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ARTICLE TYPE

Guanidine Based Self-Assembled Monolayers on Au Nanoparticles as Artificial Phosphodiesterases

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Gold nanoparticles passivated with a long chain alkanethiol decorated with a phenoxyguanidine moiety were prepared and investigated as catalysts in the cleavage of the RNA model compound HPNP and diribonucleoside monophosphates. The catalytic efficiency and the high effective molarity value of the Au monolayer protected colloids points to a high level of cooperation between the catalytic groups.

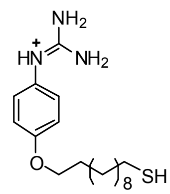
Introduction

The extreme biological relevance of phosphodiester bonds has challenged many research groups to design and synthesize artificial catalysts capable of cleaving DNA, RNA and their model compounds.[1-5] These artificial catalysts contain metal cations[1] or other functions as catalytically active components. Among them the guanidinium unit has a great importance as activating and/or anchoring group in hydrolytic reactions both in nature[6] and in artificial systems.[2-5,7]

In enzyme mimics an important role is played by the molecular scaffold that keeps the active functions at the proper distance as a result of a good compromise between preorganization and flexibility. The major issue in these multifunctional systems is the need to employ time-consuming multistep syntheses. This drawback can be overcome by relying on self-assembly of multivalent nanostructures. In particular, the self-assembly of catalytic monolayers on the surface of gold nanoparticles (Au NPs) to give gold monolayer-protected clusters (Au MPCs) is an emerging and attractive strategy.[8-11] In a recent series of seminal papers Au NPs passivated with thiols featuring catalytic moieties have been reported as catalysts for the cleavage carboxylic esters[12] and phosphoric diesters.[13]

In recent studies, we reported the synthesis and catalytic activity of compounds functionalized with two or more guanidine units.[4,5,7] It was shown that a prerequisite for catalysis is the simultaneous presence, on the same molecular framework, of a neutral guanidine acting as a general base, and a protonated guanidine acting as electrophilic/electrostatic activator. These systems turned out to be highly efficient in the cleavage of ATP[7] and of the RNA model compound 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP).[4,5] These results suggest the possibility to employ the guanidinium unit as active component in catalytic Au MPCs.

In this paper we describe the preparation of gold nanoparticles passivated with varying proportions of the catalytic active thiol **1** and inert thiol **2**, together with the results of a kinetic investigation of their catalytic activity in the cleavage of HPNP and diribonucleoside monophosphates.



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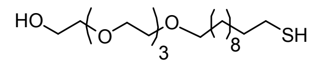

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Results and Discussion

The synthesis of thiol **1** was carried out according to Scheme 1S (ESI). The preparation of thiol monolayers on gold nanoparticles was carried out according to literature protocols.[13d,14] The procedure consisted in the preparation of Au NPs transiently stabilized with secondary amines featuring long alkyl chains. In a subsequent step the amines were replaced under mild conditions with the desired mixture of thiols (see ESI for further details). This two-step protocol offers an important advantage over other preparative protocols,[12a,16] in that only the minimal amount of thiol necessary to cover the Au NPs needs to be added. Furthermore the composition of mixed monolayers neatly reflects the composition of the mixture of thiols added with no homodomain formation.[13d]

The series of Au NPs listed in Table 1 was prepared. To ensure the same gold core size for all the nanoparticles the initial batch of amine-stabilized NPs was split into four batches to which thiols **1** and **2** were added in the mole fractions reported in Table 1. The average diameter of the gold core was determined as 1.8 ± 0.2 nm by means of High Resolution TEM (Fig. 7S, ESI). The absence of the band around 520 nm in the UV-Vis spectrum confirms that the NPs size is lower than 2 nm (Fig. 2S).[13d] The ^{1}H-NMR spectra of Au NPs **I-IV**, as well as DOSY spectra (p. 15S-18S) showed no trace of unbound additives, thus confirming that the thiols are fully bound to the metal core. The weight fractions of organic monolayer and gold core were assessed by thermogravimetric analysis (TGA, Fig. 3S-6S). Combination of TGA data and with potentiometric titrations (Fig. 1) afforded mole fractions x_1 in fair agreement with the expected values (Table 1).

Table 1. Monolayer Composition and Acidity Constants of Au NPs **I-IV**

NP batch	x_1 expected [a]	x_1 [b]	pK [c]	n_1 (μmol) [d]
I	1.00		10.21 ± 0.09	12.1
II	0.67	0.61	10.63 ± 0.08	8.3
III	0.33	0.31	10.79 ± 0.08	4.5
IV	0.00		-	-

[a]mole fraction of thiol **1** expected on the basis of the thiol ratio used in the preparation; x_2= 1-x_1. [b] determined by a combination of potentiometric titrations and TGA (p. 9S, ESI). [c] apparent pK data from potentiometric titrations reported in Fig. 1. Reported errors are standard deviations σ. [d] μmoles of thiol **1** present in 10 mg of NPs determined by potentiometric titrations (Fig. 1).

Determination of the acid-base properties of Au MPC **I-III** is a prerequisite for a meaningful investigation of their catalytic properties. A mixture of DMSO:H_2O 80:20 (v/v), hereafter referred to as 80% DMSO, was used as solvent in titration experiments. This mixture is well known to be suitable for potentiometric measurements[17] and for the investigation of phosphoryl transfer reactions.[3-5,7] The pK_w for water autoprotolysis in 80% DMSO rises to 18.4,[17] and this implies that the pH value of a neutral solution is 9.2. Solutions of Au NPs were potentiometrically titrated with a standard solution of Me_4NOH in 80% DMSO in the presence of 10 mM Me_4NClO_4. Analysis of the titration plots (Fig. 1) afforded the apparent pK values listed in Table 1.

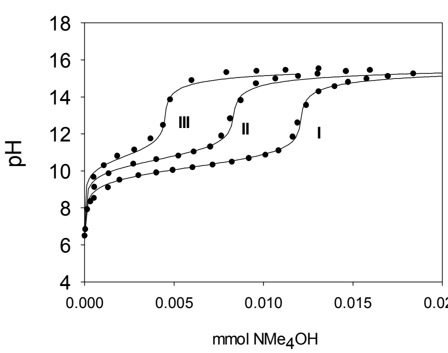


Figure 1 Titrations of Au NPs **I-III** (10 mg in 5.0 mL) with Me_4NOH in 80% DMSO, 25 °C, in the presence of 10 mM NMe_4ClO_4.

The pK values decrease upon increasing the mole fraction of x_1, the thiol provided with the phenoxylguanidinium moiety. Moreover these values are significantly lower than 11.5, the pK of the model compound N-(4-methoxyphenyl)guanidinium measured in the same solvent mixture.[4] This evidence is most probably ascribable to the repulsion of the charged units in the monolayer that facilitates the departure of a proton from a neighbouring phenylguanidinium group, as expected from electrostatic considerations.

The catalytic efficiency of Au MPC **I** was systematically investigated over a wide pH range. Partial neutralization of 200 μg/mL solutions of nanoparticles with calculated amounts of Me_4NOH afforded buffer solutions with pH values in the range of around 8−12, which were used for catalytic rate measurements. Pseudo-first-order rate constants (k_{obs}) for the transesterification of HPNP, corrected for background contributions[4] whenever appropriate (pH>11), are reported in Fig. 2. The bell-shaped pH−rate profile indicates that the maximum catalytic activity of Au MPC **I** is achieved around pH 10.2. At that pH, according to the pK value in Table 1, the same amounts of guanidinium and guanidine

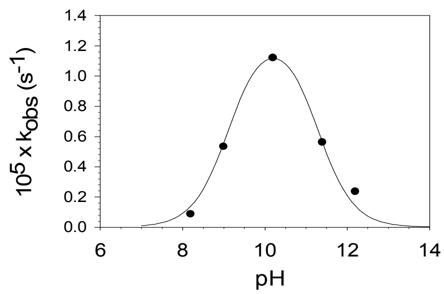


Figure 2 pH−rate profile for the cleavage of 0.10 mM HPNP catalyzed by 200 μg/mL Au MPC **I** in 80% DMSO, 25.0 °C, 10 mM Me_4NClO_4. The rate constants measured at pH > 11 were corrected for background hydrolysis (see ref 4 and note c in Table 2).

The activity of batch **I** was also investigated at different concentrations of nanoparticles at pH 10.21. The results of the kinetic experiments are graphically shown in Fig. 3 as plot of pseudo-first-order rate constants (k_{obs}, s^{-1}) for the spectrophotometrically determined liberation of p-nitrophenol versus Au NPs concentration (0-250 μg/mL). Data points could be fitted to straight line with zero intercept, showing that (i) the catalytic system works under subsaturating conditions, i.e., binding of HPNP to the catalyst is too low to affect the kinetics in the investigated concentration range, and (ii) contribution from background hydrolysis to the overall rate is, as expected,[4] negligibly small. From the slope of the straight line in Fig. 3 the following value of second-order rate constant was calculated: k_2=(4.70± 0.13)×10^{-2} s^{-1} M^{-1}.

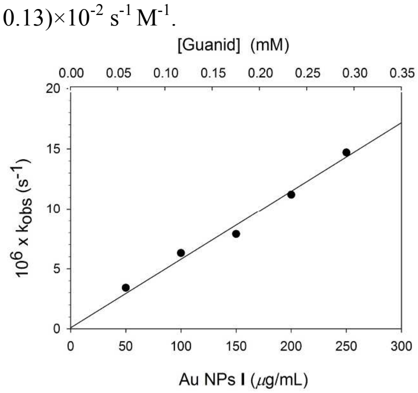


Figure 3 Plot of pseudo-first-order rate constants k_{obs} for the liberation of p-nitrophenol from 0.10 mM HPNP catalyzed by Au MPC **I** (80% DMSO, 25 °C, pH 10.21, 10 mM Me_4NClO_4) versus NPs concentration and versus guanidinium concentration (top scale).

The catalytic activity of Au NP **II** and **III** was also investigated. Solutions of Au NPs (200 μg/mL) were half-neutralized with calculated amounts of Me_4NOH, affording buffer solutions with pH values indicated in Table 2. At these pH values equal amounts of guanidine and guanidinium units are present in the monolayer.

Table 2. Transesterification of HPNP Catalyzed by Au MPCs **I-IV** (200 μg/mL) in 80% DMSO at 25.0 °C. [a]

entry	NPs batch	Guanidine/ium conc. (mM)	pH [b]	$10^7 \times k_{obs}$ (s^{-1})	k_{rel} [c]
1	**I**	0.242	10.21	112	110
2	**II**	0.166	10.63	72.9	27
3	**III**	0.090	10.79	19.2	4.9
4 [d]	**IV**	-	10.21	0.88	0.86
5 [d]	**IV**	-	10.79	4.20	1.1

[a] Pseudo-first-order specific rates k_{obs} calculated as v_o/[HPNP], where v_o is the spectrophotometrically determined initial rate of *p*-nitrophenol liberation in 0.1 mM HPNP solutions, 10 mM Me_4NClO_4. Error limit: ±10%. [b] Error limit of pH measurements ±0.04 [c] $k_{rel} = k_{obs}/k_{bg}$; the rate constant (k_{bg}, s^{-1}) for the hydroxide-catalyzed reaction as a function of pH is given by the following expression: $k_{bg} = 10^{(pH-17.2)}$ (see ref 4). [d] in the presence of 1 mM phosphate buffer.

Pseudo-first-order rate constants (k_{obs}) for the transesterification of HPNP are reported in Table 2. Au MPCs **I** is the most efficient system in the cleavage of HPNP (entry 1), with an acceleration over two orders of magnitude compared to the background HPNP transesterification at the same pH due to hydroxide catalysis.[4]
The reactivity of batches **II** and **III** is significantly lower compared to that of **I** due to the lower number of guanidine/ium units and to the lower probability for the catalytic groups to cooperate, as clearly indicated by the sigmoid-shape dependence of k_{obs} on the mole fraction of ligand **1** (Fig. 4). The activity Au NPs **IV**, passivated with the bare inert ligand **2**, was also tested in control experiments at two different pH values (entries 4 and 5, Table 2), showing no advantage over the background reaction at the same pH.

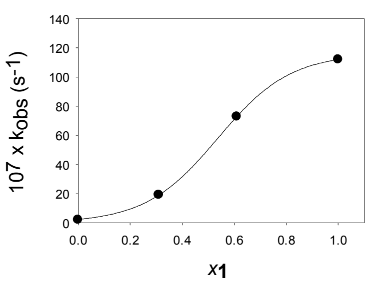
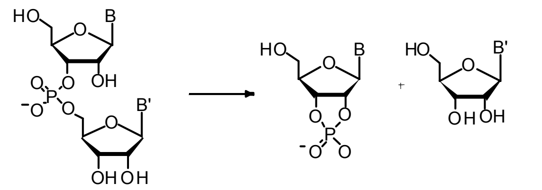


Figure 4 k_{obs} from Table 2 versus x_1, the mole fraction of thiol **1** in the monolayer of Au NP.

Since conclusions drawn from the cleavage of activated phosphodiesters do not necessarily apply to the cleavage of unactivated phoshodiesters,[18] it seemed worthwhile to investigate the catalytic activity of the most active batch AuNP **I** in the transesterification of three diribonucleoside 3',5'-monophosphates *NpN'*, eq. (1), as more appropriate RNA models.



NpN' *2',3'-Ncp* *N'* (1)

Catalytic runs were carried out under the same conditions used for the cleavage of HPNP, namely, pH 10.4, 10 mM Me_4NClO_4, 80% DMSO. The sole differences are the higher temperature, 50 °C rather than 25 °C, and the higher nanoparticle concentration (2.0 mg/mL), dictated by the slower reactivity of diribonucleoside monophosphates compared to HPNP.
The kinetics were monitored by HPLC analysis of aliquots of the reaction mixture withdrawn at time intervals in the early stages of the reaction, as previously described.[1f] Initial rates of nucleoside *N'* formation were translated into pseudo first order specific rates k_{obs} reported in Table 3.

Table 3 Cleavage of diribonucleoside 3',5'-monophosphates *NpN'* in the presence of AuNPs **I**.[a,b]

entry	*NpN'*	$10^6 \times k_{obs}$ (s^{-1})	$10^{10} \times k_{bg}$ (s^{-1})	k_{rel} [c]
1	UpU	26	5.1	5.1×10^4
2	GpU	17	5.6 [d]	3.0×10^4
3	CpA	1.1	3.6 [d]	3.0×10^3

[a] 2.0 mg/mL of AuNPs **I**, [guanidine/ium]= 2.4 mM; 0.10 mM *NpN'*, 10 mM Me_4NClO_4; 80% DMSO, pH 10.2, 50.0 °C. [b] Pseudo-first order specific rates k_{obs} calculated from initial rates of HPLC monitored nucleoside liberation. Error limits on the order of ±10%. [c] $k_{rel} = k_{obs}/k_{bg}$. [d] calculated from data in ref 19.

Table 3 shows that Au MPC **I** effectively cleaves the three investigated substrates, with a marked preference for UpU and GpU (entries 1–2).
In order to compare the catalytic efficiency of AuNPs **I** in the cleavage of diribonucleoside monophosphates *vs*. HPNP, catalytic rates relative to background (k_{obs}/k_{bg}) are required. Initial rates of the hydroxide catalyzed cleavage of CpA, GpU[19] and UpU, measured in the presence of 1.0 mM Me_4NOH (pH 15.4), gave k_{bg} values at that pH that were extrapolated to pH 10.2 under the assumption that the reaction is specific base catalyzed, on the analogy of the corresponding reaction of HPNP, that was found to be strictly first order in hydroxide concentration in the pH range 9.3–13.0.[4] The close similarity of k_{bg} values measured for CpA, GpU and UpU is consistent with the fact that rates of background cleavage of the phosphodiester bond of diribonucleoside monophosphates are affected by nucleobase identity to a moderate extent.[18a,20]
The results listed in Table 3 show that Au NPs **I** exhibit high acceleration (k_{rel} from 3 to 4 orders of magnitude compared to background) in the cleavage of diribonucleoside monophosphates. Therefore the nanoparticles are much more effective in the cleavage of diribonucleosides than in the cleavage of HPNP. Thus, replacement of a good leaving group with a bad leaving group has a favorable effect on catalytic efficiency.

Both an associative two step (A_N+D_N) mechanism and a concerted (A_ND_N) mechanism are likely possibilities for the hydrolysis of phosphate diesters.[1n,21,22] When the leaving group is poor the question of mechanism is still under debate, but there is little doubt that upon replacement of a good leaving group with a poor one the transition state becomes tighter, *i.e.* more associative in character and, consequently, bears a close resemblance to a pentavalent phosphorane dianion. Accordingly, the larger rate enhancements experienced by the reactions of diribonucleosides are understood as arising from a stronger electrophilic/electrostatic stabilization of the transition state by the guanidinium units of the multifuntional Au MPC.

Conclusions

To sum up, the kinetics confirm that in Au MPC **I**, in agreement with previous conclusions,[4,5,7] bifunctional catalysis arises from the combined action of a neutral guanidine acting as a general base and a protonated guanidine acting as a general acid, as in the mechanism schematically depicted in Fig. 5.

The ratio k_2/k_{inter} =4.7 M, (k_{inter} = 1.0×10^{-2} $M^{-2}s^{-1}$, measured with the model compound *N*-(4-methoxyphenyl)guanidine/ium in the same conditions[4]) is the effective molarity (EM) of the system that provides a measure of the high degree of synergism of the catalytic units in the stabilization of the transition state of the reaction. This value rivals the EMs of the most efficient guanidino-phosphodiesterases based on calix[4]arene[4] and diphenylmethane[5] scaffolds previously reported by us. Considering the gold core as a rigid bond, the cyclic structure in the transition state (Fig. 5) can be compared with the ring closure of a large strainless ring. The EM values expected for rings of comparable size is 0.02-0.05 M,[23] namely two orders of magnitude lower than that of Au NP **I**.

These considerations point to the existence of a high level of preorganization in the monolayer due to a reduced conformational mobility of the alkyl chains compared to the situation in solution.

Au MPC **I** were also tested in the cleavage of three diribonucleoside monophosphates showing a marked selectivity for two of them and remarkable acceleration compared to the spontaneous cleavage at the same pH.

The results presented here open the possibility to extensively employ the guanidinium unit, possibly combined with other active units, in Au MPC and other nanostructures to fabricate catalytic systems active in the cleavage of RNA oligomers and other phosphodiesters.

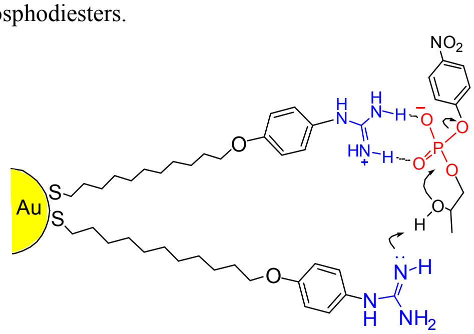


Figure 5 Mechanism of HPNP cleavage catalyzed by Au NPs **I**.

Experimental Section

Materials HPNP[24] and compound **2** [25] were prepared as reported in the literature. The synthesis of compound **1** and the procedure followed for the nanoparticle preparation are fully described in the Electronic Supplementary Information

Potentiometric titrations. Potentiometric titrations were performed by an automatic titrator equipped with a combined microglass pH electrode. Experimental details and procedure for the electrode calibration were the same as previously reported.[4] Potentiometric titrations were carried under nitrogen atmosphere, on 6 mL solutions prepared dissolving 10 mg of AuNP in 80% DMSO, in the presence of 10 mM M Me_4NClO_4, (80% DMSO, 25 °C). A 50 mM Me_4NOH solution in 80% DMSO was added to the titration vessel in small increments. Analysis of titration plots was carried out by the program HYPERQUAD 2000.[26]

UV-Vis Measurements. Kinetic measurements of HPNP transesterification were carried out by UV–vis monitoring of *p*-nitrophenol liberation at 400 nm on either a double beam or on a diode array spectrophotometer. Calculated amounts of Me_4NOH were added to the reaction mixture and the pH of the solution was checked before and after the before kinetic runs. Rate constants were obtained by an initial rate method, error limits on the order of ±10%.

Cleavage of diribonucleoside 3',5'-monophosphates *NpN'* was monitored by HPLC analyses of aliquots of the reaction mixture withdrawn at appropriate time intervals. Reactions were carried out at 50.0 °C, pH 10.2, on 0.10 mM *NpN'*, and 2 mg/mL Au NPs **I** solutions in 80% DMSO, 10 mM Me_4NClO_4. The pH of the solution was measured by a microglass pH electrode. Experimental details and procedures for the electrode calibration were as previously reported.[4] In a typical experiment, the mixture was added with a solution of Me_4NOH in 80% DMSO until pH 10.2 was reached. The mixture was thermostated at 50.0 °C for 30 min and the reaction was started by addition of a calculated small volume of a 5.0 mM solution of *NpN'* in water. At proper time intervals, aliquots (80 μL) of the reaction mixture were withdrawn and quenched with 80 μL of a 10 mM solution of $HClO_4$ in 80% DMSO. After addition of *p*-hydroxybenzoic acid (internal standard) in 80% DMSO, the solution was filtered and subjected to HPLC analysis by elution with H_2O (0.1% trifluoroacetic acid)/MeCN, linear gradient from 100:0 to 85:15 in 25 min, flow 0.9 mL/min. The pseudo-first-order rate constant for the hydroxide catalyzed cleavage of UpU was measured at 50.0 °C in the presence of 1.0 mM Me_4NOH (pOH 3.0), 10 mM Me_4NClO_4, by HPLC monitoring of the nucleoside liberation (initial rate method).

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Notes and references

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Electronic Supplementary Information (ESI) available: experimental details, spectra and Fig. 1S-7S. See DOI: 10.1039/c000000x/

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