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Cross-Reactive Sensor Arrays for the Detection of Peptides in Aqueous Solution by Fluorescence Spectroscopy

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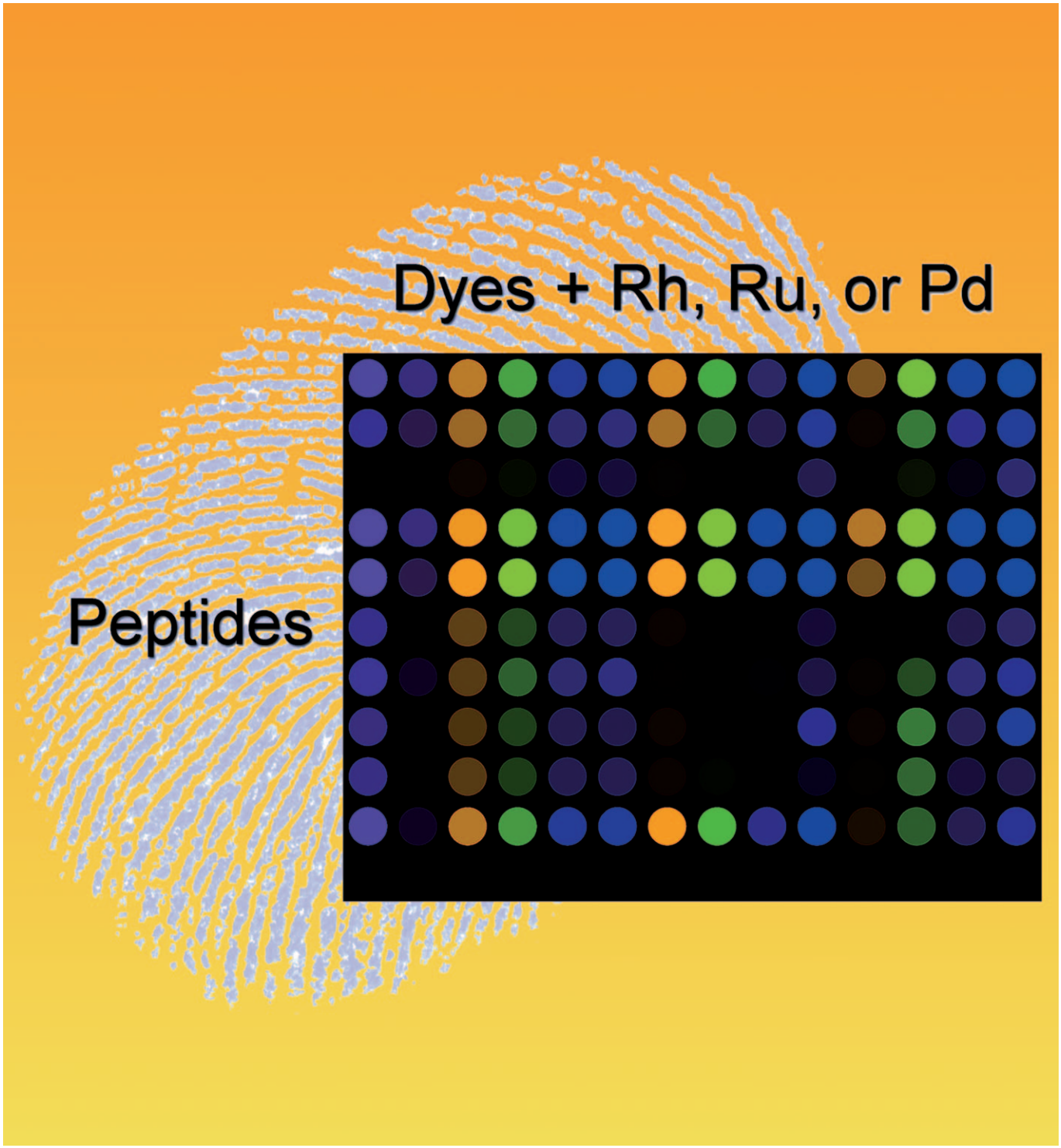

Abstract: A simple but powerful method for the sensing of peptides in aqueous solution has been developed. The transition-metal complexes $[PdCl_2(en)]$, $[\{RhCl_2Cp^*\}_2]$, and $[\{RuCl_2(p\text{-cymene})\}_2]$ were combined with six different fluorescent dyes to build a cross-reactive sensor array. The fluorescence response of the individual sensor units was based on competitive complexation reactions between the peptide analytes and the fluorescent dyes. The collective response of the sensor array in a time-resolved fashion was used as an input for multivariate analyses. A sensor array comprised of only six metal–dye combinations was able to differentiate ten different dipeptides in buffered aqueous solution at a concentration of 50 μM. Furthermore, the cross-reactive sensor could be used to obtain information about the identity and the quantity of the pharmacologically interesting dipeptides carnosine and homocarnosine in a complex biological matrix, such as deproteinized human blood serum. The sensor array was also able to sense longer peptides, which was demonstrated by differentiating mixtures of the nonapeptide bradykinin and the decapeptide kallidin.

Keywords: fluorescence spectroscopy · indicator displacement assay · organometallic complexes · peptides · sensors

Introduction

The pattern-based recognition of analytes with a collection of cross-reactive sensors represents an interesting alternative to more classical detection methods based on analyte-specific chemosensors.[1–4] A selective chemosensor requires a mechanism for recognizing the analyte of choice. In the case of complex bioanalytes such as peptides, the design and the synthesis of such a recognition unit can be a challenging task. For pattern-based sensors, on the other hand, the individual sensor units can be rather unsophisticated as long as they give a differential response upon interaction with the analyte. Furthermore, pattern-based sensors can be used for classes of analytes, whereas normal chemosensors are generally used for one particular analyte.

Cross-reactive sensor arrays can be employed to detect analytes in the gas phase or in solution. Gas-phase sensors are known as "electronic noses",[2] in analogy to the pattern-based sensing of the olfactory system. Research in the area of electronic noses is quite advanced, and sensors for different applications are commercially available.[2] The pattern-based recognition of analytes in solution is less developed, but the field has advanced substantially over the last years.[3,4] Sensors that display an electronic signalling mechanism are referred to as "electronic tongues".[3] They have found applications for the analyses of food and beverages, for example. The construction of electronic tongues typically requires the fabrication of specialized sensor hardware. This is in contrast to pattern-based sensors, which are based on UV/Vis or fluorescence spectroscopy.[4] These techniques are appealing because standard equipment can be used for the signal readout (e.g., plate readers). Cross-reactive sensors with a colorimetric or a fluorescence response have been described for the solution-based detection of simple cations[5] and anions,[6] of organic amines[7] and carboxylates,[8] of nitrated explosives,[9] nucleotides,[6b,10] steroids,[8b,11] alkaloids,[12] sugars,[13] amino acids,[14] peptides,[15,16] and proteins[15a,17] among others. Furthermore, it has been demonstrated that sensors of this kind can be used for the classification of beverages.[5b,8d,18]

For the analysis of short peptides, two conceptually different approaches have been described. Anslyn and McDevitt et al. have generated libraries of synthetic receptors containing Cu^{II} and Cd^{II} ions. The receptors were either immobilized on a silicon microchip array[15c] or used in homogeneous solution.[15b] The UV/Vis response of the sensor collection was used to differentiate tri- and tetrapeptides as well as the neurotransmitter peptides α-neurokinin (a decapeptide) and substance P (an undecapeptide). Good discrimination was achieved in buffered aqueous solution at peptide concentrations of 13[15c] or 267 μM.[15b] A drawback of these systems, however, is the fact that considerable synthetic efforts were required to prepare the receptors. We have reported colorimetric sensors that were obtained by mixing $CuCl_2$ and $NiCl_2$ with three dyes in buffered aqueous solution.[16] The resulting solutions were composed of complex mixtures of metal–dye complexes. Upon addition of the peptide analyte, a characteristic change in the UV/Vis spectrum was observed. The peptide was then identified with the help of multivariate analyses. The advantage of this method is its simplicity. However, only a single UV/Vis spectrum is used as the data input (the composite spectrum of all metal–dye–peptide complexes), which compromises the resolution that can be achieved.

Below we describe an alternative approach to sense peptides with metal–dye complexes. Sensor arrays with up to 14 individual sensor units were created by combination of

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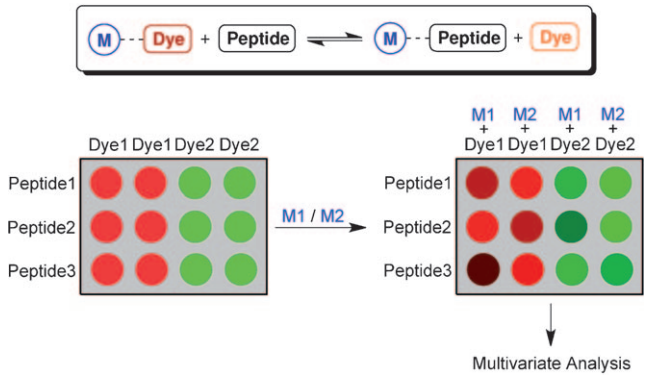
metal complexes with dyes. The dyes show strong luminescence, and fluorescence spectroscopy could be used for the analysis. Instead of 3d transition-metal ions, we have now used 4d transition-metal complexes of rhodium, ruthenium, or palladium. These complexes show high binding constants and moderately fast exchange kinetics. As a result, it was possible to perform measurements at very low analyte concentrations and in a time-resolved fashion. The sensor arrays were found to display a remarkable discriminatory power. An excellent differentiation of dipeptides was achieved at concentrations as low as 20–50 μM. This includes peptides composed of amino acids without coordinating side chains, a challenging class of analytes for metal-based receptors. Furthermore, it is shown that mixtures of the nonapeptide bradykinin and the decapeptide kallidin can be distinguished, and that the dipeptides carnosine and homocarnosine can be analyzed in a complex matrix, such as human serum.

Results and Discussion

The basic design principle of our cross-reactive sensor array is shown in Scheme 1. The peptide analytes compete with fluorescent dyes for the coordination to metal complexes.



Scheme 1. Basic principle of a sensor array based on competition reactions of peptide analytes and metal–dye complexes.

When coordinated to the metal complexes, the fluorescence of the dyes is significantly reduced. The fluorescence intensity is therefore an indication of how much metal–dye complexes are formed for a given metal–dye–peptide combination. For the minimal sensor array shown in Scheme 1, two different fluorescent dyes are combined with two metal complexes to give a 2×2 array. Larger arrays can be obtained by increasing the number of the dyes and/or the number of the metal complexes. The identification and/or quantification of the peptide analytes can be achieved by analyzing the fluorescence response of the sensor array with pattern-recognition protocols.

To implement such a sensor scheme, we had to identify metal complexes with the following characteristics: 1) they should be water soluble and inert towards oxidation; 2) they should bind to peptides with high affinity; 3) they should show reasonably fast ligand exchange kinetics; and 4) they should quench the fluorescence of metal-binding dyes. For practical purposes, we were furthermore interested to have metal complexes that are either commercially available or easy to synthesize. Simple 3d transition-metal halides (e.g., $CuCl_2$) might fulfill the above-mentioned criteria. However, we decided to focus on 4d transition-metal complexes, because they potentially show higher binding constants to the peptide analytes.

Previous experiments from our laboratory had shown that the organometallic complex $[\{RhCl_2Cp^*\}_2]$ ($Cp^* = \eta^5\text{-}C_5Me_5$) is well suited for competitive assays with dyes and peptides.[19] The commercially available rhodium complex is soluble in water (aqua complexes are formed) and the solutions are not air sensitive. Importantly, this complex shows a good binding affinity to peptides, albeit with a strong preference for those containing histidine or methionine.[19] The coordination chemistry of the arene complex $[\{RuCl_2(p\text{-cymene})\}_2]$ is known to be similar to that of $[\{RhCl_2Cp^*\}_2]$,[20] and studies by Beck and Sheldrick have shown that the {Ru(*p*-cymene)} fragment is able to bind to peptides.[21] The complex $[\{RuCl_2(p\text{-cymene})\}_2]$ was therefore used as the second metal complex for our sensor array. The third and final metal component that we chose was $[PdCl_2(en)]$ (en = ethylenediamine). Again, there was ample evidence that this metal complex is able to bind to peptides, in particular due to the detailed studies of Kostić on Pd^{II}-induced peptide hydrolysis.[22,23] To the best of our knowledge, there was no precedence for the utilization of Pd–en or Ru–*p*-cymene complexes in the context of indicator displacement assays.

The structures of the fluorescent dyes that we used for the sensor are depicted below. They are all commercially available and possess donor groups that are suited for the complexation to transition metals.

To obtain quantitative information about the binding affinities of the dyes to the three metal complexes, we performed fluorescence titration experiments in buffered aqueous solution (phosphate buffer, pH 7.0) with a fixed amount of the dye and a variable amount of the metal complex. The binding constants were obtained by fitting of the binding isotherms (for details see Supporting Information). In most of the cases, a 1:1 binding model provided a reasonably good fit. For calcein (CAL), it is likely that $[M_2(CAL)]$ complexes are formed as well, but it was difficult to obtain an accurate binding constant for the coordination of the second metal, because the fluorescence was nearly fully quenched by the first metal. When comparing the binding constants for the three different metal complexes, it is apparent that the values for a given dye generally increase in the order $K_1(Pd) < K_1(Ru) < K_1(Rh)$, except in the case of nuclear fast red (NFR; Table 1). Overall, the binding constants span more than four orders of magnitude, which is advantageous for the analysis of peptides possessing very different binding affinities to metal complexes. No pronounced quenching

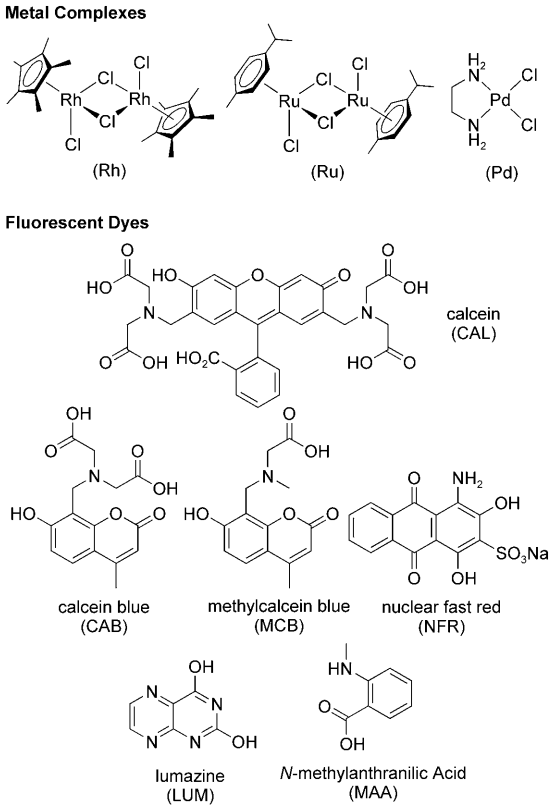


Table 1. Binding constants for the complexation of the fluorescent dyes to the metal complexes.

Fluorescent dye	Metal complex	K_1 [M^{-1}][a]
CAL	Rh	$7.6(\pm0.8)\times10^6$
CAL	Ru	$1.9(\pm0.2)\times10^6$
CAL	Pd	$6.2(\pm0.6)\times10^5$
CAB	Rh	$>10^7$
CAB	Ru	$1.6(\pm0.2)\times10^6$
CAB	Pd	$1.2(\pm0.1)\times10^5$
MCB	Rh	$>10^7$
MCB	Ru	$1.8(\pm0.2)\times10^7$
MCB	Pd	$2.2(\pm0.2)\times10^5$
NFR	Rh	$1.6(\pm0.2)\times10^6$
NFR	Ru	$>10^7$
NFR	Pd	$>10^7$
LUM	Pd	$7.8(\pm0.8)\times10^4$
MAA	Pd	$8.6(\pm0.9)\times10^3$

[a] The binding constants were calculated by numerical fitting of the binding isotherms obtained from fluorescence titration experiments. The titrations were performed in buffered aqueous solution (100 mM phosphate buffer, pH 7.0). For details see the Supporting Information.

was observed when solutions of Rh or Ru were added to lumazine (LUM) and *N*-methylanthranilic acid (MAA). Therefore, these combinations were not taken into account for our sensor array.

Next we studied the sensor response to dipeptides for some selected metal–dye combinations. For this purpose, an aqueous solution of the dye was mixed with the respective peptide. The competition reaction was then initiated by adding a stock solution of the metal complex and the fluorescence response was recorded as a function of time. The data for a mixture of $[PdCl_2(en)]$ (Pd) and lumazine (LUM) and the analytes Met-Leu, His-Ala, and Ser-Gly are shown in Figure 1. The final concentrations for these reactions were: [Pd] = [LUM] = 25 μM, [peptide] = 50 μM. In all cases,

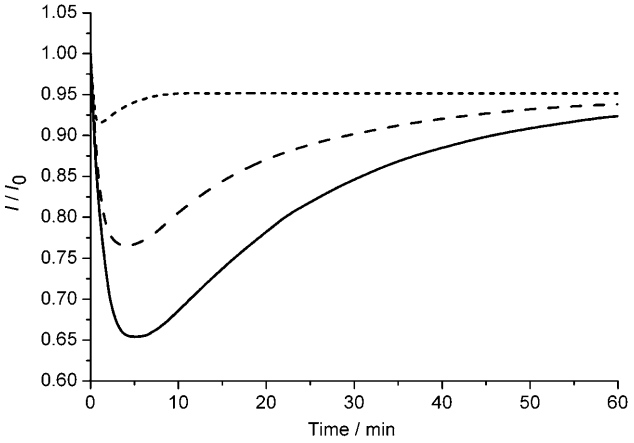


Figure 1. Relative fluorescence emission intensity at 460 nm for the reaction of $[PdCl_2(en)]$ (25 μM) and lumazine (25 μM) in the presence of Met-Leu (50 μM, dotted line), His-Ala (50 μM, dashed line), and Ser-Gly (50 μM, solid line). The data were obtained at 25 °C in H_2O (100 mM phosphate buffer, pH 7.0). The excitation wavelength was 328 nm.

an initial decrease in fluorescence intensity was observed. However, within a few minutes the fluorescence signal started to grow. After one hour, a steady state was nearly reached with a fluorescence signal that was only slightly less strong than that of the free dye. These data suggest that the Pd complex reacts faster with lumazine than with the peptides (initial decrease of the signal), but the peptides bind stronger to the metal than lumazine and ultimately displace the dye (subsequent increase of the signal).

A different situation was encountered for reactions with $[PdCl_2(en)]$, calcein blue (CAB), and the peptides Trp-Gly, Val-Phe and Phe-Pro (Figure 2). A steady decrease of the fluorescence signal was observed for all three peptides for the first 20 min. A minor increase in signal intensity occurred for Trp-Gly and Val-Phe over the last 30 min, but for Phe-Pro the signal continued to decrease. The data show that the reaction rate for the complexation of calcein blue to Pd is slower than that of lumazine, and that the peptides are less-efficient competitors for the binding to the metal. Kinetic profiles similar to that shown in Figure 2 were found for the Rh and Ru sensors (the competition reactions were slower for Ru than for Rh). Some representative examples are shown in the Supporting Information.

From the results of these competition experiments it is evident that for some metal–dye–peptide combinations, larger differences in fluorescence intensity are observed at the beginning of the reaction, whereas other combinations show more pronounced differences at the end. Consequently, the sensing of peptides was best achieved by time-resolved measurements.

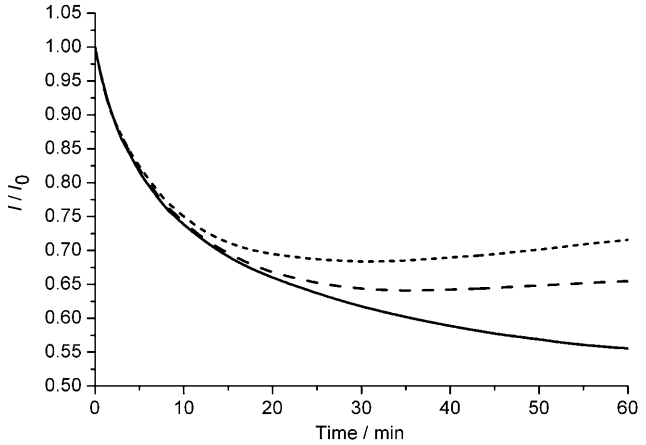


Figure 2. Relative fluorescence emission intensity at 440 nm for the reaction of [$PdCl_2$(en)] (25 μM) and calcein blue (25 μM) in the presence of Trp-Gly (50 μM, dotted line), Val-Phe (50 μM, dashed line) and Phe-Pro (50 μM, solid line). The data were obtained at 25 °C in H_2O (100 mM phosphate buffer, pH 7.0). The excitation wavelength was 336 nm.

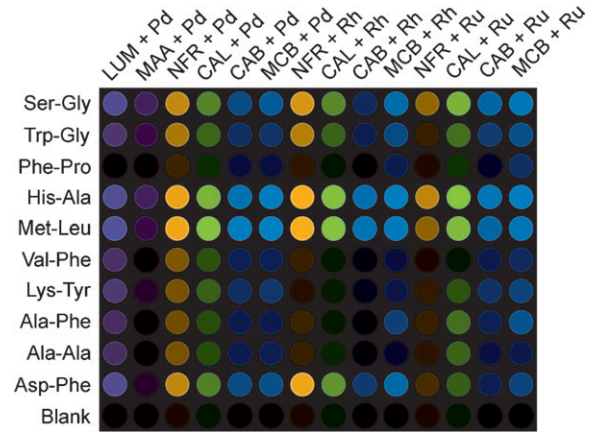


Figure 3. Graphic representation of the relative fluorescence intensities after 60 min of solutions containing the indicated dyes, peptides and metal complexes ([M] = 25 μM, [dye] = 25 μM, [peptide] = 50 μM, 100 mM phosphate buffer, pH 7.0). The colors approximately match the maxima of emission intensities, and the brightness corresponds to the fluorescence intensities of the samples. The brightness was calculated by assuming that the signal of the free dye was 100% and that of a "blank" sample without peptide was 0%.

To test the scope of our sensor array, ten different dipeptides were used. The binding kinetics of the fourteen metal–dye combinations shown in Table 1 were measured in the presence of each dipeptide. The assays were performed as follows: Metal complexes ($[M]_{final} = 25$ μM) were added to solutions containing the respective dye ($[dye]_{final} = 25$ μM) and peptide ($[peptide]_{final} = 20$ or 50 μM) to initiate the competition reactions. The fluorescence signal at the emission maximum (Table 2) was measured at regular time intervals,

Table 2. Excitation and emission wavelengths of the fluorescent dyes used in the frame of our study.

Fluorescent dye	Excitation [nm]	Emission [nm]
CAL	493	520
CAB	336	440
MCB	363	445
NFR	540	590
LUM	328	460
MAA	325	439

and each reaction was repeated four or six times. An inspection of the fluorescence response revealed that 1) the fluorescence signal before the addition of the metal complex was stable over time and independent of the peptide, thus indicating that no dye-peptide interactions occur, and 2) a pseudo-equilibrium state was reached one hour after metal complex addition in most of the cases.

For the statistical analyses, we chose to take the fluorescence intensities of each metal–dye–analyte combination at 5, 20, and 60 min as input values. This choice allowed us to take into account kinetic differences (5 and 20 min values) as well as variations of the pseudo-steady-state (60 min value). Figure 3 shows a qualitative view of the sensor response at 60 min. It is apparent that peptides containing amino acids with coordinating side chains, in particular His-Ala and Met-Leu, are able to displace a larger fraction of the dyes and thus give stronger signals. The least-coordinating peptide was found to be Phe-Pro: it does not compete efficiently with the fluorescent dyes for the complexation to the metals, resulting in almost completely quenched fluorescence signals.

To evaluate the discriminatory power of our sensor array, a principal component analysis (PCA)[24] was performed. A PCA allows us to reduce the multidimensional sensor array data to only two or three dimensions with minimal loss of variance. The resulting score plots are shown in Figure 4. The first three principal components are displayed, accounting for 94% of the total variance. All analytes are grouped in well-separated clusters. The analytes that appear close to each other are the ones that possess the weaker interaction with the metal complexes: Ala-Ala, Val-Phe, Phe-Pro, and Ala-Phe. Despite their weak displacement abilities, these analytes are still well separated from the blank sample. Interestingly, the main principal component (factor 1) correlates with the affinity of the peptide to the metal complexes: the blank sample shows the lowest values, close to those of the dipeptides possessing aliphatic side chains. As qualitatively observed in Figure 3, the peptide appearing closer to the blank on the factor 1 axis is Phe-Pro (at a concentration of 50 μM). The dipeptides with the strongly interacting His and Met amino acids have the highest values, and the dipeptides with other, weakly interacting side chains are found in between. Also of importance is the fact that the 20 and the 50 μM samples of Val-Phe and Ala-Ala appear well separated on the score plots, which underlines the possibility of obtaining information about the nature and the concentration of the analytes.

In addition to the PCA, the same data set was used to perform a linear discriminant analysis (LDA),[24] which also resulted in an extremely good separation of each analyte.

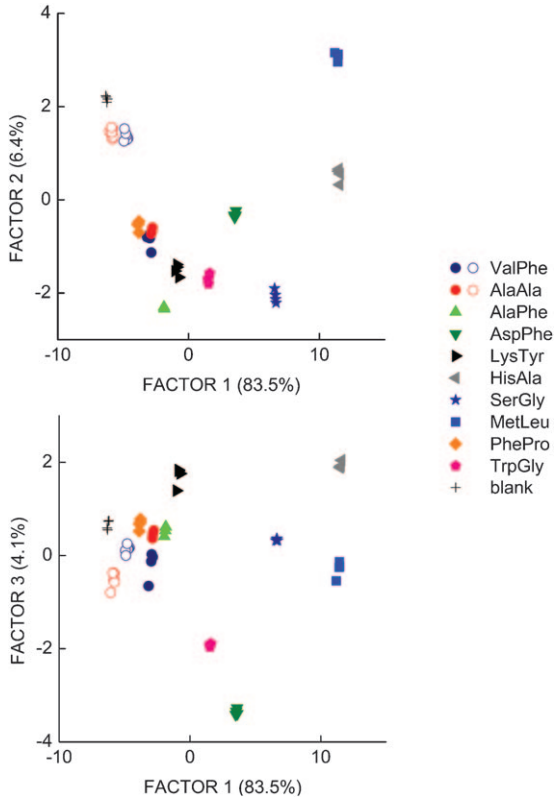


Figure 4. PCA score plots for the discrimination of ten dipeptides. The peptide concentrations were 50 μM (filled symbols) or 20 μM (open symbols). The input data for the PCA were obtained from a sensor array comprised of the 14 metal–dye combinations listed in Table 1.

The model was built by using a stepwise variables selection algorithm. A "jack-knifed matrix" validation procedure was also performed, in which one measurement at a time was randomly omitted. The remaining data were used as the training set for the LDA, and the omitted observation could be evaluated. In our case, all data were classified correctly (see the Supporting Information for detailed results).

The PCA of our data set made it possible to establish which of the fourteen metal–dye combinations brought the most information for the discrimination of the peptides. For our analysis, we focused on principal component 1 (factor 1), which contains more than 80% of the total variance. The component loadings to factor 1 of the three measurements at 5, 20 and 60 min of each metal–dye mixtures were combined, thus affording fourteen contributions (see the Supporting Information for details). A comparison of the resulting values allowed us to identify the six metal–dye combinations that contribute most significantly to the discriminating power: Pd/NFR, Pd/CAB, Rh/NFR, Rh/CAL, Pd/MCB and Rh/CAB (MCB = methylcalcein blue).[25] Sensors involving Ru were found to contribute less, plausibly because of the slower exchange kinetics.

To verify that the reduced sensor array was still able to give good separation, multivariate analyses were performed with the fluorescence data obtained from the selected six metal–dye combinations. Both PCA and LDA resulted in complete discriminations of all peptides. It is clear from the PCA score plots as well as from the contribution of the variables to the principal components that the variance was further concentrated in factor 1. However, this effect was expected (the sensors accounting most for factor 1 were selected), the discrimination still works satisfyingly, and the loss of resolution is minimal. A "jack-knifed matrix" validation procedure was performed in the same manner as in the case of the entire sensor array, and the classifications were correct in all cases. Furthermore, if only 60% of the data were used to build discriminant functions, the remaining 40% were subsequently classified with complete accuracy (see Supporting Information). Successful cross-validation procedures ensure the predictability and quality of our sensing system.

Encouraged by the results obtained for the discrimination of dipeptides, we investigated whether the sensor array was able to discriminate mixtures of longer peptides. We chose the closely related plasma peptides bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, BK) and kallidin (Lys-bradykinin, KD). Both peptides are hormones belonging to the kinin group. Kinins are important inflammatory mediators that induce pain and oedema.[26] Kallidin can be converted into bradykinin by aminopeptidases. The peptides also show vasodilatatory effects, and at the cellular level they influence glucose and chloride release as well as neurotransmitter transport.[27] Kinins are degraded by angiotensin I-converting enzymes (ACE), and it was suggested that ensuring a high micromolar level of BK in plasma by inhibition of cleavage enzymes could provide cardioprotective effects.[28] Our goal was to discriminate aqueous solutions containing various kallidin/bradykinin ratios, with the total amount of peptide being kept constant at 50 μM. It should be pointed out that this was a challenging task because both peptides lack His or Met amino acids with strongly coordinating side chains. Consequently, relatively weak signals were obtained in competition reactions with metal–dye combinations. A short pre-screening of the fourteen metal–dye combinations listed in Table 1 revealed that the following six mixtures were best at discriminating BK from KD: Rh/MCB, Rh/CAB, Rh/CAL, Pd/CAB, Pd/NFR and Pd/LUM. This selection was made by comparing the fluorescence signals obtained when the different metal–dye mixtures were reacted with BK or KD: only the combinations affording significant differences between the two analytes were chosen (see Supporting Information for details). These mixtures were then used for the time-resolved sensor array analyses. The assay was performed as follows: an aqueous solution of the respective fluorescent dye was added to a solution containing either the pure kinin or a mixture of KD and BK in the ratios 20:80, 40:60, 60:40, or 80:20. The competition reaction was then initiated by adding a stock solution of the metal complex, and the fluorescence response was recorded as a function of time. The solutions contained 50 μM of peptide (100 mM phosphate buffer, pH 7.0). For the Rh-based assays, the final concentrations were [dye] = [Rh] = 10 μM, whereas concentrations of [dye] = [Pd] = 20 μM were used for the Pd-

based experiments (the amount of dye and metal was semi-optimized in the prescreening). Each reaction was repeated four times, and the fluorescence signals measured after different time intervals were used as input for the multivariate analysis. The score plot of a PCA is shown in Figure 5. The

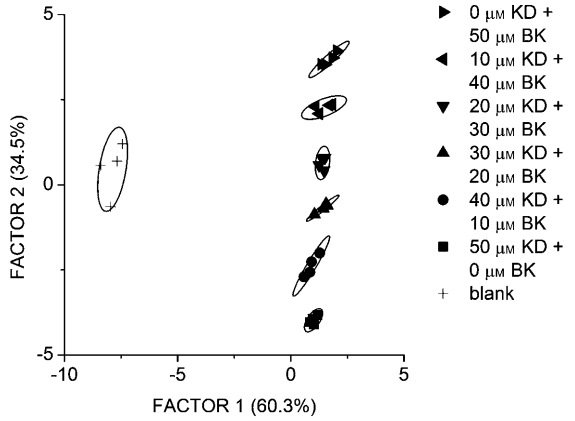
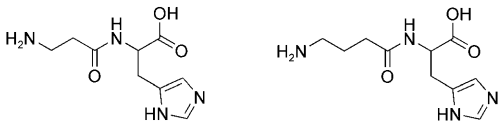


Figure 5. Two-dimensional PCA score plot for the discrimination of aqueous solutions containing different concentrations of kallidin (KD) and bradykinin (BK). The input data for the PCA were obtained from a sensor array comprised of the following six metal–dye combinations: Rh/MCB, Rh/CAB, Rh/CAL, Pd/CAB, Pd/NFR and Pd/LUM.

different peptides mixtures appear in distinct clusters. The factor 1 axis is clearly correlated with the presence or absence of any analyte, whereas the various peptide mixtures are separated along the factor 2 axis. Furthermore, the analytes are classified along the factor 2 axis according to their composition: the higher the mole ratio of bradykinin, the higher the position of the corresponding cluster along this axis. This type of correlation could be used to create calibration curves, which would allow the KD/BK ratios of samples with unknown composition to be determined. By analyzing the contribution of each sensor to the principal components, one observes that Rh-based sensors contribute mostly to factor 1, whereas Pd-based sensors are more correlated to factor 2 (see Supporting Information). To assess the predictive ability of our sensor in this case a LDA-based "jackknifed" validation procedure was applied, and this resulted in a correct classification in all cases.

Finally, we investigated whether the sensor array could be used in a more complex environment such as human serum. As analytes, we chose the dipeptides carnosine (β-alanyl–histidine) and homocarnosine (γ-aminobutyryl–histidine). Carnosine is a naturally occurring dipeptide that was initially isolated from meat extracts. Several metabolic functions are known, including antioxidant[29] and hydroxyl-radical-scavenging effects,[30] metal-ion chelation[31] and pH regulation.[32] Carnosine can be found in high concentrations in the brains and muscles of mammals, whereas the related homocarnosine can be found in cerebrospinal fluid and brain.[29b] The two dipeptides are usually very short-lived in human plasma, due to the presence of restriction enzymes (carnosinases), which degrade them to their constituent amino acids.[33] Carnosinase deficiency, resulting from a rare genetic disorder,[34] was identified as the cause of abnormal carnosine and homocarnosine levels in body fluids (micromolar instead of nanomolar concentrations in blood).[33,35] People suffering from this metabolic disorder show severe symptoms such as developmental delay and mental retardation. Besides, reduced carnosinase activity was also detected for patients suffering from Parkinson's disease, multiple sclerosis and cerebrovascular disease.[36]



Our goal was to selectively detect carnosine and homocarnosine in the micromolar concentration range. Blood serum contains large amounts of salts, sugars, hormones and proteins. The latter were expected to interfere with our metal receptors, so a simple precipitation technique with acetonitrile was used to remove them. The deproteinized serum would still contain non-negligible quantities of other potentially interfering substances, including micromolar concentrations of histidine and methionine.[37] To counterbalance the effect of these strong chelators, we decided to perform the competition reactions with slightly higher metal concentrations (200–500 μM, determined by preliminary tests). The final assays were carried out as follows: deproteinized serum samples were spiked with different quantities of carnosine or homocarnosine, an aqueous solution of the respective fluorescent dye was added, and the competition reaction was initiated by adding a stock solution of the metal complex. The reduced sensor array, which we had already used for the dipeptide analysis was employed (sensors: Pd/NFR, Pd/CAB, Rh/NFR, Rh/CAL, Pd/MCB, and Rh/CAB). As before, the fluorescence response was recorded as a function of time. The final solutions contained 25% (v/v) serum, 25% (v/v) buffered water (100 mM phosphate buffer, pH 7.0), and 50% (v/v) acetonitrile. Each reaction was repeated four times, and the fluorescence signals after 5, 20, and 60 min were used as input for the multivariate analysis. The score plot of a PCA is shown in Figure 6. The data for the two peptides at four different concentrations (25, 50, 75, and 100 μM) appear in well-separated groups with no apparent overlap. An analysis of the sensors contributions to the principal components indicates that all the sensors contribute to both factor 1 and factor 2. The peptides themselves are separated along the factor 1 axis (49.1% of the total variance), whereas most of the concentration information is described by factor 2 (36.9% of the total variance). A LDA with a cross-validation routine resulted in a correct assignment of all data when one measurement was omitted at a time and then reclassified. When only 80% of the data were

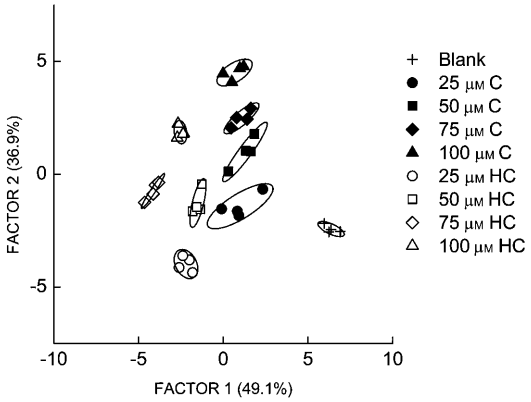


Figure 6. Two-dimensional PCA score plot for the discrimination of various concentrations of carnosine (C) and homocarnosine (HC). The input data for the PCA were obtained from a sensor array comprised of the following six metal–dye combinations: Pd/NFR, Pd/CAB, Rh/NFR, Rh/CAL, Pd/MCB and Rh/CAB.

used as the training set, the remaining data were also classified correctly (see Supporting Information).

These results demonstrate that a sensor array composed of metal–dye complexes is able to analyse structurally very similar molecules in a complex matrix. One should note, however, that the matrix in our experiments was always the same. For a real application, one would have to consider that the matrix could change (e.g., the blood composition varies from one person to another). These changes could affect the sensors response independently of the concentration of analytes of interest.

Conclusions

We have shown that cross-reactive sensor arrays can be constructed from fluorescent dyes and simple 4d transition-metal complexes. Sensor arrays comprising only six metal–dye combinations displayed a remarkable analytical power: samples containing low-micromolar concentrations of dipeptides were identified with high accuracy, and mixtures of the nonapeptide bradykinin and the decapeptide kallidin could be distinguished. Furthermore, it was possible to obtain information about the identity and quantity of the pharmacologically interesting dipeptides carnosine and homocarnosine in a complex biological matrix. A key advantage of our approach is its simplicity: all components of the sensor array are commercially available or can be easily synthesized, and the individual sensors are rapidly obtained by mixing stock solutions of the respective reagents. The modular nature of our array makes it easy to optimize a sensor for a particular sensing problem by varying the nature and/or the amount of the dyes and metal complexes. Furthermore, the approach should be well suited for parallelization and automatization.

Experimental Section

General: Calcein (Riedel-de-Haën), Calcein Blue (Fluka), Methylcalcein Blue, Nuclear Fast Red, Lumazine, *N*-Methylanthranilic Acid, Ser-Gly, Phe-Pro, Met-Leu, Ala-Phe, Ala-Ala (Sigma–Aldrich), carnosine, Asp-Phe (Acros), bradykinin, kallidin, Val-Phe, Lys-Tyr (Bachem), Trp-Gly, and His-Ala (Senn Chemicals) were used as received. Human serum was purchased from VWR. Homocarnosine[38] and the metal complexes $[PdCl_2(en)]$,[39] $[\{RhCl_2Cp^*\}_2]$[40] and $[\{RuCl_2(p\text{-cymene})\}_2]$[41] were prepared as described in the literature. Phosphate buffer (100 mM, pH 7.0) was prepared with bidistilled H_2O and used for all experiments. Stock solutions of dyes, metal complexes, and peptides were prepared in buffer and stored at 4 °C. Fluorescence measurements were recorded at 25 °C on a Varian Cary Eclipse spectrophotometer equipped with a thermostatted cell holder and a stirring mechanism.

Fluorescence assays with dipeptides: Stock solutions of the respective dipeptide and the dye were diluted with buffered water. The fluorescence signal was measured in a time-resolved fashion at the maximum emission intensity until it stabilized; this ensured the homogeneity and thermal stability of the solution. The reaction was then started by adding a stock solution of the respective metal complex. The final volume was 3.0 mL; the final concentrations were: [dipeptide] = 50 or 20 μM, [dye] = 25 μM, [metal] = 25 μM, [phosphate buffer] = 100 mM. Each of the ten dipeptides was treated with each of the 14 metal–dye combinations indicated in Table 1, and a blank (with no peptide) was also recorded. The experiments were repeated four times (when [dipeptide] = 50 μM or for the blank) or six times (when [dipeptide] = 20 μM), totalling 784 experiments. Fluorescence intensities that were recorded 5, 20, and 60 min after addition of the metal complex were used for multivariate analyses.

Reduced sensor array: see the Supporting Information for the PCA-based determination of the six most important metal–dye combinations (Pd/NFR, Pd/CAB, Rh/NFR, Rh/CAL, Pd/MCB and Rh/CAB) and the results that were obtained by multivariate analysis techniques applied on the fluorescence data recorded by these sensors.

Fluorescence assays with bradykinin (BK) and kallidin (KD)

Sensor subset determination: A selection was performed by comparing the fluorescence signals obtained when a metal complex stock solution was added to a solution containing the respective peptide (BK or KD) and dye. The final concentrations were: [metal] = 10 μM, [dye] = 10 μM (for the cases in which the metal was Rh or Ru), [metal] = 20 μM, [dye] = 20 μM (for the cases where the metal was Pd) and [peptide] = 50 μM. The measurements were repeated with each metal–dye combination (Table 1) for KD and BK. The metal–dye combinations giving the best differentiation between KD and BK were found to be Rh/MCB, Rh/CAB, Rh/CAL, Pd/CAB, Pd/NFR and Pd/LUM (Figure S32 in the Supporting Information) and were therefore used for subsequent experiments.

Differentiation of mixtures of KD and BK: stock solutions of the peptides and the dyes were diluted with buffered H_2O. The fluorescence signal was measured in a time-resolved fashion at its maximum emission intensity until it stabilized; this ensured the homogeneity and thermal stability of the solution. The reaction was then started by adding a solution of the respective metal complex. The final volume was 3.0 mL; the final concentrations were: [total peptide] = 50 μM, [phosphate buffer] = 100 mM; when the metal complex was Rh: [dye] = [Rh] = 10 μM; when the metal receptor was Pd: [dye] = [Pd] = 20 μM. Six mixtures containing different KD/BK ratios (100:0; 80:20; 60:40; 40:60; 20:80; 0:100) and a blank were measured with the six metal–dye combinations indicated above. Each reaction was repeated four times, totalling to 168 experiments. The fluorescence intensities were recorded 2.5, 5, and 10 min after the reaction was initiated when Rh was the receptor, and after 40, 50, and 60 min when Pd was the receptor. These data were used as input values for multivariate analyses.

Fluorescence assays with carnosine and homocarnosine: Serum preprocessing: two volumes of HPLC-grade MeCN were added to one volume of serum. The mixture was vigorously shaken for 5 min at RT, then centrifuged for 30 min at 4000 rpm. The supernatant was removed, filtered and stored at 4 °C.

Fluorescence measurements: The six metal–dye combinations, which were successfully used for the differentiation of the ten dipeptides were employed again (i.e., Pd/NFR, Pd/CAB, Rh/NFR, Rh/CAL, Pd/MCB and Rh/CAB). Stock solutions of the respective peptide and the dye were added to a mixture of serum (prepared as described above) and buffer. The fluorescence signal was measured in a time-resolved fashion at its maximum emission intensity until it was stabilized; this ensured the homogeneity and thermal stability of the solution. The reaction was then started by adding a solution of the respective metal complex. The final volume was 3.0 mL and contained 25% serum, 50% MeCN, and 25% buffered H_2O. The final concentrations were: [peptide] = 25, 50, 75 or 100 μM, [dye] = 25 μM, [phosphate buffer] = 25 mM. The final metal concentration was 500 μM for Pd/NFR, Pd/CAB, Rh/NFR and Rh/CAL mixtures, 300 μM for Pd/MCB mixtures, and 200 μM for Rh/CAB mixtures. Four different concentrations of each dipeptide were reacted with each of the six metal–dye combinations indicated above, and a blank (with no peptide) was also recorded. The experiments were repeated four times, totalling to 216 experiments. The fluorescence intensities that were recorded 5, 20, and 60 min after the reaction was initiated were used for multivariate analyses.

Multivariate analyses: The fluorescence data, recorded after given reaction times (indicated above for each system) at the emission maxima (Table 2), were normalized relative to the fluorescence intensity measured before addition of the metal complex (before quenching occurs). The multivariate analyses (LDA and PCA) were performed with the help of the SYSTAT software package (version 11).[42] Details about the analyses can be found in the Supporting Information.

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