Repurposing an antimicrobial peptide for the development of a dual ion channel/molecular receptor-like platform for metal ion detection†

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The presence of non-essential metals in the environment as contaminants is prone to cause hazardous health problems following accumulation in the human body and the ensuing toxic effects. This calls for continuous discovery and innovation in the realm of developing easy-to-operate, cheap and sensitive sensors. Herein, we describe the proof of concept approach for designing a molecular receptor-like, chimeric sensor based on the pore-forming peptide alamethicin (Alm), tethered via a linker with an ultrashort peptide nucleic acid (PNA) moiety, capable of generating functional ion channel oligomers in planar lipid membranes. The working principle of the sensor exploits the ability of Hg²⁺ ions to complex mismatching thymine–thymine sequences between the PNA receptor moiety on Alm oligomers and free, thymine-based, single-stranded DNAs (ssDNAs) in solution, thus creating a stable base pair at the oligomer entrance. This generates a transducing mechanism which converts the metal ion complexation into a specific electrical signature of the self-assembled Alm oligomers, enabling selective Hg²⁺ ion detection. The platform is programmable, whereby the simple exchange of the PNA sequence and its ssDNA counterpart in solution rendered the system selective for Cu²⁺ ion detection. With further optimization, the presented solution has the potential to translate into miniaturized, cost-effective biosensors suitable for the real-time, label-free and continuous detection of metal ions or other biomolecules.

1. Introduction

In addition to unquestionable economic and societal benefits, the rapid growth over the last few decades of industrial development and the advent of modern technologies have led to dire consequences on the environment through the release of nonessential toxic heavy metal ions, especially Hg²⁺ ions, as contaminants. The prolonged or systematic exposure and bioaccumulation of such non-degradable contaminants trigger harmful effects and lead to illnesses associated with the central nervous system, the immune system, various organ failure or cancer and cardiovascular diseases.1–5

The stringent demand for the design of real-time, reagent-less and noninvasive Hg²⁺ sensitive detectors remains a fundamental challenge despite the existing rich detection strategies involving spectroscopy, colorimetry, voltammetry or impedimetric approaches.6–25 Apart from their technological maturity, most of the existing detection techniques involve multistep processes for Hg²⁺ assessment (i.e., sample collection and pretreatment), rendering them complex to deploy, labor-intensive, and time-consuming and they often require advanced laboratories. These challenges preclude their use for the development of fast-tracking sensors suited for real-time monitoring and assessment in remote sites from the analytical laboratory unit. To mitigate these, there is a constant need for new generations of techniques that embody essential qualities such as cost-effectiveness, accuracy, and portability.

 Fortunately, nanopore-based, single-molecule biosensors have evolved considerably over the past decades as powerful tools and hold great promise for advancing single-molecule detection and analysis in applications covering a large variety of analytes, including but not restricted to stochastic sensing of metal ions.26–28

In one of its original, most useful configurations for metal detection purposes, the nanopore system implicated a heteroheptameric α-hemolysin (α-HL) protein containing a single metal-binding site engineered in the lumen, whose reversible interaction with metals generated stochastic blockades of the
ionic current recorded through the voltage-biased nanopore. The off-line analysis of such blockade events was useful for the multiplex detection and quantification of metal ions. As a distinct innovative strategy, various biomolecules working as chelating agents for distinct metals were coupled with nanoparticles to achieve sensitive and selective detection of metal ions. For example, it was known that DNA molecules interact with metal ions, and inspired by the specific binding of Hg$^{2+}$ ions to thymine–thymine base pairs (T–Hg$^{2+}$–T), T-rich ssDNA probes were designed, which turned into stable hairpin structures upon interaction with Hg$^{2+}$, leading to a distinct current response and metal detection.

In a different approach based on the same chemical mechanism, two DNA strands with thymine–thymine (T–T) mismatches were rationally designed, and their Hg$^{2+}$-mediated hybridization ensued a characteristic ion current blockade whilst interacting with α-HL nanoparticles, thus enabling specific Hg$^{2+}$ detection. By exploiting the metal-induced folding of guanine-rich DNA probes into G quadruplex structures, and their subsequent detection with an α-HL nanopore, the same concept proved useful for the simultaneous detection of Pb$^{2+}$ and Ba$^{2+}$ ions. Witness to its versatility, the same paradigm of employing metal chelating molecules as probes to bind specific metal ions, then interact with a single nanopore, generate specific ionic current blockades and eventually accomplish metal detection was extended to other substrates such as polypeptides, small organic molecules or enzymes.

Despite the successes in detecting and characterizing various analytes at the uni-molecular level, nanopore-based technologies are often affected by experimental drawbacks, including (i) difficulties in isolating and maintaining a single functional nanopore in a free-standing lipid bilayer and (ii) the rate at which molecules are captured, which governs the throughput of blockade events collection and hence the statistical accuracy of the target recognition, is known to be detrimentally affected by: adsorption processes, electroosmosis contributions or even the (sometimes) reduced temporal resolution, in nanopore experiments conducted with widespread amplifiers.

To alleviate these, we set to deliver a solution bridging the desirable qualities such as the elimination of the need for attaining a single nanopore in a supporting lipid membrane and clustering of active probe surfaces to trigger the engagement of multiple spatially constrained probe–target interactions, leading finally to metal detection. In line with these goals and building up on the previous work in which synthetic ion channels based on gramicidin A were proposed for analyte detection, we integrated peptide nucleic acids (PNA) on alamethicin (Alm), generating ion channels with dynamic pore sizes. The oligomerization of the resulting (Alm-PNA) chimeras was found to be controlled by conformational changes following Alm-PNA–ssDNA target recognition events, thus facilitating real-time sensing of short ssDNAs in aqueous solution.

Herein, we extend this discovery and propose a novel class of sensing elements based on structure-switching (Alm-PNA) chimeras, with the ability to detect aqueous Hg$^{2+}$ ions. At its core, the solution was based on appending ultra-short thymine (T)-rich PNA probes on Alm peptides via a flexible linker, generating chimeric Alm-PNA molecules. Under optimal conditions, such constructs successfully co-assemble in planar lipid membranes, forming diameter-variable oligomers while maintaining the PNA probe in the proximity of the oligomer opening.

As a proof of concept, we showcased that the Hg$^{2+}$-mediated hybridization of T-rich PNA moieties from Alm-PNA monomers with mismatched T-rich ssDNA targets present in the aqueous solution vigorously altered Alm-PNA chimera assembly and the mediated ionic current, thus favoring Hg$^{2+}$ detection even in the presence of other metals. By changing the sequence of the PNA moiety, this approach enabled the detection and differentiation of Cu$^{2+}$ ions. We posit that this strategy may be useful for generating highly programmable biosensors, underlining their feasibility for applications including waste management, environmental, or biological monitoring.

2. Results and discussion

2.1 Construction principles of the Alm-PNA chimera and proof of concept of active Alm_T oligomer generation via electrical recordings

We rationally designed Alm-PNA modular constructs consisting of a membrane-active domain, endowed by a modified Alm backbone, and a linker domain connecting a PNA sequence working as a probe to detect Hg$^{2+}$ ions at the Alm C-terminus (Fig. 1, I, a) (for further details see the ESI†).

For the Alm-PNA chimeras used herein, we posit a working mechanism similar to the most accepted one applicable to the native Alm, which involves the interaction of the peptide’s dipole moment with the transmembrane electric fields, leading to the transition from a surface-bound to an inserted orientation followed by the transmembrane lateral aggregation and reversible association of monomers into functional oligomers (Fig. 1, I, a). A first glimpse of encouragement came at the end of a series of screening experiments, whereby we demonstrated that the application of trans negative potentials led to the vigorous electrical behavior of the engineered Alm_T chimeras in planar lipid membranes (Fig. 1, I, b). We also concluded that the multiple conductance levels associated with the oligomer electrical activity, evidenced in Fig. 1, I, b, constituted a good proof of the continuous, reversible accretion of Alm_T monomers to an oligomer, resembling most likely the ‘barrel-stave’-like topology.

2.2 Hg$^{2+}$ metal detection with Alm_T oligomers

To delineate our detection strategy, we hypothesized that in the presence of Hg$^{2+}$ ions added on the cis side of a membrane containing functional Alm_T oligomers, the metal ions would complex neighboring intermolecular T$_5$ PNA moieties belonging to the same oligomer and alter its overall kinetics and/or
conductive properties, thus signaling the presence of Hg\(^{2+}\) ions (Fig. 1, I, a). To our dismay, the experimental evidence contradicted this hypothesis, as Hg\(^{2+}\) addition to the aqueous electrolyte bathing the Alm\(_T\) oligomers did very little to their overall kinetic and transport activity (Fig. 1, I, c). The inability of Hg\(^{2+}\) ions to mediate the formation of mismatched T–Hg\(^{2+}\)–T duplexes between adjacent T\(_5\) PNA moieties on active oligomers indicates that the relative compactness of the Gly–Gly–Ser-based linkers\(^6\) precludes the favorable intermolecular T\(_5\) PNA domain–domain interactions within individual Alm\(_T\) oligomers.

Hence, we shifted our focus and reasoned instead that Hg\(^{2+}\)-induced complexation of T\(_5\) PNA moieties on active oligomers with thymine-rich mismatched ssDNA (T\(_5\)) dissolved in the same electrolyte could be another way to achieve Hg\(^{2+}\) ion detection. The underlying hypothesis was that due to their spatial mobility in the electrolyte, the T\(_5\) ssDNA fragments would be prone to be oriented more easily and achieve efficient Hg\(^{2+}\)-induced complexation to the T\(_5\) PNA probes on the lipid membrane-embedded Alm\(_T\) oligomers (Fig. 1, II, a). In this scenario though, new control experiments were needed to shed light on any putative T\(_5\) ssDNA–Alm\(_T\) interactions, whose manifestation would interfere with actual Hg\(^{2+}\)-induced blockade events. Nevertheless, as all electrophysiology experiments were carried out at \(\Delta V\) negative potentials, as needed to trigger Alm\(_T\) activity in membranes, we had little reason to believe that the anionic T\(_5\) ssDNA fragments would be drawn electrophoretically near membrane-embedded Alm\(_T\) oligomers, and behave as non-specific blockers.

The collected data demonstrated that in the presence of thymine-rich mismatched ssDNA (T\(_5\)) added to the cis electrolyte, the Alm\(_T\) oligomer activity remained largely unchanged (Fig. 1, II, b and c), underpinning the near absence of non-specific Alm\(_T\)–T\(_5\) interactions. Remarkably, the subsequent addition of Hg\(^{2+}\) [50 \(\mu\)M] led to a considerable decrease in the Alm\(_T\) activity (d), whereas cis side addition of EDTA [100 \(\mu\)M] restores the initial activity of Alm\(_T\) oligomers. All experiments were carried out in an electrolyte containing 3 M KCl buffered at pH = 7.

**Fig. 1** Schematic representation of the detection strategy. (I) (a) Mutated variants of Rf30 alamethicin (Alm) were engineered at the C-terminus, via suitable (Gly–Gly–Ser–Gly) linkers (in purple, in the inset), with either poly(T)\(_5\) or poly(G)\(_5\) PNA segments (in green, in the inset). When added on the grounded, cis side of a lipid membrane subjected to a negative potential (\(\Delta V\)) on the trans side, Alm-PNA chimeras – denoted herein by Alm\(_T\) or Alm\(_G\) – insert across the lipid membrane with the N-terminus head-on, oligomerize under the applied potential (\(\Delta V\)), generate functional ion channel-like receptors with the PNA segments exposed to the bulk electrolyte, and mediate ionic current fluctuations across the membrane. As hypothesized, the oligomer-mediated ionic currents were likely to be affected by intermolecular T\(_5\) PNA–Hg\(^{2+}\)–T\(_5\) PNA interactions taking place on the same oligomer. (b) Selected electrophysiology traces displaying the Alm\(_T\) oligomer activity at trans positive or negative potentials, as indicated. The ionic current fluctuations through functional oligomers at negative potentials were recorded as downwardly oriented spikes. (c) Subsequent cis side addition of Hg\(^{2+}\) [50 \(\mu\)M] does not interfere with oligomer activity recorded at \(\Delta V = -140\) mV. (II) (a) The cartoon model of Hg\(^{2+}\)-induced complexation of freely added ssDNA fragments (denoted by free poly, in orange) with Alm\(_T\) oligomers, indicating that the metal-driven hybridization of T\(_5\) ssDNAs (free poly) with dangling PNA segments from functional Alm\(_{PNA}\) oligomers would trigger conformational changes on the Alm\(_T\) oligomer. Selected recordings illustrating the electrical currents mediated by Alm\(_T\) oligomers at \(\Delta V = -140\) mV before (b) and after T\(_5\) ssDNA addition at a 1:1 molar ratio, on the cis chamber (c). Subsequent cis side addition during the same experiment of Hg\(^{2+}\) [50 \(\mu\)M] leads to a considerable decrease in the Alm\(_T\) activity (d), whereas cis side addition of EDTA [100 \(\mu\)M] restores the initial activity of Alm\(_T\) oligomers. All experiments were carried out in an electrolyte containing 3 M KCl buffered at pH = 7.
cis side addition of Hg^{2+} triggered a dramatic decrease of Alm_T oligomer activity and the effect was fully reversible, as further addition of EDTA recovered the oligomer activity (Fig. 1, II, d). We concluded at this point that the recorded decrease of Alm_T activity was very likely due to the Hg^{2+}-mediated, duplex formation between the T5 ssDNAs and respective PNA moiety on Alm_T fragments. Hence, the resulting complexes affected subsequent membrane oligomerization of Alm_T chimeras and ionic transport through the formed oligomers, and thereby indirectly signaled Hg^{2+} presence.

As a next logical step, we sought to elucidate in subsequent experiments the specificity of Alm_T–Hg^{2+}–T5 interactions pertaining to successful Hg^{2+} detection. To test this, we monitored the effect of Hg^{2+} added together with guanine-containing short polynucleotides (G5) on the Alm_T activity. The data displayed in Fig. S1† demonstrate that successive cis side additions during the same experiment of Hg^{2+} and G5 ssDNA on a membrane containing Alm_T oligomers contributed little to the overall kinetic and transport activity of embedded Alm_T oligomers. This is indicative of the absence of non-specific interactions between the Alm_T oligomers and Hg^{2+} ions or G5 ssDNA. Interestingly, further addition during the same experiments of T5 ssDNA fragments led again to a vigorous decrease in oligomer activity (Fig. S1d†), demonstrating that the Alm_T–Hg^{2+}–T5 complexation occurs regardless of the presence of the non-competitive G5 ssDNA in the buffer. As a quantitative analysis, the integrated values of the power spectra of current fluctuations recorded under the conditions specified above (Fig. S1e†) and respectively the I–ΔV diagrams drawn under similar circumstances (Fig. S1f†) provide an estimate of the inhibitory effect triggered by the concomitant presence of T5 ssDNA and Hg^{2+} metals on the Alm_T activity. This is in accordance with previous findings, which established that duplex formation with the T–T mismatch upon the addition of Hg^{2+} is highly specific.63

Subsequent experiments carried out at different transmembrane potentials and over a wider range of cis-added Hg^{2+} metals confirmed unambiguously cis-added Hg^{2+} detection (Fig. 2).

Fig. 2  Hg^{2+} concentration- and voltage-driven effects on the Alm_T–Hg^{2+}–T5 activity recorded in planar lipid membranes. While bathed together with T5 fragments at a 1:1 molar ratio (a), subsequent addition of Hg^{2+} with incremental concentrations (b–d) led to the continuous decrease of Alm_T oligomer activity, as recorded at ΔV = −100 mV. The downward spikes designate the electrical currents mediated by functional oligomers. Adjacent to each recording we display representative zoomed-in excerpts and the all-point histograms of ionic current fluctuations. (e) The voltage-dependent ionic transport through the membrane residing, self-assembled oligomers, revealing disruption of Alm_T self-assembly (or channel blockage) as a function of Hg^{2+} concentration. (f) Alm_T–Hg^{2+}–T5 complex activity is dependent on the applied holding potentials and Hg^{2+} concentration. At the used transmembrane potentials (ΔV), the reported inhibition values were calculated as percentages of the integrated power spectra of ionic current fluctuations corresponding to various concentrations of the cis side added Hg^{2+} relative to the initial state ([Hg^{2+}] = 0). The sigmoidal fits of Alm_T–Hg^{2+}–T5 activity percent inhibition vs. [Hg^{2+}] yielded the mean ± S.E.M. values for the half maximal effective concentration (EC_{50}) and Hill slope (p) of the curves (see also Table 1). All experiments were carried out in an electrolyte containing 3 M KCl buffered at pH = 7.
As reflected by the zoomed-in traces and all-point histograms presented in Fig. 2a–d, the Hg\textsuperscript{2+} effect on individual conductance substrates – indicative of reversible Alm\textsubscript{T} monomer accretion to an existing oligomer under various experimental conditions – was hardly discernible, but easily seen in the I–ΔV diagrams (Fig. 2e) or via fluctuation analysis (vide infra). Thus, in order to efficiently quantify the metal detection capability in our experiments, we resorted to a simpler strategy involving the power-spectrum density analysis of the ionic current fluctuations, specified representatively in Fig. 2a–d, from which we derived the average power in the corresponding current fluctuations over the 1–5000 Hz frequency domain, in the absence and presence of various metals or reagents. We later fitted the concentration dependence of the (Alm\textsubscript{T}–Hg\textsuperscript{2+}–T\textsubscript{5}) complex activity – quantified as the average power of ionic current fluctuations – at three different applied potentials, to a dose–response curve (Fig. 2f)

\[
y = y_{\text{min}} + \frac{y_{\text{max}} - y_{\text{min}}}{1 + \left(\frac{[\text{Hg}^2\text{+}]}{x}\right)^p}
\]

In this expression, \(y_{\text{min}}\) and \(y_{\text{max}}\) refer to the lower and respectively upper asymptotic value of the sigmoidal function, respectively, \(x\) corresponds to [Hg\textsuperscript{2+}] in the bath, \(EC_{50}\) is the half maximal effective concentration and \(p\) is the Hill slope of the curve. At the fit initialization, we set \(y_{\text{min}}\) to 0, because we considered that at vanishingly small Hg\textsuperscript{2+} concentrations, the modification in the (Alm\textsubscript{T}–Hg\textsuperscript{2+}–T\textsubscript{5}) complex activity is virtually absent. The results of the best fit analysis are shown in Table 1.

As evidenced by the data shown in Table 1, the EC\textsubscript{50} of the Hg\textsuperscript{2+} interaction with the presented system lies in a range of tens of μM at the different transmembrane potentials employed, and the corresponding threshold for detection is higher (μM range) as compared to those of other sensors based on the T–Hg\textsuperscript{2+}–T coordination chemistry, lying between tens of mM to 100 nM.\textsuperscript{12,32,34}

There are a number of reasons that could explain the low sensitivity of our detection solution, indicating also the possible venues for optimization, in order to successfully compete with other established sensors, on the sensitivity scale. The most obvious one is represented by the manifestation of intrinsic current fluctuations of the Alm\textsubscript{T} oligomer activity in the absence of metals, which constitute a background noise for Hg\textsuperscript{2+} detection, particularly in the low range of the metal concentration, and precluding the reliable recognition of Hg\textsuperscript{2+}-mediated signals. The second reason is related to the rather small size of the single-stranded PNA overhang (five thymine bases) serving as a detection probe for the aqueous Hg\textsuperscript{2+} via induced stabilization of correspondingly mismatched T\textsubscript{5} ssDNA fragments in solution. A third reason may lie in the peptide linker length and constitution, which control the rotational freedom of the PNA domains engineered on Alm monomers, the steric hindrance between adjacent probes and ultimately the dynamics of changes among different conformations of the Alm\textsubscript{T} oligomers coordinated with ssDNA fragments. Last but not least, while it is known that Cl\textsuperscript{−} ions can bind Hg\textsuperscript{2+} to decrease its effective concentration in aqueous solution,\textsuperscript{34,65} we recall that in our experiments, the salt solution contained 3 M KCl, which might render the effective free Hg\textsuperscript{2+} concentration lower than the weighted value.

### 2.3 Quantifying the detection specificity of Hg\textsuperscript{2+} with the Alm\textsubscript{T}-based chimeras

An important aspect for future consideration of the proposed solution as a useful approach for Hg\textsuperscript{2+} detection was the elucidation of nonspecific interactions of other metals with functional Alm\textsubscript{T} oligomers, which could compromise the detection. To test this, we monitored in different experiments the propensity of the formation of Alm\textsubscript{T}–T\textsubscript{5} duplexes or Alm\textsubscript{T}-metal interactions, in the presence of the cis-added Al\textsuperscript{3+}, Zn\textsuperscript{2+} and Cu\textsuperscript{2+}. As demonstrated in Fig. 3, successive addition of Al\textsuperscript{3+} [50 μM] (a), followed by Zn\textsuperscript{2+} [50 μM] and Cu\textsuperscript{2+} [50 μM], to an electrolyte containing a mixture of Alm\textsubscript{T} [1 μM] and T\textsubscript{5} ssDNA [1 μM] led the activity of Alm\textsubscript{T} oligomers largely unaffected. This phenomenon remained invariant even when the concentration of the aforementioned metals was doubled during the same experiment (Fig. 3e).

To our delight, we noted that further addition of Hg\textsuperscript{2+} [100 μM] instead led to a dramatic decrease of Alm\textsubscript{T} activity (Fig. 3f) – which we attributed to the formation of Alm\textsubscript{T}–Hg\textsuperscript{2+}–T\textsubscript{5} complexes – and this effect was fully reversible upon the addition of excess EDTA (Fig. 3g). The integrated values of the power-spectra of current fluctuations recorded in the presence of various metals shown in Fig. 3a, e and f provided a quantitative assessment of the Hg\textsuperscript{2+}-induced, selective inhibition of Alm\textsubscript{T} oligomer activity and EDTA-triggered recovery (Fig. 3h).

### 2.4 Feasibility of an alternative chimera design for probing Cu\textsuperscript{2+} ions

Based on the compelling evidence suggesting the suitability of our system for Hg\textsuperscript{2+} detection, we set out to investigate the extension and modularity of the proposed paradigm to probing other metal ions. To this end, we used the same detection strategy as above, preserving the overall architecture of the chimeric receptor, but substituted the thymidine in the PNA moiety with guanine bases, which are known to selectively bind Cu\textsuperscript{2+} ions.\textsuperscript{66,67}

The representative outcome of a series of such experiments is shown in Fig. 4. As a first noticeable observation, we discovered that under the exact same conditions, Alam\textsubscript{G} chimeras generated oligomers with a considerably larger activity than

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<th>(\Delta V) (mV)</th>
<th>(EC_{50}) (μM)</th>
<th>(p) (average ± S.E.M.)</th>
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<tr>
<td>−100 mV</td>
<td>20.1 ± 1.3</td>
<td>3.3 ± 0.6</td>
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<tr>
<td>−120 mV</td>
<td>12 ± 1.2</td>
<td>2.2 ± 0.5</td>
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<tr>
<td>−140 mV</td>
<td>32.3 ± 1.9</td>
<td>2.6 ± 0.4</td>
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Table 1 The parametrization of the [Hg\textsuperscript{2+}]- and \(\Delta V\)-dependent Alm\textsubscript{T}–Hg\textsuperscript{2+}–T\textsubscript{5} activity through the Hill equation fitted to the experimentally derived dose–response curves.
Alm\textsubscript{T}, so that in order to prevent lipid membrane rupture, their added concentration on the recording setup was diminished by 10-fold.

Once we pinned down the optimal experimental conditions enabling long-lasting ionic current recordings mediated by Alm\textsubscript{G} oligomers (Fig. 4a), we continued by adding G5 ssDNA fragments during the same experiment, and noted the virtual absence of alterations in the recorded current fluctuations (Fig. 4b). As pointed out above, this experimental observation indicated the inability of G5 to interfere with either reversible formation of Alm\textsubscript{G} oligomers or ion transport mediated by them. The subsequent Cu\textsuperscript{2+} addition triggered a sharp decrease in the Alm\textsubscript{G} oligomer activity (Fig. 4c and d) and the effect was reversible upon chelating Cu\textsuperscript{2+} ions with excess EDTA (Fig. 4e). Quantitatively, these traces were analyzed by estimating the integral power spectra of current fluctuations recorded under the indicated conditions (Fig. 4f), later used to draw the concentration dependence of the (Alm\textsubscript{G}–Cu\textsuperscript{2+}–G5) complex activity (Fig. 4g). The non-linear fit of the derived dose–response curve done as in the preceding experiments resulted in EC\textsubscript{50} = 39.3 ± 2.3 \textmu M and p = 6.1 ± 2. In the absence of additional structural information, we speculate that the increase in the Hill coefficient (p) in Cu\textsuperscript{2+} detection experiments – which provides a minimum estimate of the number of binding sites involved – as compared to that of Hg\textsuperscript{2+} (\textit{vide supra}), may reflect the distinct coordination numbers (4 > 6 for Cu\textsuperscript{2+} and respectively 2 > 4.6 for Hg\textsuperscript{2+}) and metal-base complex geometries of the two ionic species (square planar for Cu\textsuperscript{2+} and respectively linear for Hg\textsuperscript{2+}).

As shown above, to test the nonspecific interactions between other reagents and the sensing element engineered in the sensor’s architecture, we undertook additional control experiments, demonstrating that Cu\textsuperscript{2+} ion detection with the (Alm\textsubscript{G}; G5 ssDNA) system is selective. Data shown in Fig. S2† reveal that \textit{cis} side addition of Hg\textsuperscript{2+} ions on a lipid membrane containing Alm\textsubscript{G} oligomers and bathed in an electrolyte containing 3 M KCl buffered at pH = 7.
Containing G₅ ssDNAs contributed very little to the overall kinetic and transport activity of the embedded oligomers (Fig. S2a and b†). By virtue of a similar rationale as in the case of the present Alm_T system, this strongly suggests the absence of nonspecific interactions between the Alm_G oligomers and Hg²⁺ ions alone and reveals the inability of Hg²⁺ ions to mediate mismatched duplex formation between the G₅ PNA moiety and the G₅ ssDNA. Subsequent addition of Cu²⁺ ions led, however, to a clear decrease in the Alm_G oligomer activity (Fig. S2c†), demonstrating that the Alm_G–Cu²⁺ complexation not only occurs, but it does so regardless of the presence of Hg²⁺ ions in the electrolyte.

To argue more strongly in favor of the validity of the presented detection strategy, we challenged our system by reversing the addition order of Cu²⁺ ions, and bathed the Alm_G sensor with T₅ instead of the G₅ sequence. As displayed in Fig. S3a and b†, the electrical fluctuations elicited by Alm_G oligomers in a lipid membrane were hardly influenced by the presence of the cis-added Cu²⁺ ions alone. The observed mild increase in the activity (see Fig. S3f† for quantitative estimations) is associated most likely with re-homogenization effects in the buffer following reagent addition and stirring. This demonstrates that by their presence alone, Cu²⁺ ions are not capable of generating inter-oligomer Alm_G–Cu²⁺ complexes and a subsequent decrease in their membrane activity. As suggested above, it still remains to be established whether a longer, more flexible linker connecting the Cu²⁺ binding moiety on the oligomers (poly G PNA) to the modified alamethicin backbone would result in a more efficient transduction mechanism, needed to convert the physical capture of Cu²⁺ binding between adjacent Alm_G monomers in an oligomer into a specific electrical signature of the sensor. Further cis side addition of the non-specific T₅ fragments leaves the activity of Alm_G oligomers virtually un-altered (Fig. S3c†). This important observation demonstrates the inability of Cu²⁺ ions to generate Alm_G–Cu²⁺–T₅ complexes, pointing to the presence of G₅ fragments as a critical prerequisite for Cu²⁺ ion detection. As expected, a decrease in Alm_G oligomer activity occurred immediately following G₅ addition (Fig. S3d†), in line with data and interpretation provided in Fig. 4, suggesting the

Fig. 4 Cu²⁺ ions mediate complexation between Alm_G oligomers and G₅ ssDNAs in aqueous solution. The ionic current signature of Alm_G chimeras self-assembled as oligomers in planar lipid membranes held at ΔV = −100 mV, in the presence of successive cis-side additions at the indicated micro-molar concentrations of G₅ ssDNAs (b), Cu²⁺ metals (c and d) and EDTA (e). In the panels beneath each representative trace, we display the all-point histograms of the corresponding current fluctuations. (f) The Fourier decomposition-based, power-spectrum analysis of the traces is shown in panels (a, b, d and e). In the legend we display the extracted average power values contained in such current fluctuations, calculated as the graph’s integrals as indicated above. (g) Alm_G–Cu²⁺–G₅ activity vs. Cu²⁺ concentration reported as inhibition values which were calculated as percentages of the integrated power spectra of ionic current fluctuations corresponding to various concentrations of the cis-side added Cu²⁺ with respect to the initial state ([Cu²⁺] = 0). The sigmoidal fits of the experimentally derived percent inhibition vs. [Cu²⁺] yielded the mean ± S.E.M. values for the half maximal effective concentration (EC₅₀) and Hill slope (p) of the curves (see the text). All experiments were carried out in an electrolyte containing 3 M KCl buffered at pH = 7.
the presented method is quite acceptable in financial terms. Furthermore, the Cu²⁺ ion-mediated formation of Alm_G-G₅ complexes, was found to be reversible upon excess EDTA cis side addition (Fig. S3e†), supporting the validity of Alm_G-Cu²⁺-G₅ complex formation as a main cause for the recorded decrease in the oligomers’ electrical activity.

3. Conclusions

To summarize, we propose a novel construct having at its core ultrashort PNA segments appended to a modified Alm peptide, which could be employed as an ion channel-like platform to detect and monitor selectively the presence of metal ions in a heterogeneous electrolyte. We demonstrate that by changing only the sequence of the PNA binding interface on the Alm-PNA chimeric construct, a different binder-containing nanopore sensor can be obtained and readily implemented into detecting specifically Hg²⁺ or Cu²⁺ in solution. Speaking in favor of the system’s robustness, its stability spans tens of minutes to hours, and under experimental conditions used herein it depends mostly on the initially added concentration of the Alm-PNA construct and the applied ΔV. As we noticed in our experiments, exceedingly larger concentration values beyond those reported (1 μM for Alm_T and respectively 0.1 μM for Alm_G) usually lead to the lipid bilayer destabilization following peptide penetration, especially under larger applied trans-negative potentials. A critical benefit of the proposed solution is facilitated by the modular nature of the sensors. By creating combinatorial libraries of tethered PNA probe binders of varying binding affinity and specificity, our sensors may be further developed to achieve new nanopore sensing systems for concurrently identifying more metal species. Taking into account the concentration threshold set for total mercury values in drinking water by the United States Environmental Protection Agency (USEPA) (2 μg L⁻¹),69 we admit that the Hg²⁺ detection sensitivity of the sensor presented herein situates below the maximum permitted levels in water. Nevertheless, we remind that the presented proof of concept solution is only a first step in the direction of devising simpler and easier to use optimized constructs, complementing traditional instruments designed for the same goal. Speaking in favor of the presented approach though, one should note that in comparison with the existing methods for Hg²⁺ detection, the paradigm presented herein has the advantage that it can be harnessed to detect and monitor the presence of metals in solution in real-time, involving a relatively simple to deploy and inexpensive setup, and with minimum preparatory steps on the active reagents. Rough estimates made regarding the cost of the most expensive ingredients needed for the detection experiments described herein, including only Alm-PNA and ssDNA fragments and excluding all other reagents (e.g., buffers, lipids, and solvents), resulted in a price of ~10 euros per detection trial. It therefore appears that the presented method is quite acceptable in financial terms.

For enhanced Hg²⁺ detection sensitivity prospects – which are applicable for other metal species as well – future steps may include, for instance, the identification of more flexible linker domains to permit metal-mediated hybridization between any two PNA moieties from Alm-PNA-generated oligomers and eliminate the need for ssDNA presence in the recording electrolyte, thus rendering the detection system even simpler. Additionally, optimized length values of the PNA sequence motifs appended to the Alm backbone to determine more stable Alm_T-Hg²⁺-T₅ complexes in the lower regime of metal concentration may boost detection sensitivity. Last but not least, coupling the presented design with microfluidic systems enabling the washout of Alm chimeras from the recording electrolyte upon oligomer formation in the lipid membrane, and therefore eliminating the generation of the bulk-manifested Alm_T-Hg²⁺-T₅ pairs, which constitute a parallel pathway for target depletion in the recording solution,60 is also expected to improve detection sensitivity.

We envision that the presented strategy is prone to generalization, whereby by tethering various receptor-like moieties, e.g. aptamers, nanobodies or monobodies,70,71 to Alm peptides, generating diameter-variable Alm-based oligomers as presented herein, will produce a library of ion channel-like biosensors whose activity becomes highly target-dependent. This, in conjunction with automated and microfluidic lipid bilayer systems72–74 and the already commercially available technologies for the portable, integrated use of lipid bilayer generation and recording systems (i.e., the bilayer lipid membrane chip from ELEMENTS SRL, Italy), has the potential to improve the stability of membranes, minimize the volume of electrolyte solutions required and provide a more robust lipid membrane-based chimeric sensor.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Data availability

The data supporting this article have been included as part of the ESL† Data are available upon request from the authors.

Conflicts of interest

The authors declare no conflicts of interest.

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