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Journal

Chemical Communications, 55(88)

ISSN

1359-7345

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Publication Date

2019-10-31

DOI

10.1039/c9cc07002h

Peer reviewed

HHS Public Access

Author manuscript

Chem Commun (Camb). Author manuscript; available in PMC 2020 October 31.

Published in final edited form as:

Chem Commun (Camb). 2019 October 31; 55(88): 13259–13262. doi:10.1039/c9cc07002h.

Sensing of Citrulline Modifications in Histone Peptides by Deep Cavitand Hosts

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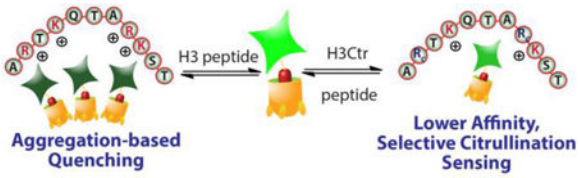
Abstract

Arrayed cavitand:fluorophore sensor complexes can selectively sense small citrulline modifications at arginine residues on post-translationally modified peptides. The sensor can differentiate between different numbers of citrulline modifications, and a simple two-fluorophore, 6-component array can effect cross-reactive discrimination between single modifications in aqueous solution.

Graphical Abstract

A host-guest fluorescence sensor array can site-selectively sense histone peptide modifications that change only a single atom in thepeptide backbone.



Peptide post-translational modifications (PTMs) are a key component of epigenetic regulation in cells,[1] and their importance in disease progression has stimulated a wide scope of study,[2] from their identification[3] to determining their downstream effects.[4] Certain peptide modifications have proved amenable to sensing with small molecule synthetic receptors, and a variety of hosts have been used to bind and sense modified peptides,[5] as well as being used in supramolecular tandem assays of PTM enzyme function.[6] These investigations have generally focused on specific types of modification that are most suitable for host-based recognition, namely lysine and arginine methylations.[5a] Some exquisite

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Conflicts of interest

There are no conflicts to declare.

examples of affinity and selectivity towards methylated lysines (Kme_2, Kme_3),[7] and different methylated arginines (Rme, Rme_2)[8] have been shown with calixarenes and cyclophanes. Resorcinarene-based deep cavitands[9] are also ideally suited for Kme_3 recognition, and we have previously shown their ability to perform site[10] and state-selective[11] recognition of peptides with Kme_3 PTMs, as well as in supramolecular tandem assays of histone methyltransferase and demethylase activity.[12]

There are, however, many more types of histone modifications that mammalian cells exploit for epigenetic regulation.[13] One such example is arginine citrullination, which describes the modification of the guanidinium group in an arginine residue to a urea group (i.e. citrulline, Figure 1). This modification is introduced by protein arginine deiminases (PADs), and over-citrullination has been linked to a number of autoimmune diseases such as lupus and Alzheimer's disease.[14] Selective sensing of these modifications and the function of their modifying PAD enzymes is of great interest in combatting these diseases. To date, this sensing has required covalent chemical probes,[15] and the application of supramolecular host sensors for these small modifications is rare. Host-based sensors almost always require a suitable binding handle that can be targeted by the receptor, one that is large enough for recognition and sufficiently distinct in structure from the unmodified sidechain to allow selectivity. Methylation modifications are ideal, as small hard NH_3^+ cations are replaced by larger, softer $NMe_xH_y^+$ cations, which allows size and cation-π effects to be exploited for selectivity.[16] Phosphorylations are also popular and important targets, as the modification causes a large change in charge and size in the sidechain which can be bound by suitably tailored hosts.[17] As citrullination only converts an NH_2^+ to an O, there is minimal change in residue size (and mass), and only a single loss of a positive charge. Binding unmethylated arginine residues in aqueous solution is challenging by itself, so detecting the conversion of those residues to citrullines in oligopeptides is even more difficult, let alone discriminating between modifications at different sites on the peptide or monitoring the enzymatic modification process. Here, we exploit the sensitivity and selectivity of water-soluble deep cavitands for this purpose: we show that multiple different recognition mechanisms can be exploited to provide cross-reactive sensing of citrulline modifications in cationic histone peptides, with differentiation between multiple modifications at different peptide sites.

Anionic deep cavitand **1** is a uniquely versatile host for peptide modifications, as it can exploit multiple different mechanisms to target different types of PTM. Kme_3 groups bind in the cavity, and fluorescence displacement assays can be exploited to sense these modifications.[10–12] The anionic upper rim of the host and overall lipophilicity also gives **1** strong affinity for long cationic peptides in aqueous solution: the binding affinity of **1** for a 21 residue histone H3 peptide (H3 (1–21)) is $K_a = 2.1 \times 10^5\ M^{-1}$ in Tris buffer at pH 7.4.[18] When the host is combined with a suitable fluorophore, changes in affinity between different peptide strands and the host:fluorophore complex can be monitored, and this has been used to analyze serine phosphorylations and kinase activity. This variable recognition relies on the strong, selective affinity of **1** for cationic peptides, which is lowered upon introduction of negative charge (i.e. phosphate groups). This change in charge from arginine to citrulline is far more subtle, however, and likely far more difficult to detect. As such, we tested a range

of water-soluble hosts and displaceable fluorophores to determine the best combination for sensing citrullination modifications in peptide strands.

Two hosts were used (Figure 1): anionic host **1**, which has shown strong affinity for cationic peptides, and water-soluble octamide host **2**, which can be synthesized in 2 steps from a known octanitro cavitand precursor.[19] The cavity of **2** is identical to that of **1**, but the upper rim amide groups are uncharged, providing a useful control to determine the mechanism of sensing of **1**. Three fluorophores were used: fluorescein guest **G1** and styryl-pyridinium guests **G2** and **G3**. These indicators were chosen as they are capable of selectively sensing different modifications when paired with host **1**: guest **G1** is selective for Kme_3 modifications,[11] whereas guest **G2** is well-suited for detection of serine phosphorylation.[18] Guest **G3** was synthesized to allow variation in the size of fluorophore, while retaining similar properties, to provide an additional element of discrimination for the small changes in peptide target required here. Finally, 4 different 11 amino acid peptides were synthesized corresponding to the Histone H3 sequence (1–11), containing variable citrullinations at either Arg 2 or Arg 8, or at both positions. The peptides were synthesized via SPPS using a CSBio peptide synthesizer, and purified via HPLC after cleavage from the Rink Amide MBHA beads.

The initial tests were to determine the binding properties and emission response between the hosts and fluorophores. The properties of **1•G1** and **1•G2** are known, so we analyzed the **1•G3** and **2•G1-G3** complexes by NMR and fluorescence spectroscopy. Guest **G1** is quenched upon binding in host **1** due to a guest-triggered aggregation and self-quenching process (K_d (**1•G1** = 1.5 μM),[11] whereas guest **G2** is a turn-on fluorophore that shows enhanced emission in cavitand hosts (K_d (**1•G2** = 50 μM) and hydrophobic environments.[18] Interestingly, while host **2** has a highly similar cavity to that of **1**, its molecular recognition properties were quite different. NMR analysis shows that **2** exists in an unfolded dimeric velcrand conformation[19] in solution, and does not bind the NMe_3^+ group in **G1**. The styrylpyridinium dyes **G2** and **G3** bind to both hosts **1** and **2**, with the NR_2 groups positioned in the cavity in each case (minimized structures are shown in Figures 1 and S-11). Guest **G3** binds strongly to both hosts, but DSMI **G2** only binds strongly to **1**: the **2•G2** host:guest complex was not kinetically stable, and fast exchange was observed in the NMR. The emission responses were also tested at the optimal host:fluorophore ratios (20:1.5 μM) - fluorescein guest **G1** shows 90 % quenching by host **1**, and guests **G2** and **G3** show 15.8-fold and 4.8-fold emission enhancement, respectively. Host **2** effects 12.6-fold emission enhancement of **G2**, but only ~30% enhancement of **G3** at the same concentrations. As expected, no quenching of **G1** was observed in **2**.

As a result, we determined the optimal host:guest sensor pairs to be **1•G1**, **1•G2, 1•G3** and **2•G2**, and we tested their responses to the various peptides to determine whether they were affected by the presence of cationic peptides, and whether they were capable of distinguishing the citrulline modifications (see Figure 2 and ESI). We also tested the effect of adding 10 μM metal ions to the system, which have been shown to bind strongly to host **1**, modulate host:peptide interactions and the fluorescence responses.[18,20]

The anionic cavitand **1** showed strong response to the peptides with all fluorophores. The data in Figure 2a clearly shows the cross-reactive quenching of **1•G1** pair upon addition of 10 μM peptide. We have previously shown that **G1** is quenched upon binding to **1** via an aggregative self-quenching process, and in the presence of either cationic peptides or chaotropic salts, the aggregation is enhanced,[18] and emission is further decreased. Upon addition of H3 to the **1•G1** pair, 60% quenching is observed. This quenching is somewhat dependent on the presence of added metal, but in all cases, the unmodified H3 peptide promotes aggregation of the sensor, and "turn-off" sensing. A suggested mechanism is shown in Figure 2 - the anionic **1•G1** complex can interact with the cationic peptides, causing additional self-aggregation and quenching. As the arginine residues are replaced by neutral citrulline residues, the affinity of the aggregated sensor for the peptide lowers, and the turn-off effect is lessened. The peptides with only one modification ($H3R2_{Ci}$ and $H3R8_{Ci}$) only slightly change the emission, but replacing both groups with citrulline modifications ($H3R2_{Ci}R8_{Ci}$) caused a large change in emission. As expected, the loss of two cationic groups had a significant effect on the peptide-**1•G1** interaction and allowing strong cross-reactive selectivity in sensing.

The **1•G2** and **1•G3** complexes behaved very differently, however. The change in fluorescence for **1•G2** was quite variable, with both fluorescence enhancement and quenching observed, depending on the combination of added metals and the differently modified peptides used (Figure 2c) and the pH of the system (Figure S-26). The mechanism of this sensing is evidently complex, but it is clear that the self-aggregation of **1•G2** is variably modulated by the different peptides. The **1•G3** sensor, on the other hand gave a very small response (<10% change in F) from addition of 10 μM peptide (Figure S-30). In the presence of metal salts, loss of fluorescence was observed upon addition of peptide, but this response was identical for all 4 peptides, so we determined that the **1•G3** complex was an ineffective sensor. In addition, the cationic cavitand **2** proved to be ineffective in discriminating the peptides. Addition of each of the H3 peptides causes a 2-fold increase in emission from the **2•G2** complex, interestingly (Figure 2c), but no selectivity was obvious between the different peptides.

The cross-reactive selectivity of the sensing was quantified by subjecting the emission responses to discriminant analysis.[21] The addition of different metal salts is essential to construct an arrayed chemical nose sensor with only a single host molecule. Figure 3 shows two principal component analysis (PCA) scores plots with the **1•G1** sensor, one with an 8-factor array containing no metal or each of the 7 metal salts tested (Figure 3a), or with a minimal 4-factor array consisting of **1•G1** in the presence or absence of the metals with the largest effect on the response, Zn^{2+}, Ni^{2+} or La^{3+} (see Figure S-24 for the raw fluorescence responses). In both cases, the scores plot is extremely one-dimensional, with >98 % of the discrimination occurring along one axis. Despite this, strong discrimination between the unmodified H3 peptide and bis-modified $H3R2_{Ci}R8_{Ci}$ was possible, with the responses well-separated along one (horizontal, PC 1) axis. Interestingly, H3, $H3R2_{Ci}$ and $H3R8_{Ci}$ were also fully discriminated within a 95% confidence range, but the response clusters for those peptides were more closely than $H3R2_{Ci}R8_{Ci}$. Still, the selectivity for small changes in

modified peptides is impressive, especially for such a simple sensor, containing only one host:guest pair and 3 metals.

To further increase the selectivity of the sensor, we combined with **1•G1** pair with **1•G2** to create an optimized array. To maintain the simplicity of the array, we reduced the number of metal components, as can be seen in Figure 4. As might be expected, considering the significant variation in response with **1•G2** and peptides (Figure 2b), the two-fluorophore array was more effective at discriminating the different peptides in two dimensions than the single **1•G1** array, as can be seen by the increased percentage of the PC 2 component in the discriminant analysis (~25% in Figure 4a). Initially a 4 component array was used that paired the **1•G1** and **1•G2** complexes with or without only La^{3+} (Figure 4a). While the scores plot was more two-dimensional, and easily differentiated between H3, $H3R2_{Ci}R8_{Ci}$ and the two singly modified peptides, this minimal array was unable to discriminate between $H3R2_{Ci}$ and $H3R8_{Ci}$. Addition of Zn^{2+} to the array conferred more power to the sensor: while the 2D plot of this 6-component shows very little difference to that of the 4-component array (Figure 4b), there is a significant third component to the discrimination, which is illustrated by the 3D plot in Figure 4c. This PC 3 component can be exploited to differentiate between the two singly modified peptides $H3R2_{Ci}$ and $H3R8_{Ci}$: this simple array is capable of cross-reactive sensing of citrulline modifications at different sites in histone peptides.

In conclusion, we have shown that cavitand:fluorophore complexes are capable of sensing small citrulline modifications at arginine residues on post-translationally modified peptides. Arrayed host:guest complexes can differentiate between different numbers of citrulline modifications, and a simple two fluorophore, 6-component array can effect cross-reactive discrimination between singly modified peptides in buffered aqueous solution, despite the modifications consisting of only changing a single NH group to an oxygen atom in the sidechain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the National Science Foundation (CHE-1707347 to WZ and RJH), and the National Institutes of Health (1R03AI139916 to MX) for support. Daphne Kim is acknowledged for initial synthesis work.

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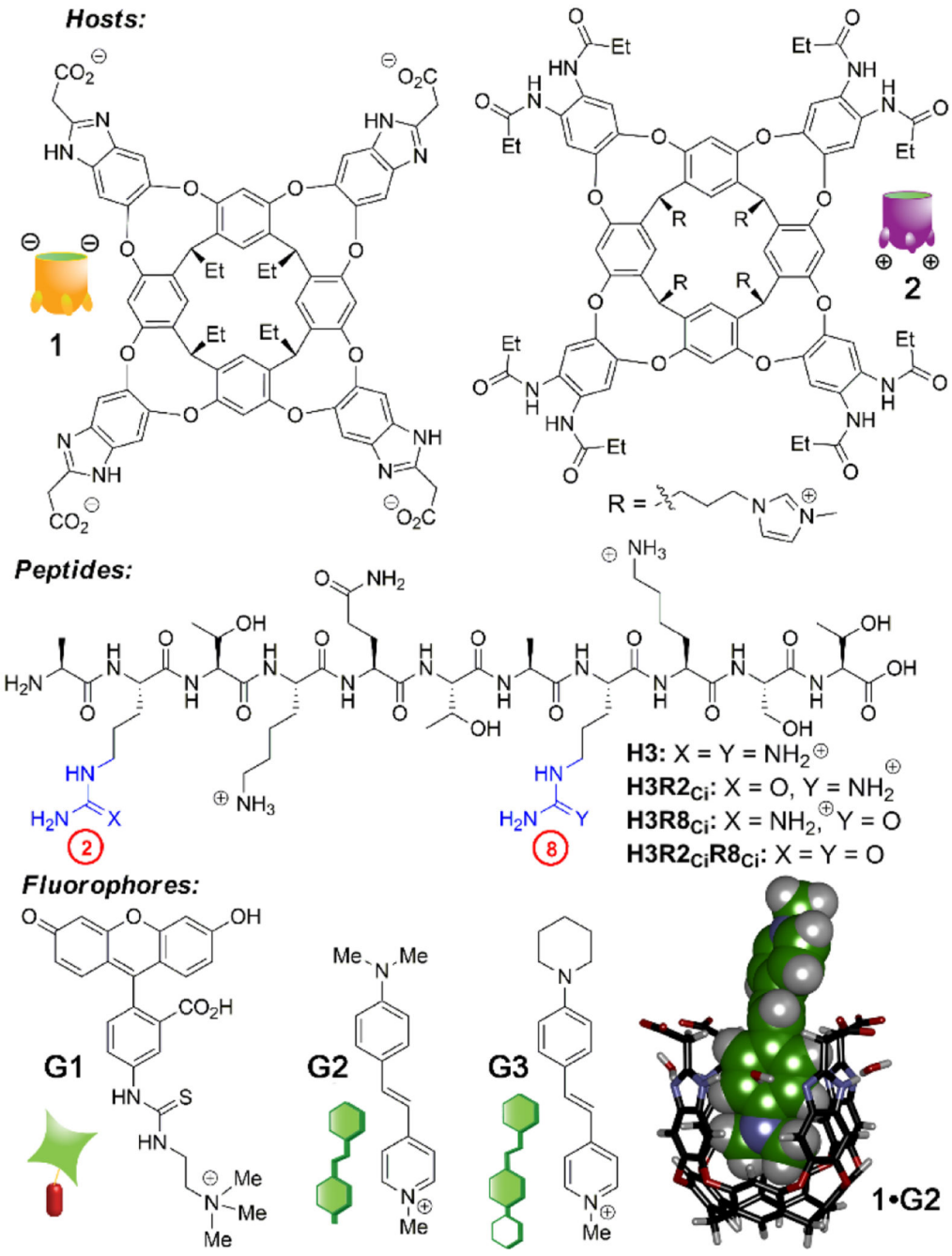

Figure 1.

Structure of hosts and fluorophores tested, as well as modified peptide sequences, and the minimized structure of the **1•G2** complex (SPARTAN, Hartree-Fock).

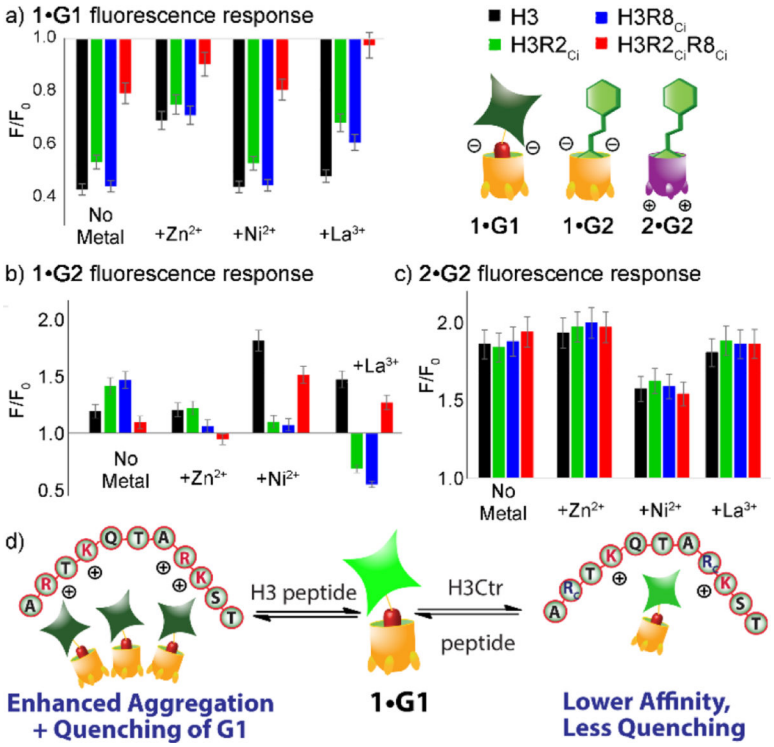

Figure 2.

Fluorescence response of host:guest complexes to citrullinated histone peptides; a) complex **1•G1**; b) complex **1•G2**; c) complex **2•G2**; d) proposed mechanism of discrimination with **1•G1**. [**1,2**] = 20 μM; [**G1–G3**] = 1.5 μM; [M^{2+}] = 10 μM; [peptide] = 10 μM, 20 mM Tris buffer, pH = 7.4.

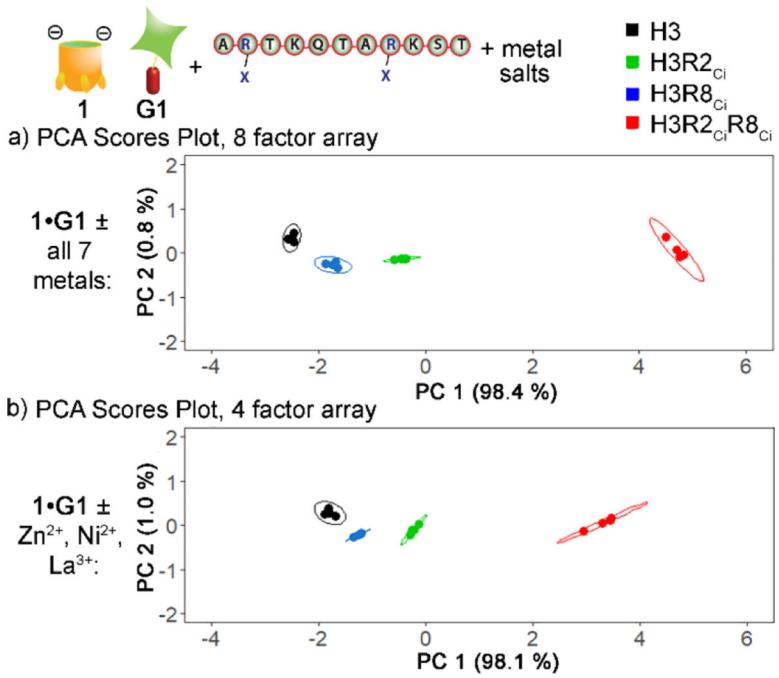

Figure 3.

PCA scores plot for single-fluorophore $H3R_{ci}$ sensing with a) 8 factor array with **1•G1** and either no metal, or 10 μM Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, La^{3+} or Ca^{2+}; b) 4 factor array **1•G1** and either no metal, or 10 μM Zn^{2+}, Ni^{2+}, or La^{3+}. [**1**] = 20 μM; [**G1**] = 1.5 μM; [M^{2+}] = 10 μM; [peptide] = 10 μM, 20 mM Tris buffer, pH = 7.4. Ellipses determined at 95% confidence in RStudio.

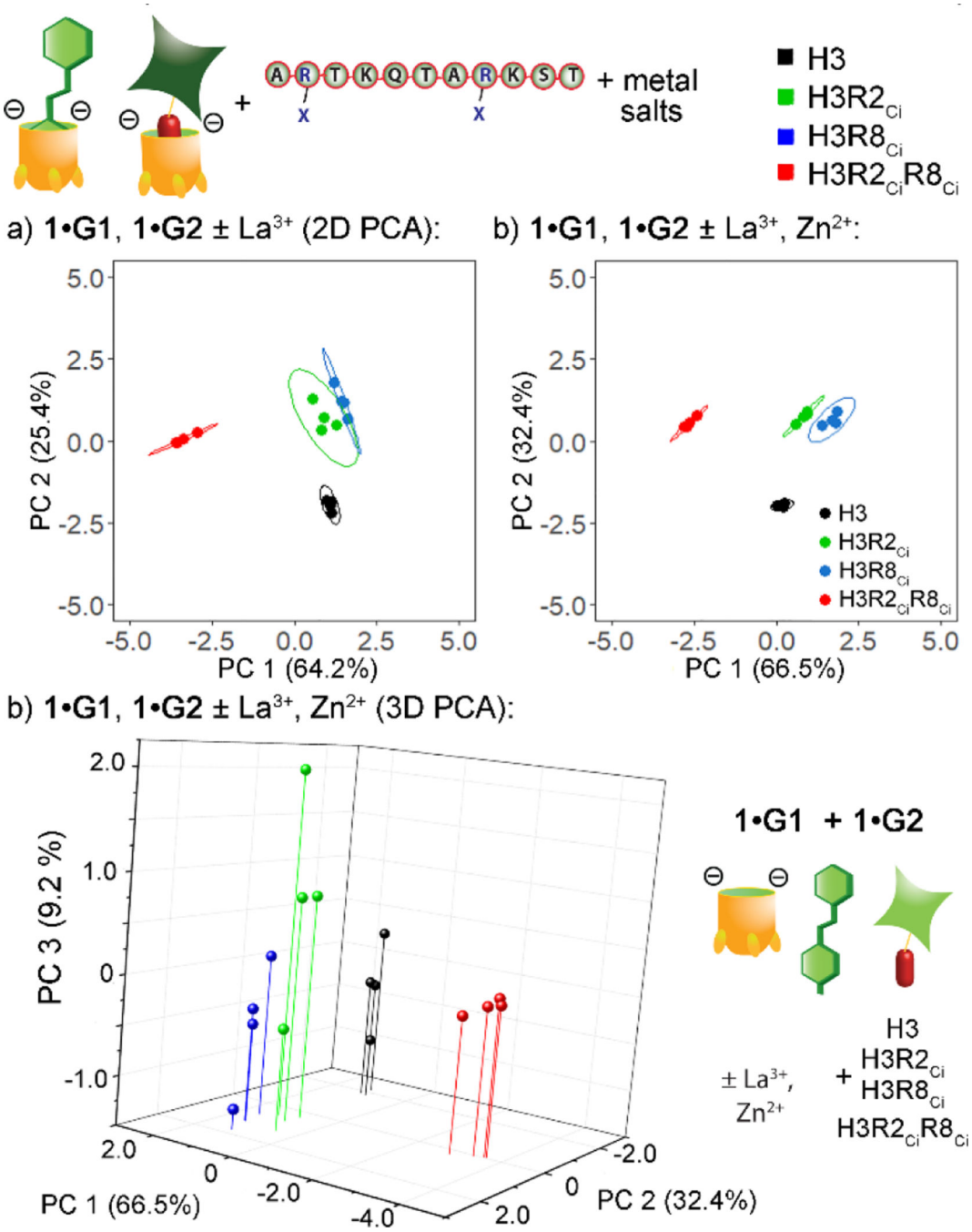

Figure 4.

Optimized $H3R_{ci}$ sensing. a) 2D PCA scores plot from a 4 factor array with **1•G1** or **1•G2** and either no metal or 10 μM La^{3+}; b) 2D PCA scores plot from the optimal 6 factor array with **1•G1** or **1•G2** and either no metal, 10 μM Zn^{2+} or La^{3+}; c) 3D scores plot of the data shown in part b). [**1**] = 20 μM; [**G1,G2**] = 1.5 μM; $[M^{2+}]$ = 10 μM; [peptide] = 10 μM, 20 mM Tris buffer, pH = 7.4. Ellipses determined at 95% confidence in RStudio.