A Single-Molecule Observation of Dichloroaurate(I) Binding to an Engineered Mycobacterium smegmatis porin A (MspA) Nanopore

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ABSTRACT: Gold(I) compounds are known to bind sulfur-containing proteins, forming the basis in the design of gold(I)-based drugs. However, the intrinsic molecular mechanism of the chemical reaction is easily hidden when monitored in ensemble. We have previously demonstrated that Mycobacterium smegmatis porin A (MspA) can be engineered (MspA-M) to contain a specialized nanoreactor to probe chemical reactions involving tetrachloroaurate(III). Here, we provide further investigations of coordination interactions between dichloroaurate(I) and MspA-M. Gold compounds of different coordination geometry and valence states are as well probed and evaluated, demonstrating the generality of MspA-M. With single-molecule evidence, MspA-M demonstrates a preference for dichloroaurate(I) than tetrachloroaurate(III), an observation in a single molecule that has never been reported. By counting the maximum number of simultaneous ion bindings, the narrowly confined pore restriction also efficiently distinguishes dichloroaurate(I) and tetrachloroaurate(III) according to their differences in geometry or size. The above demonstration complemented a previous study by demonstrating other possible gold-based single-molecule chemical reactions observable by MspA. These observations bring insights in the understanding of gold-based coordination chemistry in a nanoscale.

INTRODUCTION

Gold(I) compounds, acknowledging their unique chemical properties, have been widely used as core components in nanoparticle synthesis,8−10 gold catalysis,11 electrochemical analysis,12−14 and biomedical applications.5−7 Gold(I) compounds demonstrate a general preference for thiols or thioethers,8,9 a property widely applied in the design of gold(I)-based drugs. However, the intrinsic molecular mechanism of the chemical reaction is easily hidden when monitored in ensemble. To achieve therapeutic effect, it is generally recognized that the therapeutic mechanism of gold(I) drugs is to form complexes with sulfur-containing amino acids in proteins, by which it inhibits certain enzymatic activities to achieve therapeutic effects. However, the detailed biochemical processes and the exact therapeutic mechanism of gold(I) drugs are not fully confirmed,7 likely due to the high complexity and the small scale of the system. By engineering a biological nanopore properly, chemical reactions between a gold compound and an amino acid may be directly monitored. This strategy was previously reported, from which the chelation of metal ions by amino acids,13−18 the metal coordinating with biomolecules,17,19 and the observation of intermediates in a catalytic cycle20 were observed from differently engineered α-hemolysin nanopores.

A previous investigation led by us reported a specially engineered Mycobacterium smegmatis porin A (MspA) nanopore mutant, of which the introduced methionine to the pore (MspA-M) plays a crucial role in triggering the formation of methionine-gold coordination.17 Though MspA and α-hemolysin share common advantages such as a rigid β-barrel structure, a high stability, and an ease of preparation, the overall conical structure of MspA is more advantageous as a single-molecule nanoreactor by maximizing the amplitude of single-molecule binding events and minimizing the contribution of undesired reactive residues away from the pore restriction. MspA-M directly reports binding from tetrachloroaurate(III) ([AuCl₄]⁻) and other related reactions involving a variety of biothiols. In this work, gold(I)

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Compounds are as well studied with MspA-M, complementary to the previous investigations with only gold(III) compounds. Reactive kinetics of gold(I) compounds with different coordination configurations such as dichloroaurate(I) \( ([\text{Au}^{III}\text{Cl}_2]) \) or chloro(tetrahydrothiophene) gold(I) \( ([\text{Au}(\text{tht})\text{Cl}]^{-}) \) were as well evaluated. With single-molecule evidence, the thioether group demonstrates a clear preference for \( [\text{AuIICl}_2]^{-} \) over \( [\text{AuIIICl}_4]^{-} \), which is the first report in the single-molecule level. The above investigation of \( [\text{AuIICl}_2]^{-} \) binding to methionine has demonstrated the first quantitative and single-molecule study of Au-methionine interactions and may inspire follow-up studies to evaluate the design of new gold(I)-based drugs in a nanopore cavity or to monitor their metabolism kinetics.

**Experimental Section**

**Reagents.** Hexadecane, pentane, ethylenediaminetetraacetic acid (EDTA), Genapol X-80, chloroauric acid (HAuCl\(_4\), 99.999%), gold(I) chloride (AuCl, 99.99%), and chloro(tetrahydrothiophene) gold(I) (Au(tht)Cl) were purchased from Sigma-Aldrich. 1,2-Diphtyranoyl-sn-glycerol-3-phosphocholine (DPhPC) was from Avanti Polar Lipids. Dioxane-free isopropyl-\( \beta \)-d-thiogalactopyranoside (IPTG), tris(hydroxymethyl)aminomethane (Tris), and imidazole were from Solarbio. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was from Shanghai Yuanye Biotechnology. E. coli strain BL21 (DE3) was from Biomed. Lysogeny broth (LB) and LB agar were from Hopebio. Hydrochloric acid (HCl) was purchased from Sinopharm. Potassium chloride (KCl), sodium chloride (NaCl), \( \text{Na}_2\text{N}_4\text{dimehtylformamide (DMF)}, \text{Na}_2\text{HPO}_4\), and sodium dihydrogen phosphate (\( \text{Na}_2\text{HPO}_4\)) were purchased from Aladdin. The potassium chloride buffer (1.5 M KCl, 10 mM Tris-HCl, pH 7.0) was prepared with Milli-Q water as a stock solution (30 mM) for subsequent experiments. AuCl was dissolved in the potassium chloride buffer (1.5 M KCl, 10 mM Tris-HCl, pH 7.0) to reach a final concentration of 1 mM, while Au(tht)Cl was dissolved in DMF (final concentration 1 mM) prior to each experiment.

**MspA Preparation.** The M2MspA (D93N/D91N/D90N/D118R/D134R/E139K) and MspA-M (D93N/D91M/D90N/D118R/D134R/E139K) nanoparticles were prepared as previously published. \(^{17,21}\) The aforesaid amino acid substitutions from WT MspA \(^{22}\) are designed to neutralize original negative charges in the pore lumen for an enhanced capture rate for anions, such as DNA \(^{23}\) or possibly dichloroauroate(I). Since the M2MspA does not have methionine or cysteine on the inner surface of the pore lumen, it is a clean template to which a methionine can be introduced. Detailed protein preparation steps are as follows. First, the genes coding for M2MspA and MspA-M with an extra histidine tag on the C-terminus were custom synthesized and, respectively, constructed in pET-30a (+) plasmids (GenScript). To express either type of nanopore, the corresponding plasmid was heat-shock transformed into the E. coli strain BL21(DE3) cells. The cells were grown in LB medium. After they reached an OD\(_{600}\) = 0.7, they were induced by 1 mM IPTG and shaken overnight at 16 °C. After expression, both M2MspA and MspA-M spontaneously assemble into an octameric form. Subsequently, the cells were harvested by centrifugation (2546g, 20 min, and 4 °C) and resuspended in the lysis buffer (100 mM Na\(_2\)HPO\(_4\)/NaH\(_2\)PO\(_4\), 0.1 mM EDTA, 150 mM NaCl, 0.5% (w/v) Genapol X-80, pH 6.5) and heated to 60 °C for 10 min. The suspension was then cooled on ice for 10 min and centrifuged (16 200g, 40 min, and 4 °C) to collect the supernatant. After syringe filtration, the supernatant was applied to a nickel affinity column (HisTrapTM HP, GE Healthcare). To collect the MspA octamer, the column was first eluted with buffer A (0.5 M NaCl, 20 mM HEPES, 5 mM imidazole, 0.5% (w/v) Genapol X-80, pH = 8.0) and subsequently eluted with a linear gradient between buffer A (0.5 M NaCl, 20 mM HEPES, 5 mM imidazole, 0.5% (w/v) Genapol X-80, pH = 8.0) and buffer B (500 mM imidazole, 0.5 M NaCl, 20 mM HEPES, 0.5% (w/v) Genapol X-80, pH = 8.0). Different fractions of eluants were sequentially collected and characterized by 12% sodium dodecyl sulfate (SDS)-poly(acrylamide) gel electrophoresis. According to a previous report, \(^{17,21}\) the band corresponding to the MspA octamer is located at a position approximately equivalent to the 100 kDa marker protein. The desired fraction was aliquoted and stored at ~80 °C for long-term storage. The aliquoted fraction was directly used for all follow-up electrophysiology experiments without any further purifications. The plasmid used for the preparation of MspA-M is shared by Molecular Cloud with a request ID: MC_0010018. A citation is requested when published with this plasmid DNA.

**Electrophysiology Recording.** Electrophysiology recordings were performed on a custom-made measurement chamber as described previously. \(^{17}\) The chamber possesses a 500 μL volume on each side and is physically separated by a Teflon film (30 μm thick) with an orifice (ø = 100 μm). The orifice in the film was pretreated with 0.5% (v/v) hexadecane in pentane and air-dried thoroughly. Both chambers were then filled with the electrolyte buffer (1.5 M KCl, 10 mM Tris-HCl, pH 7.0). DPhPC was then added to the buffer. A self-assembled lipid bilayer spontaneously forms after the liquid is pipetted into the chamber up and down several times. A pair of Ag/AgCl electrodes was, respectively, placed in both sides of the chamber, respectively in contact with the aqueous buffer. By convention, the side that is electrically grounded is defined as the cis side, whereas the opposing side is defined as the trans side.

Biological nanopores (M2MspA or MspA-M) were added to the cis side. Single-channel recording was performed when a single nanopore was inserted in the bilayer. All single-channel recordings were performed by an Axopatch 200B patch clamp amplifier and digitally sampled by a Digidata 1500 B digitizer at a sampling frequency of 25 kHz (Molecular Devices) and low-pass-filtered with a corner frequency of 1 kHz. The analyte (HAuCl\(_4\), AuCl, or Au(tht)Cl) was added in the cis chamber to reach a desired final concentration. If not otherwise stated, all single-channel recordings were acquired with a +100 mV continuously applied potential.

**Data Analysis.** The recorded traces were further digitally filtered with a 200 Hz low-pass Bessel filter (eight-pole). Nanopore events were detected by the single-channel search feature in Clampfit 10.7 (Axon Instruments). Further analyses (histogram, curve fitting, and plotting) were performed in Origin 9.1 (Origin Lab).
A +100 mV potential was continuously applied to electrophoretically drive \([\text{AuICl}_2^-]\)
and anionic. Disproportionation of gold(I) chloride to AuIII
was clearly increased when the addition of gold(I) chloride was
increased (Figure 1c), indicating that the events result from
the gold(I)-methionine interaction is stochastically distributed,
and a +100 mV potential continuously applied, successive
binding events were clearly observed, in the form of reversible
pore blockages. \([\text{AuICl}_2^-]\) is an anion; when placed in cis, a
positively applied potential would electrophoretically drive \([\text{AuICl}_2^-]\) to translocate through the pore. The
engineered MspA (MspA−M) possesses methionine residues at site 91 (yellow) and is capable to bind individual \([\text{AuICl}_2^-]\). (b) The model for \([\text{AuICl}_2^-]\) binding with MspA−M. \([\text{AuICl}_2^-]\) reversibly coordinates with the thioether group on the methionine residue to form the S−Au bond. (c) Representative traces containing \([\text{AuICl}_2^-]\) binding events acquired with varying analyte concentrations. Gold(I) chloride was placed in cis with a final concentration of 0−350 nM. The two current levels correspond to the state when the pore is unoccupied (\(I_0\)) or occupied with a \([\text{AuICl}_2^-]\) (\(I_1\)). The frequency of binding events increases when the gold(I) chloride concentration is increased. (d) Kinetics of \([\text{AuICl}_2^-]\) binding. (top) A simplified model of \([\text{AuICl}_2^-]\) binding with a methionine residue on the pore. (bottom) Plots of the reciprocals of the mean inter-event intervals (\(1/\tau_{on}\)) and the mean residence time (\(\tau_{off}\)) vs \([\text{AuICl}_2^-]\) ion concentration in cis (Table S2). Means and standard deviations of \(\tau_{on}\) and \(\tau_{off}\) were from three independent experiments (\(N = 3\)) with 20 min of recording for each condition.

## RESULTS AND DISCUSSION

### Methionine Dichloroaurate(I) Coordination in MspA.

All measurements were performed in a custom nanopore measurement chamber, as described in the Experimental Section. Gold(I) chloride, when dissolved in a high-chlorine buffer, forms dichloroaurate(I) (\([\text{AuCl}_2^-]\)), which is linear and anionic.\(^{20}\) Disproportionation of gold(I) chloride to AuIII and Au is negligible at the time scale of this measurement.\(^{24}\) Experimentally, gold(I) chloride was added to the cis side of the chamber. When electrophoretically driven by a positively applied voltage, dichloroaurate(I) migrates through the nanopore. During a single-channel recording, when a proper interaction between the pore restriction and dichloroaurate(I) is established, single-molecule binding events can be observed and vice versa (Figure 1a). The mutant M2MspA (D93N/D91N/D90N/D118R/D134R/E139K),\(^{25}\) which possesses no sulfur-containing amino acids (methionine or cysteine) in its pore lumen, reports no binding from dichloroaurate(I) and thus serves as an ideal pore-engineering template (Figure S1). The MspA−M mutant (D93N/D91M/D90N/D118R/D134R/E139K), a previously reported mutant to report Au(III)-methionine binding kinetics acknowledging the introduced methionine residue,\(^{17}\) was applied all through the paper to investigate Au(I)-methionine binding kinetics acknowledging previous reports describing that Au(I) demonstrates binding to methionine residues.\(^{9}\)

Similar to that previously reported with tetrachloroaurate(III) (\([\text{AuCl}_4^-]\))\(^{17}\) \([\text{AuCl}_2^-]\) also reports clear binding events when measured with MspA−M (Figure 1a,b). Experimentally, with the addition of gold(I) chloride (50−350 nM) to cis and a +100 mV potential continuously applied, successive binding events were clearly observed, in the form of reversible pore blockages. \([\text{AuCl}_2^-]\) is an anion; when placed in cis, a positively applied potential would electrophoretically drive the \([\text{AuCl}_2^-]\) into the pore constriction, efficiently generating the appearance of binding events. The rate of event appearance was clearly increased when the addition of gold(I) chloride was increased (Figure 1c), indicating that the events result from dichloroaurate(I) intentionally added. Acknowledging the conical pore geometry of MspA, which effectively amplifies perturbations resulted from single-molecule chemistry bindings by focusing ionic flows to the pore restriction, the events of \([\text{AuCl}_2^-]\) binding report clearly noticeable current blockages (∆I), measuring 10.7 ± 0.5 pA, even if a sole \([\text{AuCl}_2^-]\) measures only few angstroms in size. The event dwell time of the gold(I)-methionine interaction is stochastically distributed, a phenomenon observed in all single-molecule chemistry studies performed by a nanopore. The mean event dwell time measures 1094.0 ± 19.5 ms, perfectly resolvable by single-channel recordings. Ten minutes of continuous recordings were performed, from which a few hundreds of events were collected to derive the results (\(N = 3\), Figure S2, Table S1). Though the mean event dwell time is mainly determined by the molecular interaction strength being probed, it may also be

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**Figure 1.** \([\text{AuCl}_2^-]\) binding to MspA−M. (a) The schematic diagram of nanopore sensing. A nanopore sensing device is separated by a lipid bilayer into the cis and the trans compartments. A single MspA−M nanopore was inserted in the lipid bilayer, forming the only pathway between the cis and the trans compartment for ions to transport through. Gold(I) chloride (yellow spheres) was added in cis reaching a desired final concentration. A +100 mV potential was continuously applied to electrophoretically drive \([\text{AuCl}_2^-]\) to translocate through the pore. The engineered MspA (MspA−M) possesses methionine residues at site 91 (yellow) and is capable to bind individual \([\text{AuCl}_2^-]\). (b) The model for \([\text{AuCl}_2^-]\) binding with MspA−M. \([\text{AuCl}_2^-]\) reversibly coordinates with the thioether group on the methionine residue to form the S−Au bond. (c) Representative traces containing \([\text{AuCl}_2^-]\) binding events acquired with varying analyte concentrations. Gold(I) chloride was placed in cis with a final concentration of 0−350 nM. The two current levels correspond to the state when the pore is unoccupied (\(I_0\)) or occupied with a \([\text{AuCl}_2^-]\) (\(I_1\)). The frequency of binding events increases when the gold(I) chloride concentration is increased. (d) Kinetics of \([\text{AuCl}_2^-]\) binding. (top) A simplified model of \([\text{AuCl}_2^-]\) binding with a methionine residue on the pore. (bottom) Plots of the reciprocals of the mean inter-event intervals (\(1/\tau_{on}\)) and the mean residence time (\(\tau_{off}\)) vs \([\text{AuCl}_2^-]\) ion concentration in cis (Table S2). Means and standard deviations of \(\tau_{on}\) and \(\tau_{off}\) were from three independent experiments (\(N = 3\)) with 20 min of recording for each condition.

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**Figure S1.** Schematic diagram of the engineered MspA shown as a ribbon model. The MspA−M mutant (D93N/D91M/D90N/D118R/D134R/E139K) possesses no sulfur-containing amino acids in its pore lumen, enabling the detection of gold(I) chloride binding. (top) Methionine residue 91 (yellow) binds gold(I) chloride, \([\text{AuCl}_2^-]\). (bottom) The molecular structure of \([\text{AuCl}_2^-]\) in cis shows that the gold(I) chloride interacts with the thioether group of methionine 91. The gold(I) chloride \([\text{AuCl}_2^-]\) is surrounded by water molecules (green) and chlorides (yellow) that bind the gold(I) chloride to the pore constriction, eventually generating the appearance of binding events.
affected by environmental conditions such as the measurement temperature, the applied potential, or the pH of the electrolyte buffer. However, to be consistent all through this manuscript, all measurements were performed in a 25 °C environment with a 1.5 M KCl, pH 7.0 buffer, and a +100 mV bias was continuously applied.

Together with the negative results acquired from the M2MspA mutant (Figure S1), the origin of this binding mechanism is clearly from the introduced methionine around the pore restriction. As previously investigated in an ensemble by spectroscopy methods,7 [AuCl]− reversibly forms a transient Au–S coordination bond with the thioester group of a methionine (Figure 1b). However, a direct, dynamic, and single-molecule observation of this coordination chemistry between [AuCl]− and a methionine were never reported, to the best of our knowledge.

Quantitatively, the mean inter-event interval (τon) and dwell time (τoff) in the concentration range of 50–350 mM were derived from the single exponential fitting results (Figure S2), with which plots of the measured rates versus [AuCl]− concentration were demonstrated (Figure 1d). The reciprocal of the mean inter-event interval (1/τon) is clearly proportional to the [AuCl]− concentration, which is consistent with a bimolecular model, in which 1/τon = koff/kon[ [AuCl]− ] and koff is the association constant. In contrast, the reciprocal of the mean dwell time (τoff) of the complex is independent of the [AuCl]− concentration, which is consistent with a unimolecular dissociation mechanism (1/τoff = koff/kon = τoff is the dissociation constant) (Figure 1d). The association constant kon = (5 ± 4) × 10^4 M^−1 s^−1, the dissociation constant koff = 1.03 ± 0.05 s^−1, and the formation constant of K = (5 ± 4) × 10^4 M^−1 (K = kon/koff) were consequently derived to quantitatively describe the observed single-molecule chemical reactions, when probed in a confined pore geometry. All measurements were performed as separate 10 min continuous recordings. To compensate the low frequency of event appearance at a 50 nM final concentration of [AuCl]−, 20 min continuous recordings were performed instead (N = 3, Table 1, Table S2).

Alternatively, tetrahydrothiophene-coordinated AuI complex chloro(tetrahydrothiophene) gold(I) (AuI(tht)Cl), of which the tetrahydrothiophene ligand is easily substituted with other strong ligands, was applied to MspA-M as well. Similar to gold(I) chloride, the addition of AuI(tht)Cl to cis with a final concentration of 50–200 nM resulted in reversible current blockages, measuring 10.9 ± 0.5 pA in amplitude (ΔI) and 934.9 ± 23.4 ms in dwell time (τoff) (N = 3, Figure 2, Table S3). The reaction kinetics constants of AuI(tht)Cl with methionine were as well derived: k_on = (1.1 ± 0.4) × 10^6 M^−1 s^−1 and k_off = 1.09 ± 0.05 s^−2; with which the formation constant is K = (1.0 ± 0.4) × 10^4 M^−1 (K = k_on/k_off) (N = 3, Table S4). The above demonstration clearly confirms the generality of MspA-M in the study of a variety of AuI compounds. When compared with results acquired from dichloroaurate(I), clearly different coordinated ligands can significantly affect the reaction kinetics between gold(I) and methionine, which may provide a reference for the design and screening of gold(I)-based drugs.
Figure 2. AuI(tht)Cl binding to MspA−M. Chloro(tetrahydrothiophene) gold(I) (AuI(tht)Cl) is a well-investigated gold(I) complex. The tetrahydrothiophene ligand can be easily substituted with other stronger ligands, a mechanism widely applied to prepare other gold compounds.31 Single-channel recording was performed with MspA−M (Experimental Section). AuI(tht)Cl was added to cis, reaching a final concentration of 0–200 nM. (a) Representative traces containing AuI(tht)Cl binding events. The frequency of AuI(tht)Cl binding was increased when the final concentration of AuI(tht)Cl in cis was raised. (b) Plots of the reciprocals of the mean inter-event intervals ($\tau_{on}$) and the mean residence time ($\tau_{off}$) vs the AuI(tht)Cl concentration in cis (Table S3). Means and standard deviations of $\tau_{on}$ and $\tau_{off}$ were from three independent measurements of 15 min of recording for each condition ($N = 3$).

Figure 3. Mean inter-event intervals ($\tau_{on}$) of [AuIIICl4]$^-$ or [AuICl2]$^-$ binding. Single-channel recordings were performed using MspA-M, as described in the Experimental Section. Chloroauric acid or gold(I) chloride was, respectively, added to cis with a 1 μM final concentration. A +100 mV potential was continuously applied. (a, b) The histogram of inter-event intervals ($\tau_{on}$) of [AuIIICl4]$^-$ (a) or [AuICl2]$^-$ (b) bindings. In (a, b), the mean inter-event interval ($\tau_{on}$) was derived from single-exponential fittings. (c) The histogram plot of the reciprocals of the mean inter-event intervals ($\tau_{on}$) of [AuIIICl4]$^-$ (green) and [AuICl2]$^-$ (red) bindings. AuIII: 0.24 ± 0.02 s$^{-1}$; AuI: 2.0 ± 0.6 s$^{-1}$. Means and standard deviations of $\tau_{on}$ were from three independent experiments ($N = 3$) with 10 min of recording for each condition.

Figure 4. A comparison between [AuIIICl4]$^-$ and [AuICl2]$^-$ binding events. Single-channel recordings were performed using MspA-M, as described in the Experimental Section. Chloroauric acid or gold(I) chloride was added to the different nanopores with the concentration of 1 μM and 350 nM, respectively. A +100 mV potential was continuously applied. (a) Molecular structures of [AuIIICl4]$^-$ and [AuICl2]$^-$: [AuIIICl4]$^-$ forms a square-planar configuration, whereas [AuICl2]$^-$ forms a linear configuration. (b) The scatter plots from [AuIIICl4]$^-$ (green) or [AuICl2]$^-$ (red) bindings. The distribution of events is plotted according to the current blockage ($\Delta I_0$) and the dwell time ($t_{off}$). (c) The histogram plots of $\Delta I_{0-1}$ of [AuIIICl4]$^-$ (green) or [AuICl2]$^-$ (red) bindings. The results indicate that the current blockades of [AuIIICl4]$^-$ and [AuICl2]$^-$ are almost indistinguishable. (d) The histogram plots of dwell time ($t_{off}$) of [AuIIICl4]$^-$ (green) or [AuICl2]$^-$ (red) bindings. The mean inter-event intervals were, respectively, derived from single-exponential fitting results.
measured by \([\text{AuIIICl}_4]\)

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in the dissociation rate between \([\text{AuIIICl}_4]\)

constant \(k\) of \([\text{AuIIICl}_4]\)

is \(5 \pm 4\) \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \) (Table 1), or 5.6 times of that measured by \([\text{AuIIIICl}_4]^–\).

When dissolved in an aqueous high-chlorine buffer, \([\text{AuCl}_4]^–\) and \([\text{AuIIICl}_4]^–\) form different coordination configurations, of which \([\text{AuIIICl}_4]^–\) is square-planar and \([\text{AuCl}_4]^–\) is linear (Figure 4a).\(^{29}\) However, when identically probed by MspA-M, the current blockages of the two gold ions produce indistinguishable event amplitude \(\Delta I\) (Figure 4b,c), whereas binding of \([\text{AuCl}_4]^–\) generates significantly time-extended dwell time, as demonstrated from the event scatter plots (Figure 4b). The comparisons of the mean dwell time \((\tau_{\text{dwell}})\) are shown in Figure 4d, which summarize the difference in the dissociation rate between \([\text{AuCl}_4]^–\) and \([\text{AuIIICl}_4]^–\) (Figure S4).

The derived dissociation rate follows a unimolecular dissociation mechanism \((1/\tau_{\text{dwell}} = k_{\text{off}})\), and the dissociation constant \(k_{\text{off}}\) of \([\text{AuIIICl}_4]^–\) is \(2.40 \pm 0.13 \text{ s}^{-1}\), while that of \([\text{AuCl}_4]^–\) measures \(1.03 \pm 0.05 \text{ s}^{-1}\) (Table 1), meaning that the M–Au complex forms a much more extended bonding lifetime than that of M–Au\(^{1+}\), when equally probed by MspA-M. \([\text{AuCl}_4]^–\) has a higher association rate and a slower dissociation rate than \([\text{AuIIIICl}_4]^–\) when binding to a throouter group, which shows that \([\text{AuCl}_4]^–\) exhibits an overall higher affinity for methionine residues than \([\text{AuIIIICl}_4]^–\).

Multibinding of Dichloroaurate(I) in a Narrowly Confined Pore Restriction. The MspA-M used in this study is a homo-octameric pore, indicating that eight identical methionines are placed axis-symmetrically around the pore restriction. When the final concentration of \([\text{AuCl}_4]^–\) or \([\text{AuIIIICl}_4]^–\) in cis is below 1 \(\mu\text{M}\), the dominant binding events are from individual gold complexes, from which a reactive kinetics constant can be derived (Table S2). However, when the final concentration of \([\text{AuCl}_4]^–\) or \([\text{AuIIIICl}_4]^–\) in cis exceeds 1 \(\mu\text{M}\), the simultaneous binding of multiple analytes may be observed. Though bindings from \([\text{AuCl}_4]^–\) or \([\text{AuIIIICl}_4]^–\) are not directly distinguishable by the blockage amplitude \((\Delta I)\), \([\text{AuCl}_4]^–\) and \([\text{AuIIIICl}_4]^–\) may differ in the total number of ions that can be simultaneously occupied in the space-limited pore restriction.

For \([\text{AuIIIICl}_4]^–\), which is geometrically larger than \([\text{AuCl}_4]^–\), we have previously proven that the pore restriction can simultaneously accommodate up to three tetrachloroaurate(III).\(^{17}\) It is expected that more \([\text{AuCl}_4]^–\), which is smaller in size, could be simultaneously accommodated in this pore restriction (Figure 5a). To experimentally verify this, the concentration of gold(I) chloride in cis was first set to 1 \(\mu\text{M}\); the simultaneous binding of three \([\text{AuCl}_4]^–\) can be observed (Figure 5b). Subsequently, the gold(I) chloride concentration was further increased to 5 \(\mu\text{M}\), and \(I_n = 4\) events

(Tables 1). However, the association constant \(k_{\text{on}}\) of \([\text{AuIIICl}_4]^–\) is \((5 \pm 4) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}\) (Table 1), ~5.6 times of that measured by \([\text{AuIIIICl}_4]^–\).
began to appear (the blockage levels were named $I_n$ where $n$ stands for the total number of $[Au^{1}\text{Cl}_2^-]$ currently in the pore). Meanwhile, since the inter-event interval ($\tau_{\text{off}}$) was decreased with the increased analyte concentration, state $I_0$ is rarely observed (Figure 5c). Finally, with a concentration of 50 $\mu$M gold(I) chloride, the simultaneous binding of five $[Au^{1}\text{Cl}_2^-]$ events were observed (Figure 5d). However, even with a 200 $\mu$M concentration of $[Au^{1}\text{Cl}_2^-]$ in cis, we still observe only a maximum of five $[Au^{1}\text{Cl}_2^-]$ simultaneously bound to the pore restriction, indicating that the pore restriction is too narrow to accommodate any more $[Au^{1}\text{Cl}_2^-]$ or that electrostatic repulsion between bound $[Au^{1}\text{Cl}_2^-]$ may exist (Figure S5). Successive binding of five $[Au^{1}\text{Cl}_2^-]$ is shown in a representative continuously recorded trace (Figure 5e). The corresponding histogram of event–amplitude ($\Delta I$) shows five resolved peaks, each fit with a Gaussian distribution corresponding to different $I_n$ blockages (Figure 5f).

**CONCLUSION**

In summary, we demonstrate dynamic and reversible binding of $[Au^{1}\text{Cl}_2^-]$ or AuI(tht)Cl to MspA-M from direct nanopore readouts. Complementary to results previously published with $[Au^{III}\text{Cl}_4^-]$, a generality of MspA-M to probe a variety of gold compounds with different coordination geometry and valence states is confirmed. In contrast to those observed with $[Au^{III}\text{Cl}_4^-]$, the reaction between $[Au^{1}\text{Cl}_2^-]$ and a thioether shows a faster association rate, a slower dissociation rate, and, consequently, an overall higher binding affinity. Different coordination complexes of AuI also show measurable differences in their binding kinetics. On the basis of the number of gold ions that can simultaneously occupy the lumen of a MspA restriction, which is a tightly and narrowly confined nanocavity, the difference in the geometric configuration between $[Au^{1}\text{Cl}_2^-]$ and $[Au^{1}\text{Cl}_2^-]$ was well observed. Other environmental parameters such as the measurement temperature, the buffer salt concentration, and the buffer pH also affect the event shape and binding kinetics. However, these combinations of experimental conditions will be studied in a series of follow-up studies. Though demonstrated in a simplified and conceptual form in this study, immediate follow-up investigations will include tests of clinically used drugs such as tetraacetyl-β-D-thioglucone gold(I) triethylphosphine (Auranofin), sodium aurothiomalate (Myocrisin), or aurothiogluscule (Solganol) with MspA-M. We foresee that these investigations may provide new insights in the understanding of gold coordination chemistry or might provide inspiration in the design of new gold-based drugs.2,30

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/10.1021/acs.analchem.0c03840.

Measurements of M2MspA with or without the presence of AuI, $[Au^{1}\text{Cl}_2^-]$ binding analysis, AuI(tht)Cl binding to MspA–M, $[Au^{III}\text{Cl}_4^-]$ binding to MspA–M, $\Delta I_{0-1}$, and $\tau_{\text{off}}$ of $[Au^{III}\text{Cl}_4^-]$ or $[Au^{1}\text{Cl}_2^-]$ bindings, $[Au^{1}\text{Cl}_2^-]$ binding to MspA–M at a high concentration, time-extended observation of $[Au^{1}\text{Cl}_2^-]$ binding to MspA–M, and tables (PDF)

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Notes
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