Nanoparticles and Catalysis

Nanozymes: Gold-Nanoparticle-Based Transphosphorylation Catalysts**

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In his review on catalysis by colloid aggregates that appeared in this journal more than ten years ago, Menger wrote:[1] "…groups of molecules, properly assembled, can obviously accomplish much more than an equal number of molecules functioning separately". This observation is becoming more and more important as evidence is mounting that many biological systems interact and function through multiple simultaneous interactions.[2] On these bases a number of multivalent synthetic systems have been designed and studied for the recognition of simple molecules or more challenging biological targets. However, multivalent catalysts in which real cooperativity between the components is observed remain elusive, particularly in the case of self-assembling systems. For instance, most of the relevant rate accelerations often observed with aggregation colloids (such as, micelles and vesicles) appear to be related to concentration effects in the reaction loci (which include effects on the local pH value as well as on reactants and catalytic units) rather than to cooperativity.[3] Nevertheless, cooperativity is a rule in biological systems, such as enzymes. The reason for this lack of cooperativity in colloidal systems is largely entropic, and is related to the mobility of the constituent units (lipids/surfactants) that does not allow a catalytic site to last the time required for the catalyzed process to occur. Synthetic, functional polymers (synzymes)[4] allowed this problem to be solved, at least in part. Indeed cooperative catalysts based on these systems have been described.[5] However, a polymer presents other problems related to the difficulty in controlling its composition and conformation in solution.[6] Note that, as in the natural systems, not only the sequence of the building blocks (amino acids or any other) but also the conformation of the polymer are key requisites for catalysis. Alternatively,

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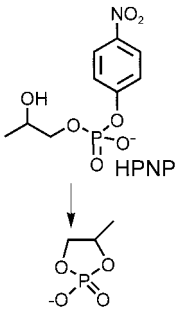
dendrimers functionalized on the periphery may constitute a suitable alternative for which. contrasting results have been reported.[7]

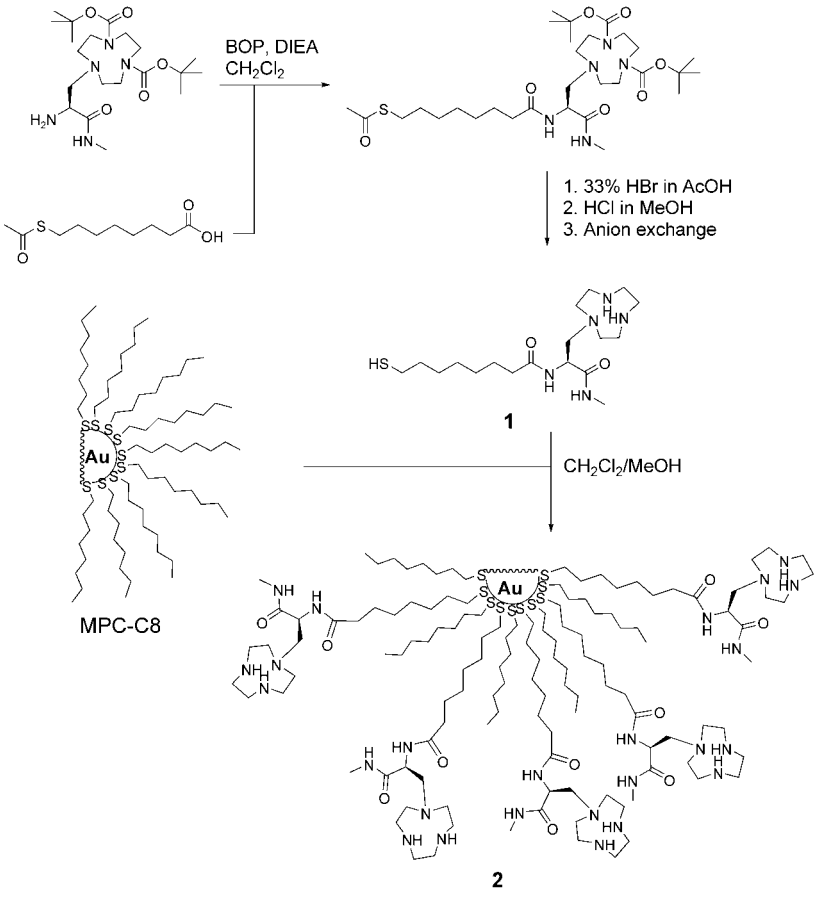
It occurred to us that by exploiting the well known ability of thiols to bind to gold nanoparticles we could have facile access to multivalent, functional systems[8,9] anchored on a support, and yet fully soluble, with limited mobility and conformationally constrained and, hence, suitable to act cooperatively in a catalytic process. To test this hypothesis we have chosen one of the most challenging reactions: the cleavage of the phosphate bond of phosphodiesters as a model of a RNase. Most of these enzymes require for their activity at least two metal ions that act cooperatively.[10]

Gold nanoparticles (MPC) protected by a monolayer of 1-sulfanyloctane (MPC-C8) were prepared by the procedure reported by Brust and co-workers[11] and optimized by Murray and co-workers.[12] They were subsequently subjected to site exchange[13] with the azacrown-functionalized thiol **1** to yield functional nanoparticles **2** (Scheme 1).[14] Proton NMR spectroscopic analysis of the monolayer composition of **2** revealed a 1:1.2 ratio of 1-sulfanyloctane and **1**, respectively. The core size of these ligand-functionalized MPC was 2.5 ± 0.7 nm as determined by transmission electron microscopy (TEM), see Supporting Information. Because of the presence of the triazacyclononane units, gold MPC **2** are expected to be able to bind transition-metal ions (such as, Cu^{II}, Zn^{II}) with high binding constants.[15] In the case of Cu^{II} the binding process can be followed spectrophotometrically at 648 nm, the maximum of the absorption band of the triazacyclonane–Cu^{II} complex. This property was used to determine the concentration of the ligand units (**1**) in stock solutions of **2**.

Transphosphorylation activity of **2** was tested with Zn^{II} ions because of the relevance of these ions in biological phosphate-cleavage catalysis.[10] A thorough analysis of the system was carried out by using 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP) as the substrate, an activated phosphate diester frequently used as a model of RNA.



With HPNP the release of *p*-nitrophenol (or *p*-nitrophenolate, depending on pH) is accompanied by the formation of a cyclic phosphate[16] and can be easily followed spectrophotometrically. Figure 1 shows the reactivity profile obtained by progressively adding Zn^{II} ions to a solution of **2** up to the saturation of the metal-ion binding subunits. This kinetic analysis reveals that a) the most active system is the one fully loaded with Zn^{II} ions, b) the sigmoidal profile of the curve supports cooperativity[18] between the metal centers because the catalytic efficiency becomes much higher after the first 30% of Zn^{II} ions is added. The plot also indicates that possible cooperativity between a metal ion and an ammonium ion[19] (owing to the presence of the uncomplexed and hence protonated azacrown) is less important than the cooperativity between Zn^{II} ions. The presence of such a contribution to the catalysis would have resulted in a bell-shaped profile, the maximum corresponding to a nanoparticle where Zn^{II} complexes and ammonium ions coexist in close proximity.



Scheme 1. Reaction scheme for the synthesis of ligand thiol **1**, and ligand-functionalized gold nanoparticles **2**; BOP = (1H-benzotriazol-1-olato-*O*)tris(*N*-methylmethanaminato)-phosphorus hexaflurophosphate, DIEA = N,N-diisopropylethylamine.

The real catalytic nature of the process was assessed by carrying out experiments with excess substrate. No formation of an intermediate was detected and first-order kinetics were observed up to the complete cleavage of all the substrate present. By varying the initial substrate concentration a kinetic profile of the reaction towards saturation was observed. These kinetics allowed the determination of the apparent Michaelis–Menten parameters $K_M = 0.93$ mM and $k_{cat} = 4.2 \times 10^{-3}\ s^{-1}$. Zinc(II)-nanoclusters **2** (Zn^{II}-**2**)[20] are, however, not selective in the binding of anions and, in fact, they also bind zwitterionic HEPES used as buffer for the experiments, which acts as an inhibitor of the catalytic process (see Supporting Information).[21]

The formal second-order rate constant for HPNP cleavage (k_{cat}/K_M) by Zn^{II}-**2** is $4.4\ s^{-1}M^{-1}$ which is more than 600-times

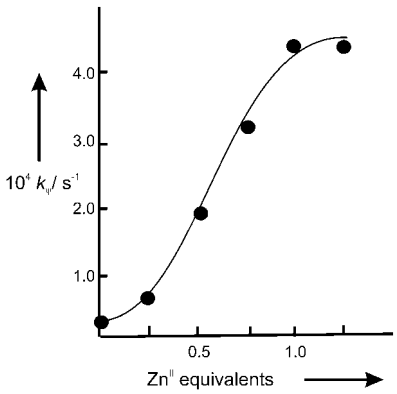


Figure 1. Dependence of the rate constant for the cleavage of HPNP by **2** on the amount of Zn^{II} ions. Conditions: 40 °C, pH 7.5 (5 mM *N*-(2-hydroxyethyl)-*N*′-(2-ethanesulphonyl)piperazine (HEPES) buffer), [**1**] = 0.1 mM.[17]

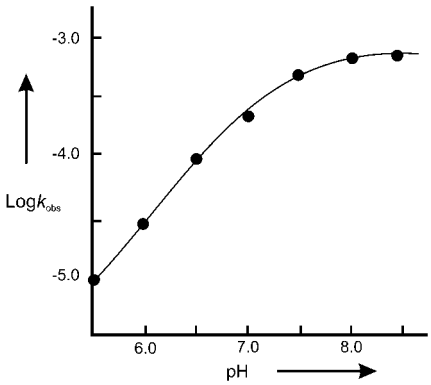


Figure 2. Dependence of the rate of cleavage of HPNP by **2**-Zn^{II} as a function of pH value. Conditions: 40 °C, [buffer] = 5 mM, [**1**-Zn^{II}] = 0.1 mM.

higher (see Table 1) than that determined under identical conditions for the mononuclear catalyst Zn^{II}-**3**, which corresponds to the "active unit" on the surface of MPC **2**.

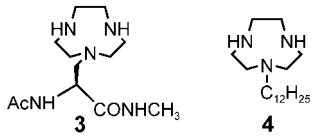


Table 1: Rate constants for the cleavage of HPNP by different Zn^{II}-based catalysts.

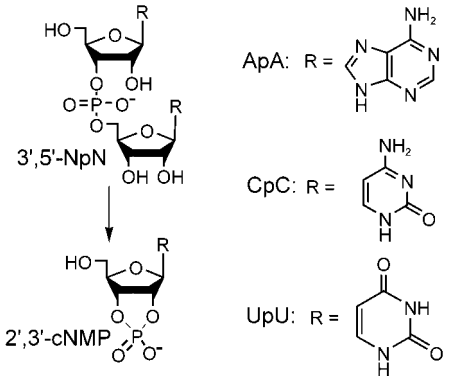
Catalyst	k_{cat} [10^{-4} s^{-1}]	k_2 [$M^{-1}s^{-1}$]	Relative rate[a]
Zn-**3**[b]	–	0.007	1
Zn-**2**[b,c]	42	4.4	629
Zn-**4**[b]	–	0.028	4
Calix[4]arene-2Zn[d,e]	7.7	43	3071
Calix[4]arene-3Zn[d,f]	24	2.9	138

[a] Relative second-order rate constant normalized for the number of metal centers present in the catalyst; [b] At pH 7.4 and 40 °C; [c] The activity of MPC-C8 (if any) could not be tested because of the insolubility of these nanoparticles in the aqueous environment; [d] At pH 7.0, 25 °C, and 50% aqueous CH_3CN; [e] Dinuclear Zn^{II} complex of 2,6-diaminomethylpyridine-functionalized calix[4]arene reported in ref. [25]; [f] Trinuclear Zn^{II} complex of 2,6-diaminomethylpyridine-functionalized calix[4]arene reported in ref. [25].

Micellar Zn^{II}-**4** scores slightly better than Zn^{II}-**3** but still the second-order rate constant determined for this catalytic system is approximately 160-fold lower than that of the nanoparticle-based catalyst. To study the origin of this impressive rate acceleration we have run kinetics at different pH values to assess the pK_a of the active nucleophile in the process (Figure 2). Available data from our[22] as well as from other[23] laboratories indicate that the close proximity of several metal centers induces the decrease of the pK_a of metal-ion-bound protic species with respect to monomeric complexes. The apparent pK_a value for the active nucleophilic species is 7.4 which is 0.4 units lower than that reported[24] for the Zn^{II} complex of triazacyclononane.

Thus part of the reactivity gain is due to a decrease of the pK_a value of the nucleophile. Allowing for this difference in pK_a value, catalyst Zn^{II}-**2** is still 380 times more effective than Zn^{II}-**3**. Indeed the k_{cat} of Zn^{II}-**2** is comparable to that of the best multinuclear Zn^{II} catalysts for HPNP cleavage reported so far (see Table 1).[25] These systems are based on calyx[4]-arene functionalized with two or three 2,6-diaminomethylpyridine units and are thus able to bind up to three Zn^{II} ions. They show a rate versus pH-value profile that goes through a maximum at approximately pH 7. This behavior indicates that cooperativity between the metal centers may be due to the occurrence of general-acid/general-base catalysis or nucleophilic catalysis and substrate binding.[26] In our case, on the contrary, the plot indicates that the role of the metal ions is in stabilizing the complexed substrate towards the transition state where a further negative charge develops and in facilitating deprotonation of the nucleophilic species.[22] Note that the efficiency of catalyst **2** was estimated on the basis of the total concentration of the active monomers, **1**, and not on that of the nanoparticles, in analogy to what is conventionally carried out with aggregation colloids. We do not know the number of Zn^{II} ions that actually take part in the catalytic process and that would define the catalytic site of the system (we estimate, on the basis of elemental analysis and particle size, the presence of about 45 thiol units **1** per nanoparticle). Accordingly, the reactivity reported is per single Zn^{II} complex and clearly underestimates the intrinsic reactivity of the active catalytic cluster. For comparison it is as if the reactivity of the calyx[4]arene systems were divided by two or three, that is, the number of metal centers present in the systems. In contrast with the nanoparticles, these latter systems are more efficient in the binding of the substrate because of the presence of the calyx[4]arene cavity.

With such an outstanding catalyst in hands we turned to more appealing substrates such as RNA dinucleotides (3′,5′-NpN), namely ApA, CpC, and UpU. Their uncatalyzed cleavage is extremely slow with rate constants (at pH 7) ranging from $9.8\times10^{-9}\,s^{-1}$ (UpU)[27] to $1.7\times10^{-9}\,s^{-1}$ (ApA),[28] that is, about two orders of magnitude less reactive



than HPNP. The cleavage process was followed by HPLC monitoring the disappearance of the substrates and the formation of cyclic ribonucleoside monophosphate (2′,3′-cNMP) and the corresponding nucleoside. As in the case of HPNP, the process is an intramolecular transesterification, in this case by the hydroxy group at the 2′-position of the ribose. At pH 7.5 (5 mM HEPES buffer) and 40 °C, Zn^{II}-**2** cleaves ApA, CpC, and UpU with second-order rate constants of $3.0\times10^{-4}\,s^{-1}M^{-1}$ (ApA), $3.6\times10^{-4}\,s^{-1}M^{-1}$ (CpC), $1.2\times10^{-2}\,s^{-1}M^{-1}$ (UpU).[29] Thus catalyst Zn^{II}-**2** is fairly active in the cleavage of RNA dinucleotides. The activity of Zn^{II}-**2** is, in this case too, subjected to inhibition by anions. We also observed relevant inhibition by the internal standard used for the HPLC analysis (sodium 3-nitrophenylsulphonate, see Supporting Information). The much higher activity observed with UpU may be related to a tighter binding of this substrate to the catalyst as suggested in the case of the above mentioned calyx[4]arene-based catalysts.[30] However, for UpU, Zn^{II}-**2** is less active the best calyx[4]arene-based catalyst.[31] Since UpU binding probably occurs by coordination to a Zn^{II} ion of the unprotonated amide of the base[32] it is possible that the geometry of binding of the substrate to the nanocluster is less appropriate than that obtained with the calyx[4]arene-based systems. These are just speculations and a more detailed analysis of the system is necessary.

In conclusion, we have shown that the self-assembly of ligand-functionalized thiols on gold nanoclusters provides a straightforward entry to a Zn^{II}-based catalyst that is extremely effective in the cleavage of phosphate diesters, such as HPNP and 3′,5′-NpN. In the case of HPNP, gold nanoclusters Zn^{II}-**2**, constitute one of the best Zn^{II}-based catalyst described to date. The facile synthesis of these systems and their outstanding catalytic properties induce us to call them "nanozymes" in analogy to the nomenclature of catalytic polymers (synzymes). Changing the nature of the functional units present on the gold-protecting monolayer may afford easy access to a variety of powerful, self-assembled catalysts (nanozymes) of which Zn^{II}-**2** is just a prototype.[33]

Experimental Section

1: 1,1-Dimethylethoxycarbonyl (Boc) diprotected ATANP methylamide[34] (152 mg, 0.35 mmol; ATAMP = (*S*)-2-amino-3-[1-(1,4,7-triazacyclononane]propionic acid) was treated with 8-acetylsulfanyloctanoic acid (91 mg, 0.36 mmol) under coupling conditions (BOP) in CH_2CH_2 to yield 97 mg of a oily product (43 %) after column chromatography (SiO_2, ethyl acetate:light petroleum 7:3). This material was treated with 33 % HBr in acetic acid (6 h, amine deprotection) and the precipitate obtained by addition of ethyl ether was treated with a solution of acetylchloride in methanol (24 h, thiol deprotection). The solid obtained was passed through an IRA-410 Amberlite resin (acetate) to give the diacetate salt of **1** in quantitative yield. ^{1}H NMR (250 MHz, D_2O): $\delta = 1.30$ (bm), 1.55 (bm), 2.17–2.31 (m), 2.76–2.98 (m), 4.65 ppm (m). ESI-MS, *m*/*z*: $[M+H]^+$, 387 $[M^+]$.

2: In a thermostated reactor kept at 28 °C MPC-C8 (26 mg), prepared according to Murray's procedure,[12] was dissolved in CH_2CH_2 (15 mL). Compound **1** (13 mg) dissolved in methanol (15 mL) was added to the solution which was then stirred for five days. Removal of the solvent and triturating the waxy solid with water gave a dark solution that was passed through a Sephadex G-50 resin eluting with water. Liophilization of the appropriate fractions gave 19 mg of MPC **2** whose authenticity was ascertained by ^{1}H NMR (300 MHz, D_2O) and IR (KBr) spectroscopy (see Supporting Information).

Kinetics: For the cleavage of HPNP, kinetic experiments were recorded by monitoring the absorbance of released *p*-nitrophenol (317 nm) or *p*-nitrophenate (400 nm) against the pH value of the solution with a Perkin-Elmer Lambda 16 instrument equipped with a thermostated cell holder. Rate constants were determined by interpolation of the absorbance versus time data using MicroMath Scientist version 2.01 software whenever the kinetics were followed to completion. For kinetics in the presence of excess substrate the initial-rate method was used monitoring at least 20 % product formation. Reproducibility within ± 15 % was observed in repeated runs. For the cleavage of ApA, CpC, and UpU the reaction was followed by HPLC by withdrawing 10 μL of the reaction solution which was mixed with 40 μL of a 10 mM solution of ethylenediaminetetraacetic acid (EDTA). Reaction vessels were carefully sterilized before use at 130 °C for 1 h. Separation conditions: column Alltech LiChrospher RP-18 (150 mm × 4.6 mm); eluent gradient (0–20 % of B in A; A = H_2O 0.075 % trifluoroacetic acid (TFA); B = 1:1 CH_3CN/H_2O 0.075 % TFA). For a typical chromatogram see the Supporting Information.

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