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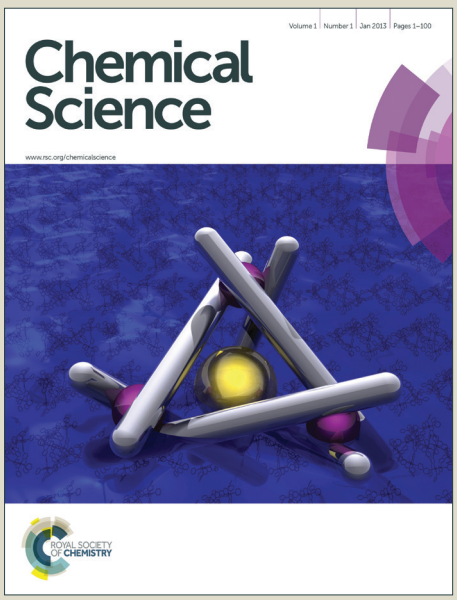

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ARTICLE TYPE

Highly sensitive and selective detection of the pyrophosphate anion biomarker under physiological conditions

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A multidentate adsorbate having a bis(carbazolyl)urea unit, as a receptor for hydrogen pyrophosphate anions, and two cyclic bidentate alkyl disulfide groups, as linkers to gold surfaces, has been designed and synthesized. Self-assembled monolayers (SAMs) on gold of this adsorbate have been obtained and characterized showing a high robustness along with an extremely large sensitivity and selectivity for hydrogen pyrophosphate anions enabling to be used as a surface plasmon resonance (SPR) sensor for the detection of such anions at the ppt concentration level under physiological conditions.

Introduction

In the field of supramolecular chemistry the topic of anion recognition and sensing has become an intense pursuit for a growing number of research groups worldwide.[1] Indeed, as a result of this effort, many excellent examples of molecular hosts for anionic species have been successfully developed.[2]

Among anions, pyrophosphate, $P_2O_7^{4-}$ (PPi), is a biologically important target because it plays an important role in the energy transduction in living organisms controlling many metabolic processes by participating in several enzymatic reactions. ATP hydrolysis, with the concomitant release of PPi, is central to many biochemical reactions, such as DNA polymerization and the synthesis of cyclic adenosine monophosphate (c-AMP) catalyzed by DNA polymerase and adenylate cyclase, respectively.[3] Furthermore, the detection of released PPi has been examined as a real-time DNA sequencing method,[4] and it has also been considered important in cancer research.[5] Indeed, telomerase activity (a biomarker for cancer diagnosis) is measured by evaluating the amount of PPi in the PCR amplification of the telomerase elongation product.[5] Furthermore, the high level of PPi in synovial fluids is correlated to calcium pyrophosphate dehydrate disease (CPDD), a rheumatologic disorder.[6] This anion could also be used as a potential biomarker for arthritis in the clinic diagnosis and therapy of arthritic diseases.[7] Consequently, the specific recognition and sensing of PPi anion under physiological conditions is of immense significance and, accordingly, the detection and discrimination of this anion has been recently the main focus of the effort of several research groups.[8] Good progress has also been made towards realizing this goal using metal-based approaches[9] or gold nanoparticles as the signal readout.[10.] However, due to the high solvation energy of PPi in water (ΔG^o = -465 KJ mol^{-1})[11] and the presence of other competitive anions, it becomes a difficult and challenging task the use of H-bonding synthetic receptors to achieve strong binding affinities in pure aqueous solution. To date, several different heterocyclic ring systems containing a pyrrolic NH group have been reported in the literature as hydrogen-bond donors to anions, as demonstrated in calixpyrroles,[12] expanded porphyrinoids,[13] pyrrole derivatives,[14] indoles,[15] bisindoles,[16] bisimidazoles,[17] carbazole derivatives,[18] and imidazole derivatives.[19] However, very few examples of effective selective fluorescent,[20] chromogenic,[21] or redox[22] chemosensors have been reported so far. Moreover, the creation of effective PPi sensors is also a demanding task as a consequence of its similarity with the phosphate anion. To date, there are only few chemical sensors reported in the literature that detect the PPi anion in pure aqueous solutions using in many cases changes in the optical properties when the anion is complexed with the receptors.[23] Among the available optical sensing techniques, surface plasmon resonance (SPR) is one of the most sensitive showing as a main advantage its use with aqueous solutions.[24] Highly specific SPR sensors are usually based on the proper modification of a metal surface, like gold, with a self-assembled monolayer (SAM) containing a ω-terminated receptor unit, as a recognizing element, located not too far away from the metallic surface.[25] To date most of the SPR-based sensors available are focused on the recognition of large biomolecules[26] and only a very few of them have been shown to work with analytes of low molecular weight.[27]

Herein we present a selective and reusable hydrogen pyrophosphate, $HP_2O_7^{3-}$ (HPPi), SPR sensor, based on self-assembled monolayers of compound **1** (Figure 1) on gold surfaces (henceforth denoted as **1·SAM**). This SPR sensor is able to perform "on flow" detection of small concentrations of this important anion in buffered aqueous solutions under physiological conditions with an unprecedented sensitivity and selectivity. Compound **1** present a rational design where a bis(carbazolyl)urea receptor unit has been modified with the purpose of ameliorating its self-assembling properties without modifying the sensing characteristics. For this purpose a multidentate adsorbate strategy has been used attaching two cyclic bidentate alkyl disulfides to the two extremes of the receptor unit to generate a robust SAM via the "chelate effect".[28] This strategy appeared properly for preparing sensors to be used

under harsh conditions, since SAMs derived from singly bound headgroups often suffer from stability issues and conformational defects.[29] Another presumed advantage of this multidentate strategy was that it might provide a higher control of the structural order of the SAM which might be critical for the anion recognition. To the best of our knowledge, a multidentate SAM approach, like **1·SAM**, for SPR sensing have not been previously attempted for the detection in aqueous media of pyrophosphate anions in the form of hydrogen pyrophosphate. In this context, it is worth mentioning that some authors have also used hydrogen pyrophosphate as a model for the detection of pyrophosphate anion.[8b, 23j]

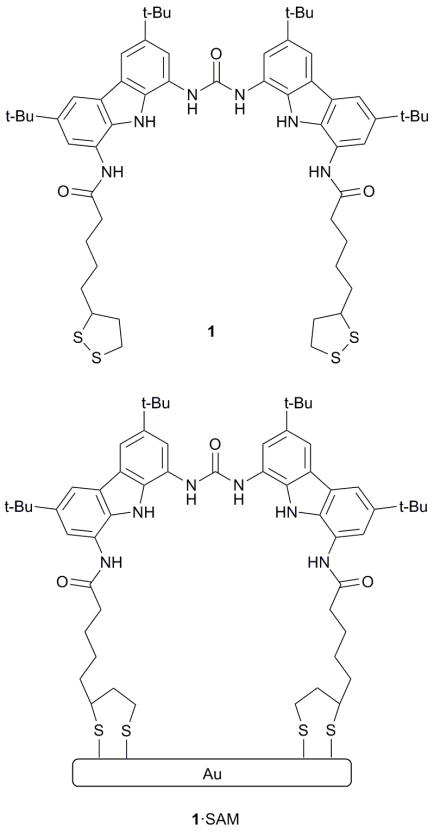

Fig.1 Multidentate receptor **1** and its self-assembling monolayer on gold **1·SAM**

Results and Discussion

The synthesis of the target molecule **1** was carried out in a one-step procedure by treatment of the 1,3-bis-(8-amino-3,6-di-*tert*-butyl-)-9*H*-carbazol-1-yl)urea[8f] with an excess of lipoic acid in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-benzotriazolol (1-BtOH) yielding the desired compound in 40% yield.

The attachment of the multidentate adsorbate **1** to a gold surface was accomplished by immersion of freshly cleaned gold substrates into 1mM ethanolic solutions of the receptor yielding robust monolayers of **1·SAM** after 24 h. Additionally, microcontact printing (μ-CP) was used to further characterize the monolayers by introducing a molecularly grafted pattern of **1** on the surface (see experimental section for details on the monolayer preparation).

A full characterization of the self-assembled monolayer was carried out by means of a multi-technique approach based on surface techniques such as contact angle (CA) measurements, polarization modulation-infrared reflection-adsorption spectroscopy (PM-IRRAS), scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (TOF-SIMS).

From the contact angle values depicted on Table 1 it can be concluded that gold surfaces modified with **1·SAM** presented a moderate hydrophobicity. The hysteresis value ($\Delta\Theta = \Theta a - \Theta b$) found for **1·SAM** (> 12º) showed that the packing of the monolayer was not vey compact which might be in agreement with the interface complexity of **1·SAM** produced by the grafting of the multidentate adsorbate **1**. Nevertheless, it is worth noting that a slight decrease in the contact angle value was detected after immersion of **1·SAM** in a HPPi solution, which indicates an increase in the hydrophilicity of the surface due to the presence of anions anchored to the receptors. On the other hand, a slight increase in the hysteresis was also detected probably due to a further loss in the molecular order of the SAM that increases the structural complexity of the interface.

Table 1 Advancing and receding contact angle values and hysteresis and XPS data of SAMs

	Contact angle[a] (º)			XPS
	θ_a (H_2O, º)	θ_r (H_2O, º)	Hysteresis, $\Delta\Theta$	S/N (exp/calcd)
1·SAM	81.7±0.3	54.5±0.2	28.1	1.25/1.52
$HP_2O_7^{3-}$@ **1· SAM**$^-$	79.6±0.4	47.4±0.1	32.2	-[b]

[a] θ_a and θ_r are referred to advancing and receding angles, respectively. [b] No phosphorous peak was detected. Probably, the washing step carried out in order to remove the excess of salts after the immersion of the substrate in the anion solution (10^{-3} M in EtOH) also removed most part of the anchored anion

XPS analysis of **1·SAM** showed the presence of all the expected elements (C, N, O and S) of the receptor **1**. Furthermore, the deconvolution of the peaks obtained for each electronic level gave rise to the energies related to the corresponding bonding of **1** corroborating the binding of the receptor to the gold surface (see ESI). It is worth to mention that the peak corresponding to unbound sulphur atoms to the gold substrate was much less intense as compared to that of the bound ones.[30] With these data, the presence of about 87% bound sulphur atoms can be estimated. This value was in agreement with the presence of a complete monolayer of **1** in which most part of the receptor molecules are using both bidentate cyclic disulfide arms for the anchoring to the surface. Furthermore, such data are in line with some degree of disorder in the SAM.

PM-IRRAS of **1·SAM** on gold showed the expected carbonyl band at 1648 cm^{-1}. Additionally, other bands assigned to ArH and NH vibrations were observed at 3104 and 3353 cm^{-1} respectively (see ESI), confirming the attachment of **1** on the gold.

TOF-SIMS with lateral resolution analysis with a positive ionization using a μ-contact printed gold substrate with the

multidentate receptor **1** revealed the presence of peaks at 1179 – 1185 $[M+Au\text{-}S]^+$, 1208 –1214 $[M+Au]^+$, 1378 –1381 $[M+2Au\text{-}S]^+$ and 1410–1415 $[M+2Au]^+$ emu (see ESI) also confirming the presence of **1·SAM**. On the other hand, lateral TOF-SIMS images indicated, not only the presence of **1·SAM** following the pattern introduced by the μCP procedure (red coloured area, Figure 2a) but also OPO_3H fragments when the substrate employed was immersed into a $HP_2O_7^{3-}$ solution in EtOH for 20 min (green coloured area, Figure 2b). These fragments were only detected in the areas where the receptor was anchored and could arise from the breakdown of the anion through one of the P-O_{bridge} bonds.

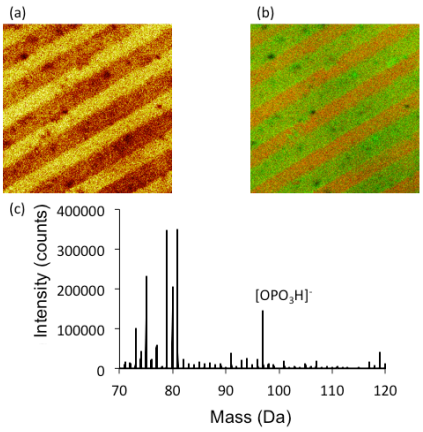

Fig. 2 TOF-SIMS with lateral resolution images: (a) μ-contact printed **1**·SAM (the receptor is located in the red coloured areas), (b) μ-contact printed **1**·SAM exposed to $HP_2O_7^{3-}$ (the OPO_3H^- fragment is located in the green coloured areas) and (c) partial spectra showing the peak of the OPO_3H^- fragment (negative ionization mode).

In order to add support to the characterization of the monolayers, we also studied the patterned substrates by means of SEM and AFM (see ESI). The pattern introduced on the substrates using the μ-contact printing technique was also visible in the SEM images as a set of alternating light and dark grey lines. A zoom in the border area of the pattern allowed us to assign the dark lines to the ones possessing the attached multidentate receptor **1**. Additionally, two different areas with a step height of around 1.2 nm corresponding to the approximate size of **1** were also observed by AFM indicating the success in the patterning and hence, the functionalization of the surface.

Having fully characterized and probed the recognition properties of **1·SAM** in the presence of HPPi anions, we proceed to establish the pyrophosphate recognition properties of the monolayer using a commercial SPR instrument equipped with a flow cell through which the control and test solutions were pumped at constant injection flows of 100 μL per minute. This technique is based on the detection of changes in the refractive index and the thickness changes near the gold surface produced by the complexation of the anion to the sensing surface. The shift in the plasmon angle during a binding experiment on a surface is proportional to the amount of bounded mass of analyte.[31] Calibration of the SPR signal to refractive index units (RIU) was performed by injecting solutions of NaCl of different concentrations with known refractive index. Equilibrium values of the SPR signal for each concentration were used to calibrate the sensor. The sensitivity experiments were first carried out in an aqueous media with a controlled ionic strength (NaCl 0.1 M). In order to ensure that no unspecific adsorption took place, control experiments with a 1-decanothiol SAM were first performed. Using solutions of HPPi anions at concentrations from 10^{-10} M to 10^{-4} M, no SPR-signal increase were detected with the later SAMs indicating that no unspecific adsorption of the HPPi anion occurred (see ESI). A similar titration procedure was followed with **1·SAM**. Sensitivity of the sensing chip was obtained from the slope of the calibration curve. In this case, even with a HPPi concentration as low as 10^{-10} M, an increase in the SPR signal was perfectly readable. An example of the response obtained is shown in ESI.

The binding kinetic analysis of the response towards HPPi was performed using the linearization method.[29a] This procedure is used to estimate the rate constants when the analyte follows a simple bimolecular interaction with the surface. In the case of attached receptors, the concentration of complex can be approximated to the surface coverage (denoted in this case as θ_{HPPi}). Additionally, since the concentration differences of HPPi during the titration are negligible, it can be assumed that this term is constant. For such systems (Eq. [1]), the rate equation can be expressed as indicates Eq. [2].

$$\text{HPPi} + 1\cdot\text{SAM} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \theta_{\text{HPPi}} \qquad [1]$$

$$\frac{d\theta_{HPPi}}{dt} = [HPPi][1\cdot SAM]k_a - \theta_{HPPi}k_d \qquad [2]$$

Since the signal measured (ΔRIU) is proportional to the surface coverage and the maximum SPR signal, equivalent to a situation where all the sites are occupied, is proportional to the initial concentration of guest, Eq. [2] can be rewritten and rearranged as Eq. [3]

$$\frac{d\Delta RIU}{dt} = k_a[HPPi]\Delta RIU_{\max} - (k_a[HPPi] + k_d)\Delta RIU \qquad [3]$$

Thus, fitting the linear parts of the response signal gives a slope, $-k_s$, where $k_s = k_a[HPPi]+k_d$.

Since the concentration of the guest is constant, plotting k_s *vs* $[HP_2O_7^{3-}]$ yields a straight line whose slope is related to the association rate constant, k_a, and the intercept corresponds to the dissociation rate constant, k_d.

The changes observed in the SPR responses for different concentrations of HPPi (see also ESI) appeared to indicate that two different processes took place during the anion recognition: the first one is dominant at low concentrations of the analyte (from 10^{-10} to 10^{-6} M) while the second one is only visualized at higher concentrations (from 10^{-6} to 10^{-4} M). A plausible explanation of these two processes could be given if one takes into account that not all receptors at the interface of the SAM are equally available to the analyte molecules due to the disorder of the receptor molecules in the SAM. Thus, at lower concentrations only the most accessible receptors having a higher association

constants are able to complex the anions while the receptors most buried or least accessible at the interface, that show lower association constants, require higher concentrations of the anion to complex them. Fitting the data at lower concentrations ([HPPi] = 10^{-10}-10^{-7} M) of the anion gave the following values for the kinetic constants: $k_a = 1.58\times10^5$ $M^{-1}s^{-1}$ and $k_d = 0.36$ s^{-1}. On the other hand, repeating the fitting procedure but with solutions of the anion at higher concentrations ([HPPi] = 10^{-6}-10^{-4} M) gave values of $k_a = 2.72\times10^3$ $M^{-1}s^{-1}$ and $k_d = 0.55$ s^{-1} for the kinetic parameters. Assuming that the binding constant, K_a, is the ratio between the association and dissociation rates, the association constants, $K_a = k_a/k_d$, for the set of the most and least accessible receptors are $4.39\cdot10^5$ M^{-1} and $4.95\cdot10^3$ M^{-1}, respectively. It is worth highlighting the remarkably high values for the association constants obtained indicating that the recognition process occurred through the formation of a very strong complex, similar to that already reported in solution for a molecule with a similar receptor unit.[8f] Furthermore, from the data obtained above, a remarkable low detection limit of 17 ppt can be obtained. This value is, to the best of our knowledge, the lowest concentration of HPPi anions detected by a synthetic receptor in aqueous media.

The selectivity of the **1·SAM** sensor was tested by the injection of solutions of several different anions with different charges, shapes and sizes in 100-fold excess. Even in such conditions, only a small response compared to that obtained with HPPi was observed for some other anions (Figure 3). Thus, under such high concentrations only trivalent anions, such as citrate and trimesate and the monovalent benzoate, show a significant response but with a magnitude which was about 60% weaker than that observed for $HP_2O_7^{3-}$. The current sensor shows a higher selectivity to hydrogen pyrophosphate over phosphate in aqueous solution. Taking into account that both anions coexist under many circumstances this constitutes a remarkable result since this discrimination is of crucial importance for assays detecting the activities of many enzymes.[9, 32]

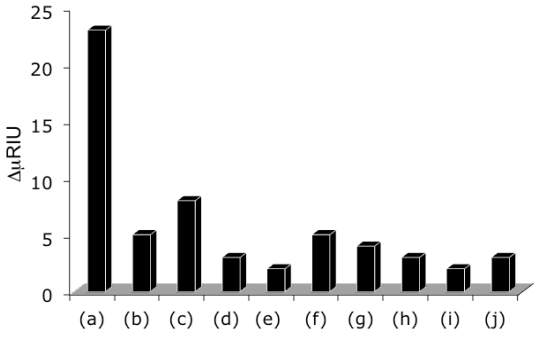

Fig.3 Selective response of the **1·SAM** sensor towards $HP_2O_7^{3-}$ anions. The concentration used was 10^{-9} M for $HP_2O_7^{3-}$ and 10^{-7} M for the rest of the anions (100-fold excess). (a) $HP_2O_7^{3-}$, (b) citrate, (c) trimesate, (d) $H_2PO_4^-$, (e) acetate, (f) benzoate, (g) chloride, (h) phthalate, (i) isophthalate and (j) terephthalate.

The reusability of a sensory system is another important feature concerning its practical applicability. Thus, regeneration tests were carried out with the same sensing chip using 10^{-9} M solutions of HPPi and carrying out several immersion/washing cycles. The response of the **1·SAM** sensor varied a 15% in average even with 7 cycles (see ESI). This result is very remarkable as it indicates the possibility to use the same sensory chip a minimum of seven times without a significant loss in the quality of the signal bringing an additional value to the feasibility of the sensor chip.

Once we stated the suitability of **1·SAM** for the recognition of hydrogen pyrophosphate in aqueous media and in order to go one step forward towards a real biomarker detection we also carried out binding experiments using a 20 mM HEPES saline buffer at pH=7.4, which constitutes a good model for normal physiological conditions. Furthermore, the high concentration of NaCl present in this buffer ensures the required high ionic strength over all the titration. Addition of different concentrations of the HPPi anion to **1·SAM** under these buffered conditions provoked similar changes in the SPR response, evidencing that the response of the monolayer was also interacting with the anions (Figure 4). Again, two different processes were detected which correspond to a kinetic profile similar to the one previously described. Accordingly, the kinetic parameters were: $k_a = 1.75\cdot10^5$ $M^{-1}s^{-1}$ and $k_d = 0.24$ s^{-1}, at [HPPi] = 10^{-10}-10^{-7}, and $k_a = 5.73\cdot10^3$ $M^{-1}s^{-1}$ and $k_d = 0.40$ s^{-1}, at [HPPi] = 10^{-6}-10^{-4}. The association constants obtained for both concentrations were $7.29\cdot10^5$ M^{-1} and $1.43\cdot10^4$ M^{-1}, respectively. These data revealed that the quality of the recognition events is maintained under simulated physiological conditions. It is noteworthy mentioning that the detection limit of HPPi under such conditions remains as low as 17 ppt.

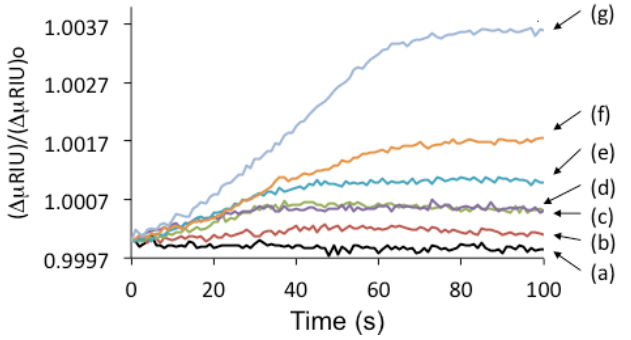

Fig. 4. Normalized SPR sensogram obtained upon addition of different concentrations of hydrogenpyrophosphate anion to **1**·SAM in 20 mM HEPES-saline buffer (pH = 7.4). (a) baseline, (b) $[HP_2O_7^{3-}] = 10^{-10}$ M, (c) $[HP_2O_7^{3-}] = 10^{-8}$ M, (d) $[HP_2O_7^{3-}] = 10^{-7}$ M, (e) $[HP_2O_7^{3-}] = 10^{-6}$ M, (f) $[HP_2O_7^{3-}] = 10^{-5}$ M and (g) $[HP_2O_7^{3-}] = 10^{-4}$ M.

Regeneration tests under these conditions showed a drop of 40% of the response in the third cycle and a new decrease to a 35% of the original signal in the sixth one, making the system unsuitable for the recognition of hydrogen pyrophosphate after three cycles (see ESI). This behaviour can be understood regarding the amount of salts present in the buffer. It is plausible that the monolayer becomes more saturated of salts after each cycle hampering the entrance of more HPPi. It is worth recalling that the salts presents in the buffer are in 10^7-fold excess compared to the lower concentration of the HPPi added.

In real practical uses it is very important to find selective sensors of phosphate species that can differentiate between different structural similar phosphate anions, such as PPi and the biologically important adenosine triphosphate (ATP) and adenosine diphosphate (ADP) anions. Thus, the selectivity of the **1·SAM** sensor in front of other phosphate anions was tested in

physiological conditions (20 mM HEPES buffer) by the injection of solutions of different phosphate anions. In such conditions PPi and HPPi gave similar responses while only a smaller response, 60-70% weaker compared to that obtained with HPPi, was observed for the ATP or ADP anions (Figure S12). Taking into account that all these anions coexist in many real samples this constitutes a remarkable result since this discrimination would be of crucial importance for practical uses.

Conclusions

In summary, we have rationally designed and synthesized a multidentate adsorbate with a bis(carbazolyl) urea derivative, as a receptor for pyrophosphate, that has attached two cyclic bidentate alkyl disulfides to the two extremes of the receptor unit in order to increase the robustness of the SAM formed on gold substrates. We carried out binding studies in water with this functionalized substrate using the SPR technique. These binding experiments showed an exceptional selectivity and sensitivity towards hydrogen pyrophosphate anion in two different buffered media (in 0.1 M NaCl and in 20 mM HEPES at pH = 7.4). Thus, **1·SAM** selectively recognizes HPPi from many other anions with different charges, shapes and sizes and differentiate from ATP and ADP anions. The detection limits reached by this new system were in the order of 17 ppt of HPPi and this result is, to date, the lowest value detected by a synthetic receptor which opens new ways for the detection of such important biomarker in aqueous physiological media. Furthermore, the reported sensing device can be reused a few times in 0.1 M NaCl and 20 mM HEPES.

Experimental section

Reagents used as starting materials were commercially available and were used without further purification. Compound **2** was synthesised following the procedure previously reported.[8f] Solvents were dried following the usual protocols (THF, Et_2O and Toluene were distilled from sodium wire with benzophenone indicator; CH_3CN and CH_2Cl_2 were distilled from $CaCl_2$; EtOH and MeOH were distilled from magnesium and stored with molecular sieves). Unless stated otherwise, all reactions were carried out under nitrogen atmosphere. Column chromatography was run with silica gel 60 Å CC 70-200 μm as stationary phase and using HPLC grade solvents. Melting points were measured in a Reichert instrument and are not corrected. ^{1}H-NMR, ^{13}C-NMR and NOESY experiments were recorded on a Bruker AV200, AV300, AV400 or AV600 instruments. Chemical shifts are referred to the residual peak of the solvent. In the experimental data "bp" stands for broad peak and "Cq" for quaternary carbon atom. Mass spectrometry was recorded on HPLC-MS TOF 6220 instrument. SEM measurements were performed in a QUANTA FEI 200 FEG-ESEM microscope equipped with two EDS (EDAX). Contact angle was measured in a Kruss DSA100 instrument equipped with a CCD camera. PM-IRRAS spectra were collected in a Brucker Vertex 70 with a PMA 50 module using a liquid nitrogen-cooled detector and an incidence angle of 80º for gold surfaces. XPS measurements were carried out in a K-alpha Thermo Scientific instrument with the Kα monochromatic radiation source of Al at 1486.68 eV and a perpendicular irradiation of samples. The SPR experiments were performed using a Reichert SR7000DC dual channel SPR instrument (Reichert Analytical Instruments, NY, USA). The setup is based on the configuration introduced by Kretschmann and Reather.[33] On top of the sample, the standard flow cell with two reaction channels was used. The sample was kept at a constant temperature coinciding with the calibration temperature (25ºC) during the whole experiment and under a constant continuous flow of 20 μl/min.

1,3-bis-(3,4-di-*tert*-butyl-8-[(5-[1,2]-dithiolan-3-yl-pentanoylamino)-9H-carbazol-1-yl] urea (1). (3-dimethylaminopropyl)-ethylcarbodiimide (EDC, 0.15 mL, 0.77 mmol) was added to a solution of lipoic acid (135 mg, 0.65 mmol) and 1-benzotriazolol (121 mg, 0.90 mmol) in dry THF (25 mL) under nitrogen atmosphere. Then, a solution of 1,3-bis(8-amino-3,6-di-*tert*-butyl-9*H*-carbazol-1-yl)urea[8f] (200 mg, 0.31 mmol) in dry THF (10 mL) was incorporated and the mixture was stirred at room temperature for 16 h. The reaction was quenched with brine (50 mL), the organic layer was separated and extracted with aqueous $NaHCO_3$ (3x25 mL), after the corresponding aqueous workup of the organic phase, the residue remaining after evaporation of the solvent was chromatographed in Hexanes/AcOEt 1:1 yielding a light Brown solid (100 mg, 32%). ^{1}H-NMR (300 MHz, DMSO-d_6); δ (ppm): 1.37-1.60 (m, 40H); 1.71 – 1.78 (m, 2H); 2.06 – 2.08 (m, 2H); 2.25 – 2.32 (m, 2H); 2.39 – 2.42 (m, 4H); 3.01 – 3.12 (m, 4H); 3.48 – 3.50 (m, 4H); 7.52 (s, 2H); 7.67 (s, 2H); 7.93 – 7395 (m, 4H); 8.91 (s, 2H); 10.02 (s, 2H); 10.11 (s, 2H). ^{13}C-NMR (75 MHz, DMSO-d_6); δ (ppm): 25.0 (CH_2); 28.3 (CH_2); 31.8 (CH_3); 34.1 (CH_2); 34.4 (CH_2); 35.9 (CH_2) 38.0 (CH_2); 39.6 (CH_2); 56.1 (CH); 111.9 (CH); 112.6 (CH); 116.2 (CH); 116.6 (CH); 122.5 (Cq); 123.0 (Cq); 124.4 (Cq); 124.5 (Cq); 130.6 (Cq); 131.1 (Cq); 141.6 (Cq); 141.8 (Cq); 171.3 (C=O). HRMS (ESI-TOF) *m/z:* $[M+H]^+$ $C_{57}H_{76}N_6O_3S_4$, found: 1021.4928; calcd: 1021.4934. mp.: 236 – 238 ºC.

1,3-bis-(3,4-di-*tert*-butyl-8-[(5-[1,2]-dithiolan-3-yl-pentanoylamino)-9H-carbazol-1-yl] urea SAM on gold (1·SAM). Gold substrates were immersed in piranha solution for 15 s. After extensive rinsing with milliQ water, the freshly cleaned substrates were immersed into a 1 mM solution of **1** overnight. Then, the substrates were rinsed thoroughly with EtOH, sonicated for 2 min and blown dry into a stream of nitrogen.

Acknowledgements

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Notes and references

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† Electronic Supplementary Information (ESI) available: NMR spectra; XPS, TOF-SIMS, PM-IRRAS, SEM and AFM data; SPR titrations of a 1-decanothiol SAM in aqueous NaCl 0.1 M; SPR titrations of **1·SAM** both in aqueous NaCl 0.1 M and 20 mM HEPES saline buffer at pH = 7.4; selectivity of **1•SAM** towards anions with three negative charges and other phosphate anions and synthesis of compound **1**. See DOI: 10.1039/b000000x

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