideration is altogether irrelevant for non-metallic nanozymes such as graphitic carbon nitride (*g*-C_3N_4).

Table 1. Enzyme and copper-based nanozyme kinetics for bioconversion of resorufin-β-D-glucuronide. *K*m – Michaelis constant, *K*cat – catalytic constant.

entry	K_m [μM]	K_{cat} [s^{-1}]	K_{cat}/K_m [mM^{-1} s^{-1}]
β-Glu	25 ± 2	2.7 ± 0.2	108 ± 2
Cu NP	41 ± 24	0.05 ± 0.02	1.2 ± 0.2

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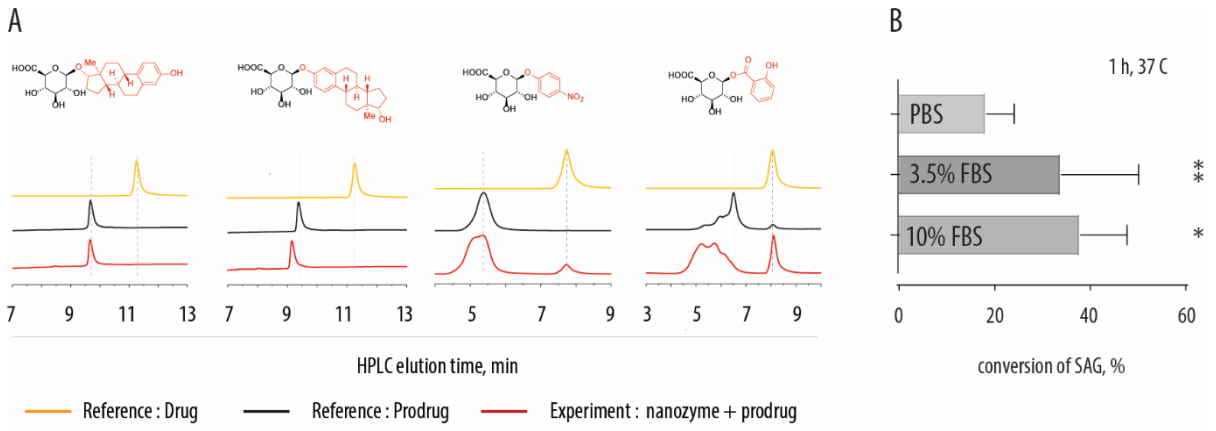

Figure 2. (A) Substrate scope of the copper nanozyme as a β-glucuronidase mimic is closely related to the quality of the leaving group in the substrate. HPLC characterization of conversion for glucuronide prodrugs of estradiol (aliphatic, estradiol 17-(β-D-glucuronide) and phenolic, estradiol 3-(β-D-glucuronide)), 4-nitrophenol, and salicylic acid acyl glucuronide (from left to right, increasing quality of the leaving group). Experimental conditions: 37 °C, phosphate buffered saline pH 7.4; prodrug concentration of 0.1 g L^{-1} and nanozyme concentration of 5 g L^{-1}. Conversion time was 24 h (all substrates) or 1 h (salicylic acid acyl-glucuronide); (B) HPLC quantitative analysis of nanozyme-catalysed hydrolysis of salicylic acid acyl glucuronide at a concentration of 0.14 g L^{-1} in phosphate buffered saline pH 7.4 optionally supplemented with fetal bovine serum (FBS) (3.5% and 10% v/v% FBS) over 1 h incubation at 37 °C. Statistical significance is calculated relative to the negative control (non-catalyzed decomposition of SAG) using a one-way ANOVA test followed by a Dunnett's multiple comparisons test.

We next performed a screen of selected nanozymes for mimicry against 20 enzymes (esterases, phosphatases, peptidases, cytochromes, glycosidases, *etc*), in each case using commercially available chromogenic or fluorogenic probes (Figure 1D). The threshold activity used to distinguish between positive and negative mimicry was set using statistical significance of the catalytic output compared to a non-catalyzed substrate decomposition. The striking observation from this map is that for each enzyme considered in this work (except phosphatase, for which nanozymes have been reported previously[9]), there was at least one nanomaterial with an apparent enzymatic activity evidenced by the conversion of the enzyme-specific substrate into its corresponding product. Furthermore, nanozymes revealed apparent pan-glycosidase activity in this screen, a feature hardly observed in nature.

Results in Figure 1D are surprising and illustrate that the same nanozyme readily accepts multiple, diverse enzyme-specific substrates for conversion. The substrates used in Figure 1D are employed as enzyme-specific probes in the overall majority of studies on enzymes, nanozymes, and other types of enzyme mimics.[1, 10] However, it appears that these substrates alone cannot provide sufficient evidence to postulate specific enzyme mimicry. It appears that nanozymes do not obey the substrate specificity of the natural enzymes and therefore enzyme mimicry most likely does not draw origin from the specific substrate recognition. To validate this conclusion, we performed inhibition studies and observed that glucuronic acid expectedly inhibits activity of the glucuronidase enzyme (via product-mediated competitive enzyme inhibition) but has no such effect on the copper nanozyme, Figure S3. Instead, we observe that all substrates used in Figure 1D are engineered to contain an enzyme-specific affinity ligand attached to a "good leaving group" (4-nitrophenol, 4-methylumbelliferone, resorufin, fluorescein) thus constructing the weakest bond in the molecule. In contrast to the proteinaceous enzymes, for nanozymes it appears that the "leaving group", not the enzyme affinity ligand in the reporter molecule, defines the substrate scope.

We further scrutinized the substrate scope for nanozymes focusing on glucuronides. To this end, we performed glucuronidase mimicry tests using prodrugs with varied quality of the leaving group,[11] increasing from aliphatic alcohol to phenol (pKa > 10) to 4-nitrophenol (pKa < 10) and carboxylic acid (Figure 2A). The former two prodrugs were glucuronides of estradiol, while the latter was a salicylic acid acyl glucuronide (SAG). Using HPLC to monitor prodrug conversion in phosphate buffered saline over 24 h, we observed that the copper nanozyme afforded negligible conversion of the estradiol glucuronides. In contrast, conversion of 4-nitrophenyl glucuronide was confirmed by HPLC. Finally, conversion of the acyl glucuronide was pronounced even within 1 h of observation (accompanied by broadening of peaks which we attribute to the well-documented acyl migration phenomenon[12]). These experiments confirm that the substrate scope for Cu nanozymes with regards to their glucuronidase mimicry is closely related to the quality of the leaving group in the prodrug. We also found that conversion of SAG was enhanced by the presence of sodium chloride, as was the case for the fluorogenic substrates, suggesting that ion-mediated enhancement of nanozyme activity is specific to the nanoparticle rather than to the substrate (Figure S4). To our knowledge, results in Figure 2A present the first example of the use of nanozymes applied to convert the privileged glucuronide prodrugs into corresponding therapeutic (salicylic acid).

Nanozymes used in the experiments presented above were based on commercial copper nanoparticles and proved to be active as glycosidases in aqueous buffered solutions but failed to exhibit catalytic activity in complex biological media. Specifically, these nanozymes were inactive in the presence of serum proteins (data not shown). Such activity is indispensable with the view of practical applications of the nanozymes in biomedicine. To achieve this, we performed nanoengineering of copper-based

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nanozymes using several literature protocols. [13] Poly(vinyl pyrrolidone)-stabilized Cu nanocubes were particularly active as artificial glycosidases, Figure 2B. These nanozymes successfully mediated catalytic decomposition of SAG in phosphate buffered saline and in the presence of increasing amounts of serum, reaching the serum content as typically used in cell culture medium. These results illustrate a successful engineering of nanozymes to optimize their activity as artificial glycosidases.

Next, we applied the knowledge on the nanozyme substrate scope to perform *de novo* engineering of glucuronide prodrugs. Specifically, we used commercially successful antibiotics, ciprofloxacin and moxifloxacin, and synthesized corresponding prodrugs that would fall within the substrate scope for nanozymes.

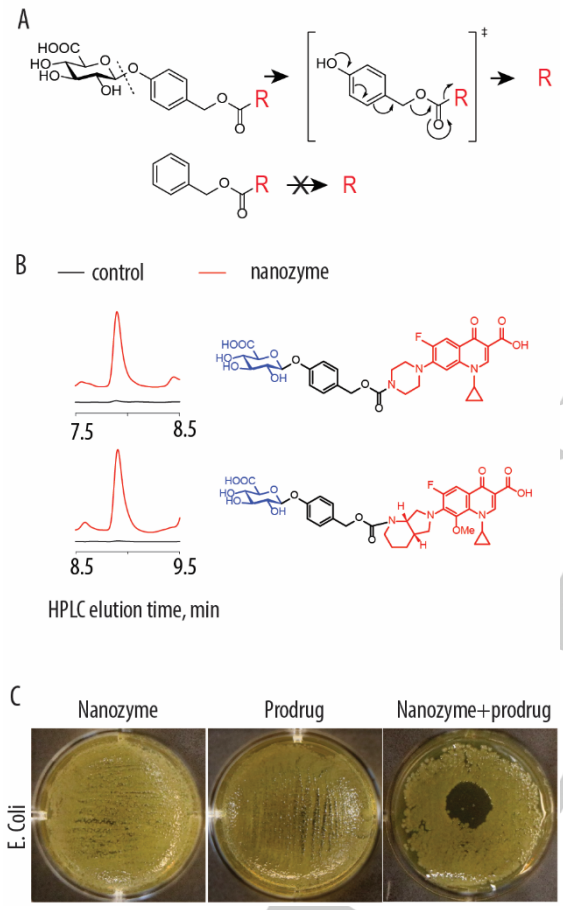

Figure 3. Antibacterial prodrugs and nanoengineered copper nanozymes afford strong antibacterial effect (zone of inhibition). (A) Proposed mechanism of drug release from the glucuronide prodrugs containing 4-hydroxybenzyl alcohol self immolative linker and ciprofloxacin/moxifloxacin as antibiotics, and the chemical structure of carboxybenzyl carbamate analogue that showed no release of antibiotics in the presence of the nanozymes. (B) HPLC traces illustrating release of antibiotics from the synthesized prodrugs through β-glucuronidase – like activity of the copper nanozyme at 100 μM prodrug concentration and 5 g L^{-1} Cu-nanozyme concentration in PBS, pH 7.4 after 24 h incubation; controls were performed under the same conditions in absence of the nanozyme, (C) "zone of inhibition" experiment revealing that nanozyme and the ciprofloxacin prodrug individually have no antibacterial effect against E.Coli. but together produce a strong zone of bacterial growth. Experimental conditions: 0.9 mg L^{-1} /1.5 μM ciprofloxacin glucuronide (corresponding to the 10x minimum inhibitory concentration of the corresponding drug against E.Coli) in tryptic soy broth, 24 h growth phase. For full details, see supporting information.

[14] While conversion of acyl glucuronides is encouraging, the latter are unstable and are prone to spontaneous decomposition.[12] We aimed to broaden the scope of nanozymes to non-acyl derivatives with inferior quality of the leaving group but with a higher stability in aqueous solutions. To achieve this, we used 4-hydroxybenzyl alcohol (PHBA) as a self-immolative linker between the glucuronic acid and the drug (Figure 3). This linker is typically used to enhance the accessibility of the scissile bond to the enzyme and is already found in marketed drugs (Brentuximab vedotin).[15] We envisioned that the phenolic nature of the leaving group coupled with an irreversible reaction character (Figure 3A) would make the prodrugs engineered around this linkage fit into the desired substrate scope for nanozymes. HPLC monitoring was used to validate the stability of the glucuronide prodrugs and their conversion by the nanoengineered Cu nanozymes, Figure 3B. Prodrugs revealed minor if any spontaneous decomposition over 24 h of incubation in absence of nanozyme (which is pivotal for applications in EPT). In contrast, nanoengineered Cu nanozymes as used in Figure 2B successfully converted synthesized glucuronides into the corresponding antibiotics – thus fully validating the proposed prodrug design. The carboxybenzyl carbamate analogues (lacking glucuronide group and the glycosidic linkage) showed no release of antibiotic in presence of the nanozyme (Figure S5) providing evidence that hydrolysis of the glucuronide prodrugs occurs at the anomeric position rather than the benzylic. In this regard, copper nanozymes are true mimics of the natural enzyme, glucuronidase.

We then performed the zone of bacterial growth inhibition (ZOI) assay as a cell culture test for the engineered catalytic nanoparticles and the glucuronide prodrugs as a nanozyme-mediated antibacterial measure (Figure 3C). Nanoengineered copper nanozymes, despite prior evidence of antibacterial activity of copper, revealed no ZOI and bacterial growth was non-inhibited. As expected, prodrugs on their own at the tested concentration (10×minimal inhibitory concentration of the corresponding drug, ciprofloxacin) also had no antibacterial effect. In contrast, a combination of the nanozyme and the prodrug achieved a pronounced antibacterial effect and afforded a significant ZOI of bacterial growth. We believe that this is the first example of nanozyme-mediated conversion of glucuronide prodrugs applied as "nanozyme-prodrug-therapy" to mediate antibacterial measures. It is important to note that prodrug design using the PHBA linker is modular: keeping the scissile bond between glucuronic acid and proximal end of the linker the same, it is possible to attach to the distal end virtually any drug with an amine or a hydroxyl functionality (thus being the overall majority of drugs on the market). [5]

Taken together, this work presents the identification of apparent pan-glycosidase activity in nanozymes, the realization of the substrate scope for nanoparticle-based biocatalysts, and the development of "nanozyme prodrug therapy" as an antibacterial treatment. These achievements became possible through a combination of successful selection of the nanozymes with a rational design of prodrugs. Current nanozymes, already highly powerful, provide mimicry to a narrow range of enzymes, mostly associated with the redox reactions. Our work goes well beyond the state of art, presents unique nanozymes and guidelines for

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the design of associated substrates, and in doing so opens up vast novel opportunities for nanozyme engineering and applications.

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Keywords: nanozyme • glucuronide • enzyme-prodrug therapy • prodrug • enzyme mimicry

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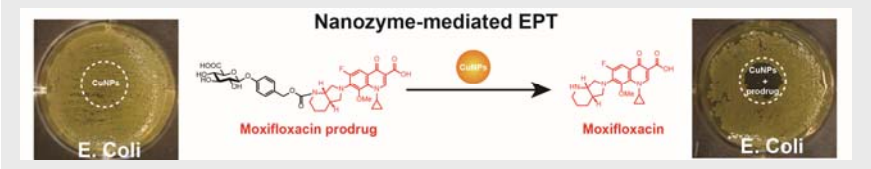

Unexpected apparent pan-enzymatic activity is discovered of non-proteinaceous materials. Identification of the substrate scope for these nanozymes led to the development of efficient mimics for β-glucuronidase that convert glucuronide prodrugs for localized synthesis of anti-inflammatory and anti-bacterial drugs.

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