Sensing Methodology for the Rapid Monitoring of Biomolecules at Low Concentrations over Long Time Spans

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ABSTRACT: Studies on the dynamics of biological systems and biotechnological processes require measurement techniques that can reveal time dependencies of concentrations of specific biomolecules, preferably with small time delays, short time intervals between subsequent measurements, and the possibility to record over long time spans. For low-concentration biomolecules, these requirements are very challenging since low-concentration assays are typically slow and require new reagents in every assay. Here, we present a sensing methodology that enables rapid monitoring of picomolar and sub-picomolar concentrations in a reversible affinity-based assay, studied using simulations. We demonstrate that low-concentration biomolecules can be monitored with small time delays, short time intervals, and in principle over an endless time span.

KEYWORDS: monitoring, sensing, low concentration, fast response, high precision, biomolecules, affinity kinetics

Biological systems and biotechnological processes exhibit time dependencies that are at the most basic level regulated by the dynamics of constituting biomolecules, such as small molecules, hormones, proteins, and nucleic acids. This calls for measurement technologies that allow the evaluation of biomolecular concentrations, for instance, to serve fundamental research on biological and biomedical dynamics,1−6 to enable the development of patient monitoring strategies based on real-time biomolecular data,7−10 and to enable the development of closed loop control strategies in biotechnological applications.11−15 Desirable characteristics of a generic monitoring technology are (1) precise and specific measurements, (2) small time delays between sampling input and data output, (3) short time intervals between successive measurements, and (4) a long total time span over which time-dependent biomolecular concentration data can be recorded.

It is a fundamental challenge to develop a technology that can rapidly monitor low-concentration biomolecules over long time spans. Sensitive assays are available, such as ELISA and flow cytometry,17−20 but in these assays, new reagents are needed for every sample that is taken. The repeated consumption of reagents complicates applications where biomolecular concentrations need to be monitored over long time spans. On the other hand, sensing technologies that can operate without consuming reagents, such as surface plasmon resonance,21 redox cycling,22 and quartz crystal microbalance,23 have not been designed for monitoring biomolecules at low concentrations, such as in the picomolar and sub-picomolar range.

A generic principle to measure specific biomolecules at low concentrations is by using the biochemical affinity between specific binder molecules (such as antibodies and aptamers) and the biomolecule that is to be detected (the analyte). The specificity originates from molecular interactions such as charge, hydrogen bonding, van der Waals forces, and hydrophobic and steric effects.24 To be able to measure biomolecules at low concentrations with high precision, binder molecules are needed that have strong interactions with the analyte, which corresponds to high binding energies, low equilibrium dissociation constants $K_d$ and low dissociation rate constants $k_{off}$.25 However, this conflicts with the wish to have small time delays because low dissociation rate constants would imply a need for long incubation times to reach equilibrium.24−26 Furthermore, low dissociation rate constants result in a slow reversibility, which conflicts with the wish to enable short time intervals between successive measurements.

In this paper, a sensing methodology is presented that enables rapid monitoring of low-concentration biomolecules in the picomolar and sub-picomolar range, studied using simulations. The method is based on the use of binder molecules with a high affinity in a limited-volume assay, with a...
fully reversible detection principle and time-dependent sampling of the analyte of interest. The system allows optimal trade-offs between time characteristics and precision. We present the measurement concept, time dependencies of sensor signals, and a comprehensive analysis of the achievable time characteristics and precision as a function of sensor design parameters. We demonstrate that the sensing methodology enables precise quantification of low biomolecular concentrations, with time delays and interval times that are much shorter than the time dictated by the dissociation rate constant of the binder molecules. Furthermore, due to the reversible detection method, measurements can in principle be performed over an endless time span.

## BASIC CONCEPT OF THE SENSING METHODOLOGY

The basic concepts of the sensing methodology are sketched in **Figure 1**. The sensing system features time-dependent sampling of the analyte of interest, provided by a time-controlled analyte exchange between a biological or biotechnological system of interest and a measurement chamber (see **Figure 1a**). The measurement chamber contains specific
Table 1. Standard Parameter Values Used in the Finite-Element Simulations

| parameter | value | description |
|-----------|-------|-------------|
| input $H$ | 200 μm | measurement chamber height |
| $D$ | $10^{-16}$ m$^2$ s$^{-1}$ | diffusion coefficient of the analyte molecule |
| $\Gamma_b$ | $10^{-3}$ mol m$^{-2}$ (600 μm$^{-2}$) | binder density |
| $k_{diff}$ | $10^{-1}$ s$^{-1}$ | dissociation rate constant |
| $k_{on}$ | $10^9$ M$^{-1}$ s$^{-1}$ | association rate constant |
| $C_{in}$ | 0.1 pM | input analyte concentration |
| derived $\Gamma_b,H/D$ | 400 s | characteristic diffusion time |
| $\Gamma_b,H/D$ | $k_{diff} = H$ | characteristic reaction time for limited-volume assay with $C_{in} \gg C_{off}$ and $C_{in} \gg K_d$ |
| $C_{eq} = \Gamma_b/H$ | 5 nM | effective volumetric binder concentration |
| $K_d = k_{on}/k_{off}$ | 100 pM | equilibrium dissociation constant |
| $\alpha = \Gamma_b/K_d = C_{in}/K_d$ | 50 | acceleration factor: reduction factor of the time-to-equilibrium of a limited-volume assay with $\tau(H, \Gamma_b)\text{,}$ compared to an infinite-volume assay with $\tau(k_{diff})$ |
| Da = $\tau_{eq}/\alpha$ | 2 | Damköhler number |

“Details about the simulations are described in Note S4. Additional standard parameter values are given in Table S1 (see Note S1).”

binder molecules from which signals are recorded. The data are translated into concentration—time profiles, which should resemble as close as possible the true concentration—time profile of analyte molecules in the system of interest. During the exchange of analyte molecules, various processes occur, such as mass transport by advection and diffusion and association and dissociation of analyte molecules to binder molecules (see Figure 1b). To illustrate the concept, a generic rectangular measurement chamber is assumed with height $H$, width $W$, and length $L$. The bottom surface is provided with binder molecules, where association and dissociation of analyte molecules occurs. The rates of association and dissociation depend on the association rate constant $k_{on}$, the dissociation rate constant $k_{off}$, the density $\Gamma_b$ of binder molecules, and the analyte concentration $C_a$ at the sensor surface. These processes result in a time-dependent density $\gamma_a$ of analyte—binder complexes, also represented as a fractional occupancy $f$ of binder molecules occupied by analyte molecules: $f = \gamma_a/\Gamma_b$. Variables $\gamma_a$ and $f$ are changing as a function of analyte concentration and time. In an affinity-based sensor, the observed sensor signal scales with $f$, therefore, $f$ is used in this paper as the sensor readout parameter to determine the analyte concentration. Analyte exchange between the system of interest and the measurement chamber is facilitated by diffusion or a combination of diffusion and advection. A net diffusive molar flux $J_a$ (orange gradient) is caused by concentration differences between the system of interest and the measurement chamber and by concentration differences within the measurement chamber. Advecive mass transport of analyte molecules into the measurement chamber is facilitated by a developed laminar flow profile with flow rate $Q$ and mean flow speed $v_m$ (black arrows). Here, it is assumed that diffusive transport occurs in both the longitudinal ($x$-direction) and the lateral direction ($y$-direction) and scales with the diffusion coefficient $D$, while advective transport occurs only in the longitudinal direction and scales with the mean flow speed $v_m$.

Figure 1c sketches two different sensor designs, namely, an infinite-volume assay and a limited-volume assay. The graphs visualize the fractional occupancy $f$ of binder molecules occupied by analyte molecules as a function of time, with the corresponding characteristic time-to-equilibrium $\tau$, defined as the time needed to attain 63% of the difference between the starting level and the equilibrium level of $f$ (see Note S2). In an infinite-volume assay, continuous analyte exchange is enabled between the system of interest and the measurement chamber, where the system of interest is assumed to be much larger than the measurement chamber. The continuous analyte exchange could, for example, be facilitated by diffusive analyte transport across a contact area between the system of interest and the measurement chamber, while another configuration may involve a continuous flow of sample fluid provided into the measurement chamber from the system of interest. When the analyte exchange is effective and gives negligible time delay, then the analyte concentration at the sensor surface ($C_a$) is equal to the input analyte concentration ($C_{in}$). In the case of low analyte concentrations ($C_{in} < K_d$), the infinite-volume assay condition leads to a characteristic time-to-equilibrium $\tau \equiv 1/k_{diff}$ (see Note S2). This implies that the time-to-equilibrium is determined by the dissociation rate constant $k_{diff}$ so that the time is long when the binder molecules strongly bind to the analyte molecules.

The sensor design with a limited-volume assay has very different properties. Here, analyte exchange between the system of interest and the measurement chamber is enabled for a limited time. After this analyte exchange, the binder molecules in the measurement chamber interact with only a limited sample volume and therefore with a limited amount of analyte molecules. Due to this limited volume, we can now define an effective volumetric concentration of binder molecules $C_{in} = \Gamma_b/H$, which is based on the number of binder molecules in the measurement chamber and the volume of the measurement chamber. When $C_{in}$ is high, with $C_{in} > C_{off}$ and $C_{in} > K_d$ then the time-to-equilibrium $\tau$ of the assay becomes dominated by the high concentration of binder molecules. When diffusional transport delays can be ignored, then the time-to-equilibrium of the assay equals $\tau \equiv 1/(k_{diff}C_{in})$ (see Table 1 and Notes S1 and S2). Thus, the time-to-equilibrium of the limited-volume assay is determined by the association rate constant and the effective volumetric concentration $C_{in}$ of binder molecules, which leads to equilibrium timescales that are much shorter than the time-to-equilibrium of the infinite-volume assay.

In monitoring applications, it is preferred to record measurements with one and the same sensor over long time
spans. To realize the limited-volume assay principle in a monitoring application, the sensor needs to be switched between two different conditions: an open condition and a closed condition. In the open condition, analyte molecules are exchanged effectively between the system of interest and the measurement chamber, as sketched in Figure 1a,b (also see Note S5). In the closed condition, analyte molecules are not exchanged between the system of interest and the measurement chamber, causing a limited-volume incubation in the measurement chamber, as sketched in the bottom graph of Figure 1c. We refer to the switching concept between the open and closed condition as “time-controlled analyte exchange”. Figure 1d illustrates the operating principle for a sensor where time-controlled analyte exchange is realized by a modulated flow. Phase 1 is the exchange phase, where the measurement chamber is supplied with the sample fluid so that the starting concentration in the chamber equals C_{a,0}. Phase 2 is the incubation phase, where the exchange process is stopped so that the limited-volume assay condition is provided. During incubation under the limited-volume condition, the analyte concentration in the measurement chamber decreases over time (depletion) or increases over time (repletion), depending on the initial fractional occupancy f_{init} of binder molecules by analyte molecules. When f_{init} is low, the concentration of analyte molecules in the measurement chamber decreases over time, corresponding to the depletion of analytes. When f_{init} is high, the concentration of analyte molecules in the chamber increases over time, corresponding to the repletion of analytes. For known f_{init} the supplied analyte concentration C_{a,0} can be derived from the measured time-dependent fractional occupancy f(t) during the incubation phase. At least two measurements need to be performed to determine the initial analyte concentration C_{a,0} for example, a measurement at the initial value f_{init} and a measurement at the final value f_{end} as indicated in the graph.

By sequentially applying cycles of open condition and closed condition, discrete samples with a limited volume are serially measured and result in time-dependent data that relate to the condition, discrete samples with a limited volume are serially measurement causes a varying nonzero initial fractional occupancy f_{init} of binder molecules by analyte molecules. When f_{init} is low, the concentration of analyte molecules in the measurement chamber decreases over time, corresponding to the depletion of analytes. When f_{init} is high, the concentration of analyte molecules in the chamber increases over time, corresponding to the repletion of analytes. For known f_{init} the supplied analyte concentration C_{a,0} can be derived from the measured time-dependent fractional occupancy f(t) during the incubation phase. At least two measurements need to be performed to determine the initial analyte concentration C_{a,0} for example, a measurement at the initial value f_{init} and a measurement at the final value f_{end} as indicated in the graph.

In the next sections, we will study how sensor design parameters influence the time characteristics and precision of the sensing methodology. The time characteristics are quantified by finite-element simulations of mass transport in the sensor and reaction processes at the sensor surface, and the precision is quantified by calculating the stochastic variabilities in the measurements. The simulations and calculations are verified by experiments using a sensing technique with a single-molecule resolution, called biosensing by particle mobility (BPM, see Note S7).
RESULTS AND DISCUSSION

Timescales of the Limited-Volume Assay. Figure 2 shows simulation results of the time-to-equilibrium of the limited-volume assay, for sensor designs with different measurement chamber heights, different binder densities, and different flow rates, assuming standard parameter values, as listed in Table 1. Figure 2a shows how the time-to-equilibrium \( \tau \) depends on the measurement chamber height \( H \) for a sensor with instantaneous analyte exchange (see Note S6 for the influence of analyte exchange on the sensor performance). The arrow on the \( x \)-axis indicates the height as listed in Table 1. The data show that the time-to-equilibrium increases with the measurement chamber height. At small \( H \), this increase is caused by a decrease in the effective volumetric binder concentration, while at large \( H \), this increase is caused by diffusive transport limitations. The inset shows the same data, plotted as a function of the Damköhler number \( Da = \frac{t_{\text{d}}}{t_{\text{L, LV}}} = k_m \Gamma_b H/D \) (see Table 1); low \( Da \) means that the kinetics are limited by the reaction, and high \( Da \) means that the kinetics are limited by diffusion. To achieve a fast time-to-equilibrium, the sensor should be designed with a large \( C_{b,0} \), so a small \( H \).

Figure 2b shows how the time-to-equilibrium depends on the binder density \( \Gamma_b \) for a sensor with instantaneous analyte exchange. The arrow indicates the density, as listed in Table 1. For small \( \Gamma_b \), the time-to-equilibrium is long and determined by the dissociation rate constant \( \tau \approx \frac{1}{k_{\text{diss}}} \). For \( \Gamma_b > HK_d \approx 20 \, \mu m^{-2} \), the time-to-equilibrium decreases, until it stabilizes due to diffusive transport limitations \( \tau \approx \frac{t_{\text{d}}}{H^2/D} \). The inset shows the same data plotted as a function of \( Da \). To achieve a fast time-to-equilibrium, the sensor should be designed with a large \( C_{b,0} \), so a large \( \Gamma_b \).

Figure 2c shows how analyte exchange by advection contributes to the time-to-equilibrium per measurement cycle. The exchange phase involves a temporary flow of fluid into the measurement chamber, with flow rate \( Q \) and duration \( t_{\text{exch}} \) (see Note S6 for the influence of analyte exchange on the sensor performance). In the simulations, \( t_{\text{exch}} \) was chosen to be equal to the characteristic advection time \( \tau_a = \frac{HLW}{Q} \) (see Table 1), which means that a total fluid volume equal to the volume of the measurement chamber is displaced. The time-to-equilibrium \( \tau \), which now includes a contribution \( t_{\text{exch}} \) related to the exchange, is shown as a function of flow rate, for several values of the chamber aspect ratio \( \lambda = L/H \). The arrow indicates the flow rate, as listed in Table 1. For small \( Q \), the observed \( \tau \) is limited by \( t_{\text{exch}} \), that is, the advective transport time of analyte molecules from the inlet toward the point of sensing at a distance \( L/2 \) from the inlet, as sketched in Figure 1d. For increasing \( \lambda \), that is, increasing \( L \) with a fixed \( H \), the time-to-equilibrium increases since \( \tau_a \) (and thus also \( t_{\text{exch}} \)) increases. For increasing \( Q \), the time-to-equilibrium decreases, until it stabilizes at a level where the reaction and diffusion times determine the observed \( \tau \). The inset shows the same data
both curves would approach \( f_{\text{eq,IV}} = 1 \), which equals the equilibrium value when an infinite volume is supplied (see Table S1).

(b) Fractional occupancy \( f \) as a function of time where cycles of analyte exchange and incubation are applied every 15 min with alternatingly \( C_{\text{a,0}} = 0.1 \text{ pM} \) and \( C_{\text{a,0}} = 0.05 \text{ pM} \). The curve saturates at \( f_{\text{eq,IV}} = 10^{-4} \), which equals the infinite-volume equilibrium value for the average concentration value \( C_{\text{a,0}} = 0.1 \text{ pM} \) (see Table S1). Dashed lines: continuous supply of \( C_{\text{a,0}} = 0.05 \text{ pM} \) yields \( f_{\text{eq,IV}} = 5 \times 10^{-4} \) and \( C_{\text{a,0}} = 0.15 \text{ pM} \) yields \( f_{\text{eq,IV}} = 1.5 \times 10^{-4} \) (see Table S1). The right panel shows zoom-in images of three sections of the solid curve, each representing four cycles of instantaneous analyte exchange and subsequent incubations of 15 min. In zoom-in 1 \((t = 0-1 \text{ h})\), all curve segments show depletion behavior. In zoom-in images 2 \((t = 12-13 \text{ h})\) and 3 \((t = 42-43 \text{ h})\), depletion is seen for \( C_{\text{a,0}} = 0.15 \text{ pM} \), since \( f_{\text{init}} < f_{\text{eq,IV}}(C_{\text{a,0}} = 0.15 \text{ pM}) \), and repletion is seen for \( C_{\text{a,0}} = 0.05 \text{ pM} \), since \( f_{\text{init}} > f_{\text{eq,IV}}(C_{\text{a,0}} = 0.05 \text{ pM}) \). For all curve segments, the time-to-equilibrium \( \tau = 340 \text{ s} \). The vertical scale bars indicate \( \Delta f = 10^{-4} \).

Figure 4. Experimental study of a limited-volume assay with varying binder concentrations using BPM. (a) Sketch of the measurement chamber in a BPM measurement (see Note S7) without (top) and with (bottom) supplemented binders with concentration \( C_{b,\text{suppl}} \). For simplicity, the particles of the BPM sensor are not shown in the sketch. (b) Experimentally observed time-to-equilibrium \( \tau \) (left) and normalized signal change \( \Delta S \) (right) as a function of supplemented binder concentration \( C_{b,\text{suppl}} \) in a BPM measurement. The model assay is based on DNA–DNA interactions, with ssDNA analytes at a concentration of 200 pM (see Note S7). Left: the dashed line shows the fitted curve \( \tau = p_1/(p_2 + C_{b,\text{suppl}}) + p_2 \), where \( p_1 = 1/\Gamma_b \) (\( \Gamma_b \) is assumed to be equal for all binders), \( p_2 = \Gamma_b/H \), and \( p_2 \) is the delay contributed by diffusion (see Figure 2b) and experimental steps (see the Experimental Section). Assuming \( H = 200 \text{ μm} \) (see Table 1), the fit gives \( \Gamma_b = (3 \pm 1) \times 10^{-10} \text{ mol m}^{-2} \), which is comparable to the standard parameter value, as listed in Table 1. The fitted association rate constant is \( k_{\text{ass}} = (1.5 \pm 0.4) \times 10^{12} \text{ M}^{-1} \text{ s}^{-1} \), which is in the range of values reported in the literature for comparable DNA–DNA hybridization reactions.\[33,34\]

Right: In the depletion condition \( (f_{\text{init}} < f_{\text{eq,IV}}) \), the fractional occupancy scales according to \( f \propto 1/C_{b,\text{tot}} = C_{b,0}/(C_{b,0} + C_{b,\text{suppl}}) \). The dashed line shows the fitted curve \( \Delta S = p_1/(p_2 + C_{b,\text{suppl}}) \), where \( p_1 \) scales the change in fractional occupancy to signal change and \( p_2 = \Gamma_b/H \). For \( H = 200 \text{ μm} \), it was found that \( \Gamma_b = (7 \pm 4) \times 10^{-10} \text{ mol m}^{-2} \), which is comparable to the previously found value for \( \Gamma_b \) and the standard parameter value, as listed in Table 1. The insets show the same data on linear-logarithmic scales. The errors reported in the figure (smaller than the symbol size) and the caption are fitting errors based on a 68% confidence interval.
Supplemented with $Da = 2$ (reaction-limited) and $Da = 20$ (diffusion-limited), plotted as a function of the longitudinal Péclet number ($Pe_L = \tau_d/\tau_A = Q/\lambda Dw$, see Table S1); low $Pe_L$ means that the analyte exchange is limited by advection, and high $Pe_L$ means that the analytic exchange is limited by diffusion. A low $Pe_L$ causes a long time-to-equilibrium due to slow mass transport by advection. Increasing $Pe_L$ results in a decrease in the time-to-equilibrium due to rapid filling of the chamber, until it stabilizes at a $\tau$ value equal to the value indicated in Figure 2a. Figure 2c shows the flow rate required to minimize the influence of the exchange process on the time-to-equilibrium. In the following sections, exchange with a high $Pe_L$ is assumed, that is, rapid filling of the measurement chamber without influence of the flow rate on the time-to-equilibrium.

**Limited-Volume Assay with Time-Controlled Analyte Exchange.** Figure 3 shows simulation results for a limited-volume assay with time-controlled analyte exchange. The analytic exchange is assumed to be instantaneous and the incubation phase includes mass transport by diffusion and

![Figure 5](https://pubs.acs.org/acsensors/article-pdf/2021/6/4477.pdf)

Figure 5. Analytical performance of the limited-volume assay, derived from simulations of a single measurement cycle. (a) Fractional occupancy at the end of the incubation $f_{end}$ as a function of analyte concentration $C_{a,0}$ for different initial fractional occupancies $f_{init}$. The right y-axis indicates the number of surface-bound analyte molecules at the end of the cycle $\gamma_{ab, end}$. (b) Absolute change in fractional occupancy $\Delta f$ as a function of $C_{a,0}$ for various $f_{init}$. The right y-axis indicates $\Delta \gamma_{ab}$. A positive $\Delta f$ and $\Delta \gamma_{ab}$ indicate depletion; negative values indicate repletion. Note that the lines for $f_{init} = 10^{-3}$ and lower are overlapping. The inset shows the same data on a linear–linear scale. (c) Coefficient of variation $CV_c$ with which the analyte concentration $C_{a,0}$ can be determined as a function of analyte concentration $C_{a,0}$ for various initial fractional occupancies $f_{init}$. $CV_c$ scales as $1/C_{a,0}$ for low $f_{init}$ and high $C_{a,0}$; $CV_c$ scales as $1/C_{a,0}$ for high $f_{init}$ and low $C_{a,0}$. The horizontal dashed line indicates the 10% CV level that defines the LoQ (limit of quantification). (d) $CV_c$ as a function of measurement chamber height $H$ (top) and binder density $\Gamma_b$ (bottom) for various initial fractional occupancies $f_{init}$ and $C_{a,0} = 0.1$ pM. The arrows on the x-axes indicate the standard parameter values for $H$ and $\Gamma_b$, as listed in Table 1. (e) $CV_c$ as a function of the observed time-to-equilibrium $\tau$ when varying the measurement chamber height $H$ (left) or binder density $\Gamma_b$ (right) for various initial fractional occupancies $f_{init}$ and $C_{a,0} = 0.1$ pM. The sketches above the graphs visualize a measurement chamber with an increasing height or a decreasing binder density. The arrows on the x-axes indicate the obtained time-to-equilibrium using the standard parameter values for $H$ and $\Gamma_b$ as listed in Table 1.
reaction kinetics within the measurement chamber itself but no analyte exchange between the system of interest and the measurement chamber. Figure 3a shows data for repeated incubations with $C_{a,0} = 0.1$ pM. The analyte concentration $C_a$ in the measurement chamber (brown line) and the fractional occupancy $f$ of the binders by analyte molecules (orange line) are plotted as a function of time, for conditions of analyte depletion (left) and analyte repletion (right). The time-to-equilibrium $\tau$ of each incubation equals approximately 340 s (see Figure 2), having contributions from reaction ($r_R = 200$ s) and diffusion ($r_D = 400$ s). The contribution from the reaction to the time-to-equilibrium is much smaller than $1/k_{off} = 10^6$ s, the value that would have been observed in the case of an infinite-volume assay (cf. Figure 1c). In the absence of diffusion limitations, the acceleration that can be achieved with a limited-volume assay compared to an infinite-volume assay equals $\alpha = \frac{1/k_{off}}{R_{LV}} = \frac{k_{on} f^*}{k_{off}} = C_{b,0}/K_d$, which clarifies how the speed of the assay is directly related to the ratio between effective volumetric binder concentration and the equilibrium dissociation constant.

Figure 3b shows the response of a limited-volume assay with time-controlled analyte exchange for an analyte concentration that varies in time. As an example, the sensor is incubated with time-controlled analyte exchange for an analyte concentration $C_a$ and $d_{init}$ molecules to specify the particles are transducers that record the binding of analyte molecules at specific times. For the BPM sensor, the particles are transducers that record the binding of analyte molecules at specific times. For example, the sensor can be made with binders immobilized in the measurement chamber. This will be a topic for future research.

**Analytical Performance of the Limited-Volume Assay.** Figure 5 shows how the analytical performance of the limited-volume assay depends on the sensor design. The results are based on numerical simulations with parameters, as listed in Table 1. The analyte exchange is assumed to be instantaneous and the incubation includes mass transport by diffusion and reaction kinetics within the measurement chamber only. All panels show curves for different values of the initial fractional occupancy $f_{init}$ of the binder molecules.

Figure 5a shows the fractional occupancy of binders by analyte molecules at the end of the incubation ($f_{end}$) as a function of the initial analyte concentration $C_{a,0}$. For $f_{init} = 0$ (dashed black line), $f_{end}$ scales linearly with the analyte concentration, which makes the sensor suitable for analyte quantification. For larger values of $f_{init}$, the curves start with a rather flat segment, from which one might erroneously conclude that under those conditions, low analyte concentrations cannot be determined. Interestingly, the limited-volume assay has a linear dependence on concentration by focusing not on the absolute value of $f_{end}$ but rather on the change in fractional occupancy $\Delta f$ (see Note S2)

$$\Delta f = f_{end} - f_{init} \approx \frac{H}{K_b} (C_{a,0} - K_d f_{init})$$

This equation shows that $\Delta f$ depends linearly on $C_{a,0}$ independent of the value of $f_{init}$. This fact is also illustrated by the simulation results in Figure 5b. The response scales linearly with concentration $C_{a,0}$ and is downshifted for increasing values of $f_{init}$ in agreement with eq 1 (note that the steep increase in the curves relates to the logarithmic $x$-axis). Positive values of $\Delta f$ relate to depletion behavior and negative values to repletion. The curves cross the $x$-axis ($\Delta f = 0$) when $f_{init}$ corresponds to the equilibrium condition, that is, when there is no net association or dissociation during incubation because $f_{init}$ is equal to the equilibrium fractional occupancy $f_{eq,IV}$.

Figure 5c shows the precision of the concentration output of the sensor, that is, the precision with which the analyte concentration in an unknown sample can be determined for a signal collection area of 1 mm$^2$. The precision is calculated based on Poisson noise, which gives the fundamental limit of the precision that is achievable with a molecular biosensor due to stochastic fluctuations in the number of analyte molecules (see Note S3 and S8). To calculate the precision, a sensor with initial fractional occupancy $f_{init}$ is provided with a sample with analyte concentration $C_{a,0}$, resulting in a $\Delta f$ with...
variability $\sigma_{\text{f}}$, which via the slope of the calibration curve, given in Figure 5b, leads to a variability $\sigma_C$ in the concentration output of the sensor (see Note S3). The precision is indicated as the concentration-based coefficient of variation $\text{CV}_C = \sigma_C/\mu_C$, with $\sigma_C$ being the variability and $\mu_C$ being the mean of the concentration output. Figure 5c shows how the concentration precision depends on the analyte concentration and the initial fractional occupancy $f_{\text{init}}$. For $f_{\text{init}} = 0$ (dashed line), the CV$_C$ scales as $1/\sqrt{C_{\text{tot}}}$, in agreement with number fluctuations in a Poisson process (see Note S3). For higher $f_{\text{init}}$ a stronger dependency is observed (CV$_C$ or $1/C_{\text{tot}}$), caused by the smaller relative change in the fractional occupancy (see Note S3). The graph indicates the 10% precision level that is used to define the limit of quantification (LoQ) of the sensor (see Figure 5c, dashed horizontal line). The results show that analyte concentrations in the sub-picomolar range can be measured with a precision better than 10%, even for high initial fractional occupancies.

Figure 5d shows the precision of the concentration output of the sensor as a function of two design parameters, namely, the measurement chamber height $H$ (top panel) and the binder density $\Gamma_b$ (bottom panel), at an analyte concentration $C_{\text{tot}} = 0.1 \text{ pM}$, for an initial fractional occupancy $f_{\text{init}}$ between 0 and 0.1. The arrows indicate the height and density, as listed in Table 1. For the increasing $H$, a decrease in CV$_C$ is observed, caused by an increase in the number of analyte molecules present in the measurement chamber. The CV$_C$ is the smallest for $f_{\text{init}} = 0$ and increases for increasing $f_{\text{init}}$ since the absolute change in fractional occupancy decreases. The CV$_C$ decreases for increasing $\Gamma_b$ caused by an increase in the number of analyte molecules captured from solution. The CV$_C$ reaches a plateau for $f_{\text{init}} = 0$ due to a limited number of analyte molecules in the measurement chamber. For larger $f_{\text{init}}$, the absolute change in fractional occupancy decreases and causes a less precise concentration determination; this effect is in particular visible at high $\Gamma_b$ where the absolute number of analyte–binder complexes increases due to $f_{\text{init}}$.

The trade-off between precision and time-to-equilibrium is illustrated in Figure 5e, for sensors with different heights of the measurement chamber (left) and different binder densities (right). The arrows indicate the time-to-equilibrium that results from the height and density, as listed in Table 1. The left panel shows that an increase in $H$ gives, on the one hand, a slower sensor response (due to a larger diffusion distance) but, on the other hand, a lower CV$_C$ due to a larger number of analyte molecules present in the measurement chamber. At low $H$, the CV$_C$ strongly depends on $f_{\text{init}}$ due to the low number of analyte molecules in the solution. The right panel shows again that the CV$_C$ decreases for a slower sensor response, now controlled by decreasing the binder density $\Gamma_b$. At high $\Gamma_b$, the time-to-equilibrium is diffusion-limited (resulting in $\tau = 130 \text{ s}$). At low $\Gamma_b$, the time-to-equilibrium is reaction-limited with $\tau = 1/k_{\text{on}} = 10^4$ (see Figure 2b). At high $\Gamma_b$, the CV$_C$ increases for increasing $f_{\text{init}}$ due to the larger amount of analyte molecules on the sensor surface. At low $\Gamma_b$, the CV$_C$ strongly increases due to the small absolute change in the number of analyte–binder complexes during the incubation phase.

## CONCLUSIONS

We have presented a sensing methodology suitable for monitoring low-concentration biomolecules with high precision, with small time delays and short time intervals, over an endless time span. The sensing methodology is based on a limited-volume assay, using high-affinity binders, a fully reversible detection principle, and time-controlled analyte exchange. We studied by simulations how the kinetics of the sensor depend on mass transport and on the surface reaction in the measurement chamber, and how time-controlled analyte exchange determines the system response and enables precise measurements of analyte concentration. Experimental results show the ability to control the sensor response time by tuning the total binder concentration in the measurement chamber. Finally, simulations show that the sensing principle allows picomolar and sub-picomolar concentrations to be monitored with a high precision over long time spans.

Approaches described in the literature for measuring low-concentration biomolecules have focused primarily on assays in which every concentration determination involves consumption of reagents.20,36,37 When numbers of assays become high, due to frequent measurements over long time spans, then reagent-consuming approaches are complex and costly. The sensing methodology described in this paper is based on a fully reversible assay principle, without consuming reagents with each newly recorded concentration data point, enabling measurements with high frequency over an endless time span. The described assay principle can be implemented on several sensing platforms, for example, based on optical, electrical, or acoustical transduction methods, where especially sensing platforms with single-molecule resolution seem suitable since these allow digital measurements with very high precision, limited only by the number of observed molecular interactions. The sensing method is suited for the monitoring of a wide variety of analytes, including small molecules, proteins, and viral particles (see Note S9). Furthermore, the sensing methodology can be combined with various sampling methods, including remote advection-based sampling through a sampling line or a catheter and proximal diffusion-based sampling methods for on-body and in-body monitoring devices. The presented sensing principle warrants further experimental studies, for example, to investigate trade-offs between time characteristics and precision, for various transduction methods, sampling methods, measurement chamber geometries, binder types, and for various complex biological matrices. Due to its sensing performance and generalizability, we believe that the limited-volume assay with time-controlled analyte exchange will enable research on time dependencies of low-concentration biomolecules and novel applications in the fields of dynamic biological systems, patient monitoring, and biotechnological process control.

## ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.1c01991.

Algebraic derivations of the used equations, finite-element simulations, time-controlled analyte exchange, and BPM (PDF)

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R.M.L. and M.W.J.P. conceived and designed the methodology and measurement system. R.M.L. conceived, designed, and performed the simulations and the corresponding data analysis. M.H.B. performed the BPM experiments. R.M.L. and M.H.B. performed the BPM data analysis. R.M.L., A.M.d.J., and M.W.J.P. interpreted simulated data and discussed simulated results. All authors interpreted BPM experimental data and discussed BPM experimental results. R.M.L., A.M.d.J., and M.W.J.P. co-wrote the paper. All authors approved the submitted version of the manuscript.

Notes
The authors declare the following competing financial interest(s): M.W.J.P. and R.M.L. are listed as inventors on a filed patent application relating to the data presented in this paper. M.W.J.P. is co-founder of Helia Biomonitoring. All authors declare no further competing interests.

Data Availability: All data supporting the findings of this study are available from the corresponding author upon reasonable request.

Code Availability: All data analysis and simulation codes used for this study are available from the corresponding author upon reasonable request.

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