

Exclusive recognition of sarcosine in water and urine by a cavitand-functionalized silicon surface

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A supramolecular approach for the specific detection of sarcosine, recently linked to the occurrence of aggressive prostate cancer forms, has been developed. A hybrid active surface was prepared by the covalent anchoring on Si substrates of a tetraphosphonate cavitand as supramolecular receptor and it was proven able to recognize sarcosine from its nonmethylated precursor, glycine, in water and urine. The entire complexation process has been investigated in the solid state, in solution, and at the solid–liquid interface to determine and weight all the factors responsible of the observed specificity. The final outcome is a Si-based active surface capable of binding exclusively sarcosine. The complete selectivity of the cavitand-decorated surface under these stringent conditions represents a critical step forward in the use of these materials for the specific detection of sarcosine and related metabolites in biological fluids.

molecular recognition | phosphonate cavitand

Early stage detection of aggressive prostate cancer has been recently linked to the presence of sarcosine in urine (1). Sarcosine forms when the enzyme glycine-*N*-methyltransferase transfers a methyl group from *S*-adenosylmethionine to glycine. Given that sarcosine is one of the cancer biomarker candidates (2, 3), effective diagnostic tools for the detection of sarcosine directly in urine are highly desirable. The basic methodology for sarcosine determination is gas chromatography–mass spectroscopy of volatile derivatives (4) which, although highly sensitive and reliable, is hardly applicable for a widespread screening of this pathology. A different approach for sarcosine determination consists in the use of a fluorometric assay (BioVision Research Products). However, this approach requires various reaction steps and it is prone to interference due to unspecific reactions with other (unknown) urinary analytes (2), making it unsuitable for sarcosine measurements directly in the urine. A diffuse screening of prostate cancer requires easy and fast methodologies which minimize sample manipulations, number of reagents, and costs. An important step in this direction could be the development of an interactive surface akin to DNA chips, able to perform the recognition process directly in biological fluids.

In chemical terms, the preparation of a sarcosine detection chip requires (i) the design of a receptor capable of binding exclusively *N*-methylated amino acids in the presence of overwhelming amounts of amino acids plus many other metabolites in urine, and (ii) the grafting of this receptor on a suitable solid surface, retaining the molecular recognition properties at the solid–liquid interface.

Cavitands, defined as concave organic molecules capable of molecular recognition (5), are particularly versatile synthetic receptors, whose complexation properties depend on size, shape, and functionality of the preorganized cavity (6–8). Among them, tetraphosphonate cavitands are outstanding: Their complexation ability spans from positively charged inorganic and organic species (9) to neutral molecules (10). This diverse complexation abil-

ity is the result of three interaction modes, which can be activated either individually or in combination by the host according to the guest requirements: (i) multiple ion–dipole interactions between the inward facing P=O groups and the positively charged guests (11); (ii) single or dual H bonding involving the P=O groups (11, 12); and (iii) CH– π interactions between a methyl group present on the guest and the cavity of the host (13).

The complexation properties of tetraphosphonate cavitands anchored on silicon surface toward *N*-methyl pyridinium and *N*-methyl ammonium salts in organic solvents have been recently investigated (14). Tetraphosphonate cavitand ability to distinguish between amino acids and their *N*-methylated analogues remains to be explored, as well as the possibility to transfer their complexation properties to aqueous and biological environments.

In the present work, we report a comprehensive investigation of the molecular recognition properties of a silicon surface decorated with phosphonate cavitands (Fig. 1) toward glycine and sarcosine in water and urine. The entire complexation process has been investigated in the solid state, in solution, and at the solid–liquid interface to determine and weight all the factors responsible for the observed specificity. The final outcome is a Si-based active surface capable of binding exclusively sarcosine and other *N*-methylated amino acids in water and urine.

Results and Discussion

Sarcosine Complexation in the Solid State. At first, the crystal structures of the complexes formed by tetraphosphonate cavitand Tiiii[C₃H₇,CH₃,Ph] (15), from now onward referred to as Tiiii (16), with glycine methyl ester and sarcosine hydrochlorides were solved to define and compare type, number, and geometry of host–guest interactions present in the solid state in the two cases. Suitable crystals of both complexes were obtained under the same conditions (i.e., via slow evaporation of a methanol/water solution containing the host in the presence of an excess of guest). The complex Tiiii•methanol•glycine methyl ester hydrochloride (the use of glycine hydrochloride led to nondiffracting crystals) features a molecule of methanol into the cavity and the protonated amino acid methyl ester perching on top of the cavity (Fig. 24). The affinity of this class of cavitand toward methanol

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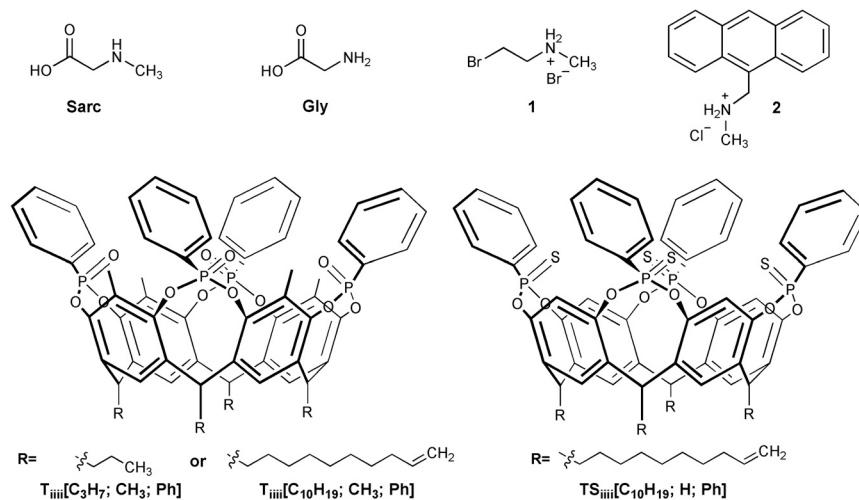


Fig. 1. Chemical structure of the guests and cavitands. The short chain footed $T_{iiiiii}[C_3H_7, CH_3, Ph]$ cavitant was used for solid state and solution experiments. The double bond-terminated long-chain footed $T_{iiiiii}[C_{10}H_{19}, CH_3, Ph]$ and $TS_{iiiiii}[C_{10}H_{19}, H, Ph]$ cavitands were grafted on silicon wafers.

has been previously reported (13), and also in this case the alcohol is stabilized within the cavity by a hydrogen bond with one P=O group at the upper rim and by two CH- π interactions between two methyl hydrogens of the guest and two aromatic rings of the host (13). The results indicate that methanol is preferred by the cavitant over glycine methyl ester hydrochloride; the latter could be expected to interact through dipolar interactions between the P=O groups and the positively charged nitrogen atom. But, although the methanol can exploit the synergistic effect of both CH- π interactions and hydrogen bonding, this is not the case for glycine methyl ester hydrochloride, whose interaction with the cavity is mediated by the solvent. The NH_3^+ group of the amino acid forms a network of hydrogen bonds both with the methanol hosted inside the cavity and with the three lattice water molecules (*SI Appendix, Fig. S1*).

The situation is completely different for the complex T_{iiiiii} •sarcosine hydrochloride (Fig. 2B): In this case, all three interaction modes with the guest described in the Introduction are activated. Sarcosine enters the cavity with its methyl group forming two CH- π interactions with two aromatic rings of the host. The complex is further stabilized by two hydrogen bonds involving the positively charged NH_2 moiety and two adjacent P=O groups. Methanol does not interact with the cavity even if it is present in the crystal lattice. The chloride ion is located among the four alkyl chains at the lower rim of the cavitant, separated by a dis-

tance of 7.136(4) Å from the positive nitrogen atom, forming C-H...Cl⁻ interactions with the four α CH₂ residues. For a detailed description of all geometrical parameters of the complexes see the *SI Appendix*.

The different behavior of the two guests toward the cavitant can be attributed to the presence of the methyl residue on the nitrogen in the sarcosine. Its CH₃- π interaction with the cavity triggers the formation of the two H bonds and the setting of cation-dipole interactions, which further stabilize the complex. Therefore, sarcosine is preferred over methanol for cavity inclusion.

Density Functional Theory (DFT) calculations (see *SI Appendix* for computational details) have been performed to estimate the energetic differences between T_{iiiiii} •sarcosine and T_{iiiiii} •glycine complexes because the theoretical model allows the exclusion of the effects due to the presence of specific solvents. The stabilization introduced by the additional CH₃- π interaction has been evaluated in 3.8 kcal mol⁻¹. In both cases, the formation of H bonds between charged NH₂ groups and P=O apical fragments is pointed out by the elongations of the P=O bonds with respect to the noninteracting T_{iiiiii} ($\Delta = +0.02$ Å) and the parallel elongation of the N-H bond with respect to the noninteracting guest ($\Delta = +0.02$ Å). Regarding the T_{iiiiii} •sarcosine adduct, the distance between the C atom of the sarcosine N-methyl group and one benzene centroid ($Ar_{centroid}$) of the cavitant (3.65 Å) as well as the C-H... $Ar_{centroid}$ angle (138.5°) are compatible with a CH- π inter-

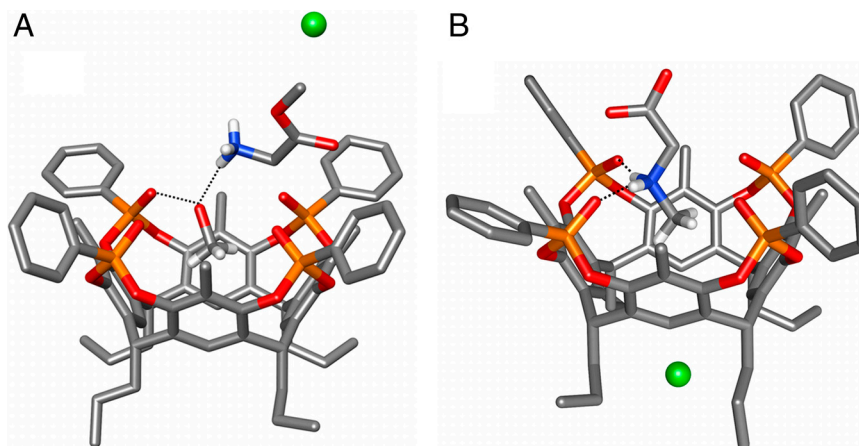


Fig. 2. Crystal structures of complexes T_{iiiiii} •methanol•glycine methyl ester hydrochloride (A) and T_{iiiiii} •sarcosine hydrochloride (B). C, gray; O, red; P, orange; N, blue; Cl, green; H, white; H bonds, black dotted lines. For clarity, the H atoms of the cavitant and those not involved in complexation of the guests have been omitted.

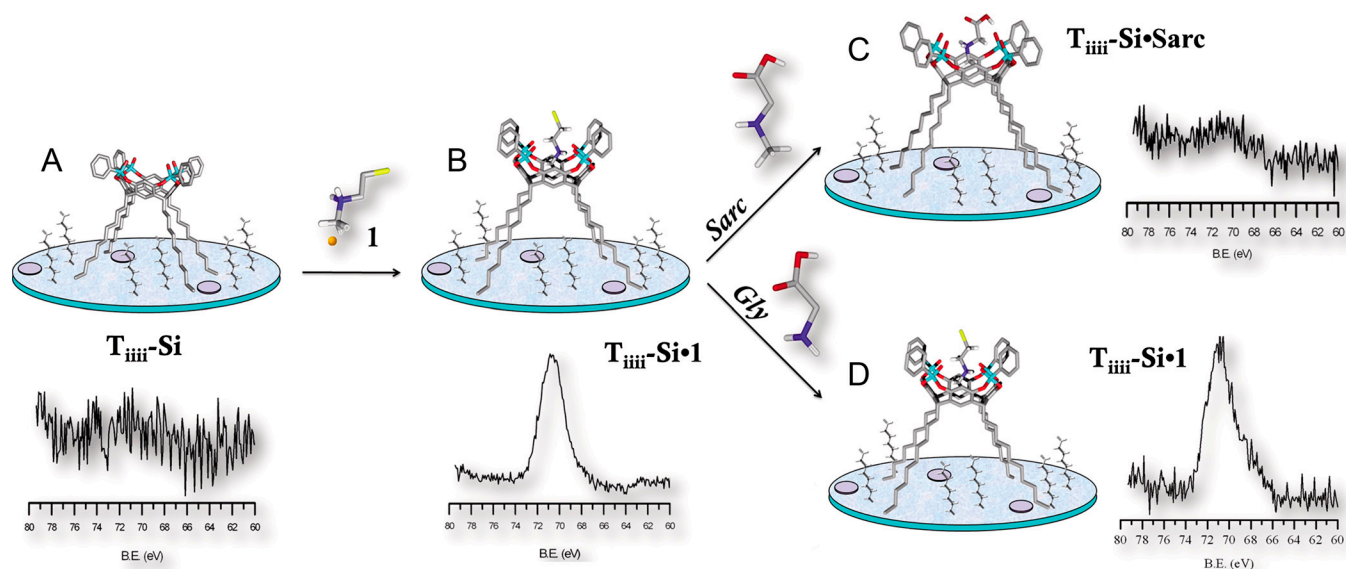


Fig. 4. XPS analysis of Br 3D region along all steps of the sarcosine recognition protocol in water. (A) pristine Tiiii-Si wafer and its XPS spectrum. (B) Tiiii-Si•1 and its XPS spectrum after exposure of the wafer to a water solution of **1**. (C) Tiiii-Si•Sarc and its XPS spectrum after exposure of the wafer to a water solution of sarcosine. (D) Tiiii-Si•1 and its XPS spectrum after exposure of the wafer to a water solution of glycine.

decomplexation can be rationalized by recalling two surface effects: (i) the apparent pK_a of surface groups (22), and in particular of surface bound amines ($pK_a \sim 4$) (23) are much lower than their intrinsic value in solution ($pK_a \sim 10$); (ii) guest deprotonation, decomplexation, and diffusion into the bulk solution are not balanced by the reverse reaction because the guest concentration in the solution resulting from surface decomplexation is negligible.

Then, the exchange reaction between sarcosine/glycine and Tiiii-Si•1 was monitored by XPS to test the selectivity of Tiiii-Si surface toward *N*-methylated amino acids. Two Tiiii-Si•1 wafers were exposed to water solutions of sarcosine and glycine, respectively. Both solutions were at the same concentration (1 mM) and pH (0.7). XPS analyses performed on the two surfaces after the treatment showed that the Br signal disappeared only in the wafer dipped in the sarcosine solution (Fig. 4C). Sarcosine completely replaced guest **1** on the Tiiii-Si surface, whereas glycine was totally ineffective (Fig. 4D). Therefore, the behavior of Tiiii at the silicon–water interface reflects exactly its conduct at the organic–water interface.

Sarcosine Detection in Urine. The procedure based on the surface exchange reaction was adopted to identify sarcosine directly in urine. A human urine sample was filtered to remove traces of proteins which can clog the surface, then acidified to pH 0.7 and divided into two portions. Sarcosine was added to one of the two portions to simulate its biological occurrence due to prostate cancer (1 mM). The same procedure was repeated adding sarcosine before filtration to mimic the real sampling conditions. Sarcosine concentration before and after filtration remained unchanged as proven by GC-MS analyses (4) (SI Appendix, Fig. S15). Tiiii-Si•1 wafers were exposed to the two urine samples

and analyzed via XPS to verify the presence of complexed guest **1** on the surface through its diagnostic Br 3D signal (Fig. 5).

XPS measurements showed no differences if the sarcosine was added before or after the work-up. The sarcosine-laced urine displaced **1** from the surface, whereas the untreated one did not. None of the potential ionic interferents present in urine (protonated amino acids, NH_4^+ , Na^+ , K^+ , Mg^{2+} , and Ca^{2+} , just to mention the major ones) has sufficient affinity for the cavity to replace *N*-methyl ammonium salts. The resulting exquisite selectivity demonstrated by Tiiii-Si is unprecedented for a synthetic receptor operating at interfaces.

A fluorescence-based detection mode was then developed to prove the potential of Tiiii-Si as active surface for sarcosine detection with optical devices. Sarcosine presence in urine was monitored via fluorescence dye displacement (24), using (9-anthrylmethyl)methyl ammonium chloride **2** as indicator dye (25). Guest **2** binds efficiently to Tiiii-Si retaining its fluorescence, and it can be replaced only by molecules with comparable affinity for the cavity (sarcosine in our case). Tiiii-Si wafers complexed with guest **2** (Tiiii-Si•2) were exposed to three portions of human urine sample, two of them added with sarcosine at different concentrations before filtration (1 and 0.1 mM, respectively). The wafers were then analyzed via fluorescence spectroscopy to detect the presence of residual complexed guest **2** on the surface through its diagnostic fluorescence signal (Fig. 6). The fluorescence results confirmed the XPS experiments because both sarcosine added samples displaced fluorescence guest (traces C and D), whereas the untreated one did not (trace B). Results also indicated that guest **2** displacement depends on sarcosine concentration because a residual fluorescence signal was observed for the sample exposed to the urine with lower sarcosine content (0.1 mM).

Conclusions

In the present work, we report the use of a silicon active surface for the specific recognition of sarcosine in water and urine using tetraphosphonate cavitand Tiiii as receptor. At the molecular level, the recognition process has been dissected in its three interaction modes and investigated in the solid state, in solution, and at the solid–liquid interface. In aqueous environment, the enhanced role played by the $CH_3-\pi$ interactions leads to complete selectivity toward sarcosine versus glycine. The energy stabilization due to the

Table 1. Br/P atomic concentration ratio from XPS data and complexation yield calculated from Eq. 1 as a function of pH

	As prepared*	pH 7–1 [†]	pH 0.7 [†]	pH 0 [†]
Br/P ratio	0.13	<0.02	0.14	0.13
Complexation yield, %	54	/	56	54

*Before water dipping.

[†]After water dipping.

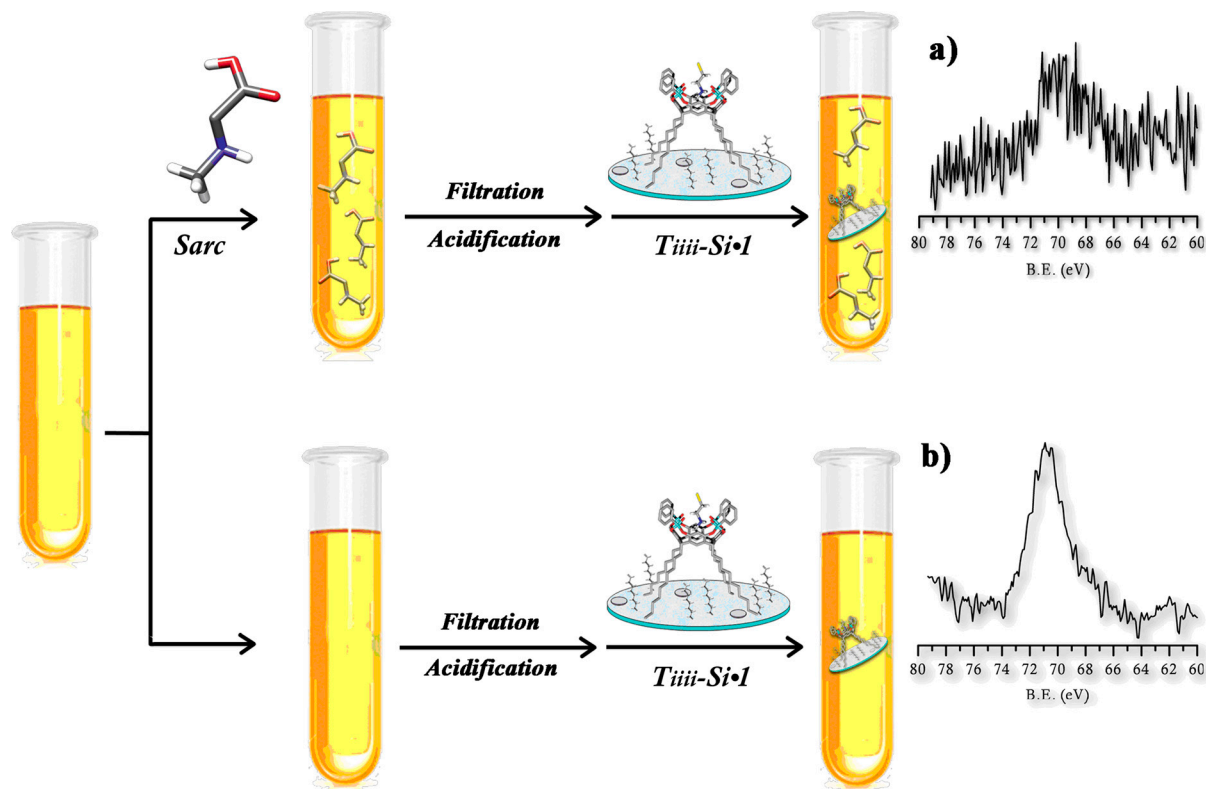


Fig. 5. Scheme of the detection procedure of sarcosine in human urine: The sample was divided into two portions, one them was added with sarcosine, and after filtration, centrifugation, and acidification, was tested with Ti_{iii}-Si•1 (upper scheme). The other portion, after filtration, centrifugation, and acidification, was tested (control sample) with Ti_{iii}-Si•1 (lower scheme). The XPS Br 3D spectra of Ti_{iii}-Si•1 slides dipped (A) in the sarcosine-added sample and (B) in the control sample are reported sideways.

CH₃- π interactions of sarcosine with the cavity has been estimated in 3.8 kcal/mol from DFT calculations.

The complexation properties of Ti_{iii} have been transferred to Si wafers with high fidelity and the exquisite ability of Ti_{iii}-Si to specifically detect sarcosine has been extended to urine, where several potential interferences are present. Complementary XPS and fluorescence guest displacement tests have demonstrated the selectivity of Ti_{iii}-Si under these stringent conditions. In particu-

lar, the fluorescence detection mode represents a fundamental requirement for the prospective application of these materials in devices for biomedical diagnostics. These results allow us to envision the use of the Ti_{iii}-Si surface for the specific detection of sarcosine as a marker of the aggressive forms of prostate tumor. Although the present fluorescence dye displacement approach is not yet suitable for sensing sarcosine at the low biological concentrations ($1-4 \times 10^{-5}$ M) (2, 26), we think that this work represents an important step in this direction.

Moreover, given the wide variety of biologically relevant compounds presenting N-CH₃ groups (drugs, neurotransmitters, painkillers, antidepressants, etc.), we believe that the use of Ti_{iii} cavitants can be extended to the detection of such compounds (27, 28).

Materials and Methods

Synthesis of Ti_{iii}[C₁₀H₁₉(CH₃-Ph)]. To a solution of resorcinarene (29) (1 g, 0.91 mmol) in freshly distilled pyridine (10 mL) dichlorophenylphosphine (0.504 mL, 3.72 mmol) was added slowly, at room temperature. After 3 h of stirring at 80 °C, the solution was allowed to cool at room temperature and 4 mL of 35% H₂O₂ was added. The resulting mixture was stirred for 30 min at room temperature, then the solvent was removed under reduced pressure, and water added. The precipitate obtained in this way was collected by vacuum filtration and profusely rinsed with diethyl ether to give the product in a quantitative yield. The detailed physical data of the product are shown in the *SI Appendix*.

Ti_{iii}/TSi_{iii} Grafting on Si. For grafting monolayers, cavitant/1-octene mixtures ($\chi_{\text{cav}} = 0.05$) were dissolved in mesitylene (solution concentration = 50mM). Cavitant solutions (2.0 mL) were placed in a quartz cell and deoxygenated by stirring in a dry box for at least 1 h. A Si(100) substrate was dipped in H₂SO₄/H₂O₂ (3:1) solution for 12 min to remove organic contaminants, then it was etched in a hydrofluoric acid solution (1% vol/vol) for 90 s, and quickly rinsed with water. The resulting hydrogenated silicon substrate was immediately placed in the mesitylene solution. The cell remained under UV irradiation

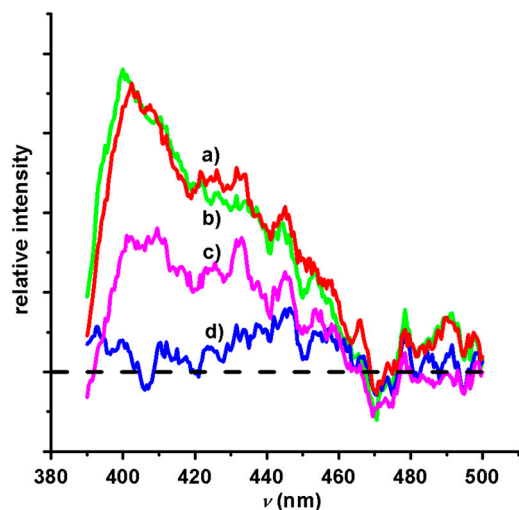


Fig. 6. Luminescence spectra of Ti_{iii}-Si•2: (A) before urine exposure (red line); (B) after dipping for 5 min in urine (green line); (C) after dipping in 0.1 mM sarcosine-added urine (magenta line); (D) after dipping in 1 mM sarcosine-added urine (blue line). Ti_{iii}-Si spectrum was subtracted from all signals. Excitation wavelength $\lambda_{\text{ex}} = 340$ nm.

(254 nm) for 2 h. The sample was then removed from the solution and sonicated in dichloromethane for 10 min to remove residual physisorbed material.

Tiii-Si Complexation Studies in Water. Complexation of Tiii-Si surface with guest 1 was performed in a 1 mM aqueous solution of guests 1 for 30 min and then the wafer was sonicated in CH₃CN for 10 min to remove any physisorbed material. Sarcosine recognition was carried out dipping Tiii-Si•1 wafers in a 1 mM sarcosine solution in water at pH 0.7 for 10 min. As a blank experiment, Tiii-Si•1 wafers were dipped in a 1 mM glycine solution in water at pH 0.7 for 10 min. All experiments have been repeated three times for consistency, without significant differences.

Urine Treatment. For XPS detection, individual human urine samples (15 mL) were divided in two portions and one of them was added with solid sarcosine up to 1 mM. Samples were loaded onto 15-mL Vivaspin filters with a molecular weight cutoff of 3,000 Da and centrifuged at 8,000 × g at 15 °C, and then the urine portions were acidified at pH 0.7. Urine filtration does not alter sarcosine concentration in the laced samples, as proven by GC selected ion monitoring MS analyses (4) (*SI Appendix, Figs. S15 and S16*). Note that the addition of sarcosine to urine before or after the filtration step led to the same results. Tiii-Si•1 wafers were then dipped in both urine samples for 10 min, washed in water at pH 0.7 for 1 min, and analyzed by XPS.

For fluorescence dye displacement detection, Tiii-Si•2 wafers were prepared by dipping of Tiii-Si surface in a 5 mM ethanol solution of guest 2 for 1 h. Then the wafer was sonicated in water at pH 0.7 for 5 min. Urine samples were divided into three portions, two of them were added with sarcosine up to 1 and 0.1 mM, respectively, and then were filtered, centrifuged, and acidified as above described. Tiii-Si•2 wafers were dipped in all urine samples for 5 min, washed in water at pH 0.7 for 1 min, and then analyzed by fluorescence spectroscopy. All experiments have been repeated three times for consistency, without significant differences.

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