**Toward the Development of Rapid, Specific, and Sensitive Microfluidic Sensors: A Comprehensive Device Blueprint**

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**ABSTRACT:** Recent advances in nano/microfluidics have led to the miniaturization of surface-based chemical and biochemical sensors, with applications ranging from environmental monitoring to disease diagnostics. These systems rely on the detection of analytes flowing in a liquid sample, by exploiting their innate nature to react with specific receptors immobilized on the microchannel walls. The efficiency of these systems is defined by the cumulative effect of analyte detection speed, sensitivity, and specificity. In this perspective, we provide a fresh outlook on the use of important parameters obtained from well-characterized analytical models, by connecting the mass transport and reaction limits with the experimentally attainable limits of analyte detection efficiency. Specifically, we breakdown when and how the operational (e.g., flow rates, channel geometries, mode of detection, etc.) and molecular (e.g., receptor affinity and functionality) variables can be tailored to enhance the analyte detection time, analytical specificity, and sensitivity of the system (i.e., limit of detection). Finally, we present a simple yet cohesive blueprint for the development of high-efficiency surface-based microfluidic sensors for rapid, sensitive, and specific detection of chemical and biochemical analytes, pertinent to a variety of applications.

**KEYWORDS:** Microfluidic biosensors, surface-based immunoassays, biomolecule immobilization, rapid biomarker detection, analytical sensitivity, analytical specificity, label-free detection, label-based detection

**1. INTRODUCTION**

The emphasis on early disease diagnosis and treatment has spurred the development of new technologies, ranging from portable point-of-care (POC) devices\(^1\) to robust high-throughput screening systems (HTS),\(^2\) aimed at creating faster, reliable, and affordable biomarker screening systems. Here, the biomarkers are typically proteins, chemical analytes, or metabolic markers present in a patient’s blood, saliva, or urine that are indicative of the patient’s health. These analytes exist at homeostatic concentrations under normal conditions, whereas their concentrations fluctuate from these levels when the patient is afflicted with a disease. By employing bioanalytical assay systems, these fluctuating disease-specific analyte concentrations are detected and quantified from purified biofluids, enabling subsequent diagnosis.\(^7\)\(^8\)

The advent of microfluidic technology has allowed us to develop POC and HTS systems that can serve as all-inclusive platforms capable of processing the biofluid samples, performing biochemical analysis to detect the analytes, and displaying the detection results, from small sample volumes and within a short period of time, typically within 30–60 min.\(^9\)\(^11\) While the “speed” of these systems is certainly an attractive feature, the adoption of these systems in healthcare facilities is ultimately determined by the “sensitivity” and “specificity” of analyte detection.

Consequently, immense effort has been devoted to develop enhanced microfluidic bioanalytical systems to address this need. Of the available microfluidic technologies,\(^12\)\(^15\) surface-based microfluidic sensors are increasingly rising to the forefront, owing to the fabricational simplicity, ease of integration with simple fluid delivery systems, and compatibility with a wide range of detection techniques.\(^16\)\(^20\) In these systems, the bioanalytical receptor–analyte reaction is localized in well-defined patterns on microchannel surfaces, making it easier to track the binding of analytes in real-time. Although there have been numerous studies focused on aspects related to the mass transport and reaction kinetics in these systems, there is a gap between fundamental engineering principles and how they relate to the experimentally achievable features such as speed, sensitivity, and specificity in an actual microfluidic sensor. In this perspective, our aim is to provide a new outlook on the development of surface-based microfluidic sensors by relating the imposed transport and reaction limits with the exper-

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mentally achievable limits of analyte detection. In what follows, we provide a general overview of these systems and present a comprehensive breakdown of the benefits and pitfalls experienced while developing high efficiency microfluidic sensors.

2. THE IDEAL SURFACE-BASED MICROFLUIDIC SENSOR

The unique fluid behavior at the microscale has allowed researchers to tailor semi- to fully automated microfluidic systems capable of processing and analyzing chemical and biological analytes with reduced reagent volumes and small processing times. Typically, these systems consist of micron-sized channels that enable the transport of fluids containing analytes which, in close proximity, are subsequently captured by highly specific receptors also confined in the microchannels. In surface-based microfluidic sensing systems, the receptors are immobilized onto one or multiple walls of the microfluidic channel, thereby allowing localized detection of the receptor—analyte reactions.

In an ideal microfluidic sensor, different kinds of analytes should be detectable within a few seconds to minutes by using a few microliters of the analyte fluid at large dynamic concentration ranges (pM to M) (Figure 1). In the quest to create an ideal system, researchers are most often forced to make a conscious decision to make a trade-off between either time, sample volume, or detectable analyte concentration ranges, depending on the specific application. In the following sections, we explain the rationale behind these decisions by critically analyzing the driving mechanisms that determine the overall efficiency of surface-based microfluidic sensors.

To limit the scope of this perspective, we do not delve into open space microfluidics and narrow our focus to pressure-driven closed microfluidic systems and their integration with various analyte—receptor reaction platforms. In what follows, we first briefly describe the classical transport reaction model and its controlling dimensionless parameters that highlight the extreme limits of the microfluidic sensors. We then apply these dimensionless parameters as guidelines to comparatively review the recent progress made toward the improvement of three key sensor efficiency factors: (1) analyte transport, (2) receptor functionality, and (3) mode of receptor—analyte reaction detection.

3. THE CONVECTION—DIFFUSION—REACTION MODEL

In every microfluidic sensor, there are typically three competing phenomena at any given time: (1) analyte convection due to fluid motion, (2) analyte diffusion, and (3) analyte—receptor binding reaction. To encapsulate these phenomena in a model system, we consider the simplest possible microfluidic sensor, as illustrated in Figure 2: a straight rectangular microfluidic channel of height $H$ and width $W$, consisting of receptors (blue sticks) immobilized on the bottom channel wall, as a well-defined rectangular strip of length $d$, spanning the entire width of the channel. This receptor-coated strip, termed as the reaction site, is placed at a sufficient distance away from the microchannel inlet to ensure that the flow is fully developed before entering the receptor-rich region. An incompressible biofluid containing an initial concentration ($C_0$) of target analyte molecules (red spheres in Figure 2) with constant analyte diffusivity $D$ is flowed through the channel with a constant volumetric flow rate $Q$. The characteristic geometric and molecular parameters that are employed to characterize this model system are listed in Table 1. Here, assuming that the flow is steady, the velocity field in a Cartesian coordinate system is defined as $U = u\mathbf{e}_x + v\mathbf{e}_y + w\mathbf{e}_z$, where $u$, $v$, and $w$ are the $x$, $y$, and $z$ components of the velocity field, and $\mathbf{e}_x$, $\mathbf{e}_y$, and $\mathbf{e}_z$ are the unit normal vectors in the $x$, $y$, and $z$ directions, respectively.

In this model system, the width of the channel is assumed to be sufficiently larger than the channel height (i.e., $W/H > 10$), entailing two-dimensional (2D) flow with negligible variations...
Table 1. Dimensional Parameters Defining the Microfluidic Bioassay System

| parameter | definition | units |
|-----------|------------|-------|
| \(H, W, L\) | microchannel height, width, length | m |
| \(d\) | reaction site length | m |
| \(\delta\) | depletion layer thickness | m |
| \(Q\) | volumetric flow rate | m³/s |
| \(\gamma\) | shear rate | s⁻¹ |
| \(\rho\) | density of carrier fluid | kg/m³ |
| \(\mu\) | dynamic viscosity of carrier fluid | kg/(m·s) |
| \(D\) | analyte diffusivity | m²/s |
| \(k_{an}\) | reaction association constant | M⁻¹·s⁻¹ |
| \(K_D\) | equilibrium dissociation constant | M |
| \(c_o\) | initial analyte concentration | mol/m³ |
| \(b_o\) | close to reaction site | mol/m² |
| \(b_{a,t}\) | bound analyte–receptor complex formation | mol/m² |
| \(b_{a,t}\) | bound analyte–receptor complex concentration | mol/m² |
| \(r_e\) | reaction equilibrium time | s |
| \(C_{	ext{crit}}\) | critical analyte concentration | mol/m³ |

in flow velocities along the width of the channel. Typically, even at the highest flow rates employed in a microfluidic sensor, the micron-scale dimensions ensure that the flow in the channel is laminar (Reynolds number, \(Re = \frac{\bar{U}d}{\mu} \ll 1\)), where \(\rho\) and \(\mu\) are the fluid density and dynamic viscosity, respectively. Here, \(\bar{U} = Q/(WH)\) is the average fluid velocity. In addition, it is assumed that the biofluid is Newtonian in nature; any changes in physical properties of the fluid due to temperature fluctuations are insignificant, and the effects of gravity are neglected owing to the micron-scale channel dimensions. Consequently, the steady, unidirectional, axial flow through the microchannel has a parabolic velocity profile \(u(y) = (6Q/(WH^2))y(H - y)\), as described by the Hagen–Poiseuille law.

3.1. Analyte Transport

Assuming constant analyte diffusivity and 2D incompressible flow, with no sources or sinks, the spatiotemporal evolution of the analyte concentration field \(c(x, y, t)\) in the microchannel is governed by the additive effect of molecular diffusion and convection due to flow that can be described by the convection–diffusion equation:

\[
\frac{\partial c}{\partial t} = D \nabla^2 c - \bar{u}(y) \frac{\partial c}{\partial x}
\]

where \(\nabla^2 = \frac{\partial^2}{\partial x^2} + \frac{\partial^2}{\partial y^2}\). To be able to effectively capture the physics in the microfluidic system, the variables can be normalized by characteristic temporal and spatial scales to obtain key dimensionless parameters.

3.1.1. Systems Operating at Low Flow Rates. The first set of key dimensionless numbers arise from the non-dimensionalization of the convection–diffusion equation (eq 1), where the analyte concentration is scaled by the initial analyte concentration \((C_o)\), the axial distance by the length of the reaction site \((d)\), transverse distances by the microchannel height \((H)\), and time by a characteristic time \((eq 2)\). At low flow rates, only those analytes that diffuse across the channel height toward the reaction site are able to bind to the immobilized receptors. Hence, the characteristic time that emulates this phenomenon, is the time \((t_{0} = H^2/D)\) taken for the analytes to diffuse across \(H\) to reach the reaction site. The dimensionless variables are indicated by stars through the article.

\[
\frac{c}{C_o} = \frac{c^*}{C_o}, \quad x = \frac{x}{d}, \quad y = \frac{y}{H}, \quad t = \frac{t}{t_D} = \frac{Dt}{H^2}
\]

Thus, the nondimensionalized expression of eq 1 is

\[
\frac{\partial c^*}{\partial t^*} = \beta^2 \frac{\partial^2 c^*}{\partial x^*^2} + \frac{\partial^2 c^*}{\partial y^*^2} - (6\beta Pe_H)y^*(1 - y^*) \frac{\partial c^*}{\partial y^*}
\]

where \(\beta = H/d\) is the dimensionless size of the reaction site size and \(Pe_H = Q/(WD)\) is the bulk Peclet number, which is the ratio of convective to diffusive analyte transport in the microfluidic system. In this low flow rate regime, the analytes are primarily transported through the microchannel by diffusion if \(Pe_H \ll 1\) and by convection if \(Pe_H \gg 1\).

3.1.2. Systems Operating at High Flow Rates. At high flow rates, the analytes are flushed downstream before they have a chance to diffuse across the channel height. In this case, the reaction site does not experience the effect of the full parabolic flow profile but is most affected by the linear flow profile \((u(y) = \gamma y)\) up to a certain distance close to the receptor-coated channel wall. Here, \(\gamma = 6Q/(WH^2)\) is the shear rate at the reaction site \((y = 0)\). Under these flow conditions, the diffusive analyte flux is independent of \(H\) but dependent on the thickness of a steady depletion zone \((\delta)\) that is formed above the reaction site as the supplied analytes are steadily captured by the receptors. As illustrated in Figure 3, at \(\delta\), those analytes that are convected by

![Figure 3. Schematic illustrating the 2D steady analyte depletion zone (thickness \(\delta\)) formed close to the reaction site surface as the analytes are captured by the receptors. At \(\delta\), the analytes are able to convect along the length of the reaction site at a time scale \(t_s\) and diffuse across the depletion zone at a time scale \(t_d\). Here, \(u(y)\) is the linear velocity profile close to the reaction site surface, \(c^*\) is the analyte concentration close to the reaction site, \(C_o\) is the bulk analyte concentration, and \(d\) is the reaction site length.](https://doi.org/10.1021/jacsau.1c00318)

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Thus, the new nondimensionalized expression of eq 1 that incorporates the effect of linear flow in the boundary layer is
\[ \frac{\partial c^*}{\partial t^*} = \left( \frac{1}{Pe_0} \right) \left[ \frac{1}{3} \frac{\partial^2 c^*}{\partial y^2} + \frac{\partial c^*}{\partial y} - \gamma c^* \frac{\partial c^*}{\partial x} \right] \]

where
\[ Pe_0 = \frac{\gamma \overline{D}^2}{D} = 6Pe_{el} \frac{H}{\beta^2} \] (6)

Equation 6 defines the local Peclet number (Pe_0) that enables us to quantify the competition between diffusive analyte transport and transport of analytes by linear convective flow in the local vicinity of the reaction site.

Furthermore, the imposed transport boundary conditions ensure that (i) the fluid introduced into the microchannel has an initial analyte concentration \( c_0(x_{0, y}) = \frac{C_0}{\gamma} \) and (ii) no analytes diffuse out of the microchannel walls (\( n \cdot \nabla c = 0 \)), where \( n \) is defined as the unit normal vector directed out of the surface. These boundary conditions are nondimensionalized using the scaling parameters listed in eq 4 to obtain (i) \( c^* = 1 \), and (ii) \[ n \left[ \frac{1}{Pe_0} \right] \left( \frac{\partial c^*}{\partial y} - \frac{\partial c^*}{\partial x} \right) = 0 \], respectively.

3.2. Receptor–Analyte Reaction Kinetics

When in close proximity, the analyte molecules reversibly bind to the receptor molecules at the liquid–solid reaction site interface. Several reaction models have been proposed to describe the receptor–analyte binding mechanisms.²⁶⁻²⁹ In this model system, we assume that the receptor–analyte binding reaction occurring on a solid surface is analogous to the reaction between adsorbates in a fluid and adsorbents on a solid surface. Given this assumption, the receptor–analyte binding mechanism can be described by the Langmuir adsorption isotherm model.²⁷ This binding model has been described as a majority of the adsorption/desorption mechanisms in molecular biology.²⁸ Specifically, the Langmuir model describes a pseudo-first-order binding reaction between an analyte in solution and a receptor immobilized on the reaction site surface given that (1) the analyte molecules reversibly bind to a finite number of receptor molecules on the reaction site, (2) one receptor molecule binds to one analyte molecule equivalently for all binding sites under isothermal conditions, and (3) the receptor–analyte binding saturates at equilibrium:

\[ \text{receptor} + \text{analyte} \rightleftharpoons \text{complex} \]

where \( k_{on} \) and \( k_{off} \) are the association and dissociation constants, respectively (refer to Figure 2). Correspondingly, the bound complex concentration \( b \) evolves exponentially as
\[ \frac{db}{dt^*} = k_{on}c_{y}^*(b_{max} - b) - k_{off}b \]

where the rate of formation of the receptor–analyte complex \( (\partial b_*/\partial t^*) \) is highly dependent on the (i) fixed surface concentration of receptors on the reaction site \( b_{max} \), (ii) the reaction kinetic constants \( k_{on} \) and \( k_{off} \), and (iii) the concentration of analytes close to the reaction site, i.e., \( c_{y} \) at \( y = 0 \). In most cases, the rate of replenishment of \( c_{y} \) by diffusion or convection proves to be the limiting factor that controls the speed of receptor–analyte binding.²⁸ This relationship can be encapsulated by the reaction–flux balance boundary condition imposed on the reaction site surface:
\[ \frac{\partial b}{\partial y} = -D \frac{\partial c^*}{\partial y} \quad \text{at} \quad y = 0 \] (9)

If the analytes are consumed faster than they are replenished, the analyte detection speed is “transport-limited”. On the contrary, if the analytes are replenished faster than they are consumed by the receptor–analyte binding reaction, the speed of analyte detection becomes “reaction-limited”. Consequently, the kinetic equations and boundary conditions can be nondimensionalized with appropriate spatial and temporal scales to derive the final key dimensionless parameter: the Damköhler number, which describes the balance between the transport and reaction time scales.

3.2.1. Transport-Limited Regime. First, we consider a microfluidic system consisting of receptors immobilized at high surface densities (large \( b_{max} \)) and high reaction affinities (large \( k_{on} \) and small \( k_{off} \)), where the analytes are instantaneously captured by the receptors when they are in close proximity. If the analyte supply is much slower than the reaction speed \( (Pe_{hl} \ll 1 \text{ and } Pe_2 \ll 1) \), the concentration of analytes close to the reaction site \( (c_y) \) steadily decreases as the reaction proceeds, thereby slowing down the receptor–analyte binding reaction. In this scenario, the time taken to detect the analytes is ultimately limited by the time taken for the analytes to diffuse across the microchannel height \( H \) and replenish the consumed analytes close to the reaction site, i.e., the diffusive time \( t_d = H^2/\overline{D} \).

Intuitively, the characteristic length \( H \) and time scale \( t_d \) naturally arise in this scenario. Under these assumptions, eq 8 can be nondimensionalized using the following conventional spatial and temporal scales as described by Gervais et al.²⁸ and Squires et al.²⁹

\[ \frac{b^*}{b_{max}^*}, \quad c^* = \frac{c}{C_0}, \quad t^* = \frac{Dt}{H^2}, \quad y^* = \frac{y}{H} \] (10)

Consequently, the nondimensionalized expression of eq 8 becomes
\[ \frac{\partial b^*}{\partial t^*} = D_{\theta1}[c^*(1 - b^*) - K_{\theta1}^*b^*] \] (11)

where \( D_{\theta1} = C_gk_{on}H^2/\overline{D} \) is the diffusive Damköhler number representing the balance between the reactive and diffusive flux for the receptor–analyte reaction. In the regime of \( Pe_{hl} \ll 1 \text{ and } Pe_2 \ll 1 \), it takes longer for an analyte to react with a receptor molecule when compared to the time taken for the analyte to diffuse to the reaction site when \( D_{\theta1} \ll 1 \). Conversely, when \( D_{\theta1} \gg 1 \), it indicates that the reaction time is much shorter than the analyte diffusion time. Additionally, we obtain the dimensionless reaction dissociation coefficient, \( K_{\theta1}^* = k_{off}/k_{on}C_0 \).

In parallel, eq 9 is nondimensionalized using the same spatial and temporal scales to obtain the dimensionless reaction–flux balance boundary condition:
\[ \frac{\partial c^*}{\partial y^*} \bigg|_{y^*=0} = -\frac{1}{\epsilon}(D_{\theta1}[c^*(1 - b^*) - K_{\theta1}^*b^*]) \] (12)

where \( \epsilon = C_gH/b_{max}^* \) is the dimensionless binding fraction that allows us to estimate the total quantity of receptors \( b_{max}^* \) required to effectively capture all analyte molecules when delivered at
different initial concentrations $C_0$ in a microchannel with height $H$. Here, it is important to note that $Da_H$ and $e^*$ only encapsulate those characteristic variables that affect the speed of analyte detection when the microfluidic system is operated at low flow rates. However, these dimensionless parameters fail to account for key spatial and temporal scales that dominate under high flow rate conditions.

3.2.2. Reaction-Limited Regime. In microfluidic systems either operating at high flow rates, i.e., at $Pe_H \gg 1$ and $Pe_d \gg 1$, or at moderate flow rates but fast analyte diffusivity, the analytes are transported through the microchannels much faster than they are captured by the receptors immobilized on the reaction site. As a consequence the concentration of analytes close to the reaction site is the same as the bulk analyte concentration (i.e., $c_i \approx C_0$). In this scenario, the analyte detection time is no longer dependent on the diffusive time, but is limited by the time taken for an analyte to react with a receptor molecule, i.e., the “on-rate”, $k_{on}$. Consequently, the naturally arising characteristic length and time scales in fast flow scenarios are the depletion zone thickness ($\delta$) and receptor–analyte association time, $t_{as} = (k_{on}C_0)^{-1}$, respectively.

As described by Hansen et al., $\text{eq } 8$ can be non-dimensionalized using the following spatial and temporal scales

$$b^* = \frac{b}{b_{max}}, \quad c^* = \frac{c}{C_0}, \quad t^* = k_{on}C_0t, \quad y^* = \frac{y}{\delta}$$

(13)

to derive the dimensionless receptor–analyte reaction kinetic equation

$$\frac{\partial b^*}{\partial t^*} = c^*(1 - b^*) - K_{DD}b^*$$

(14)

and the dimensionless reaction–flux balance boundary condition

$$\frac{\partial c^*}{\partial y^*} = -Da_D[c^*(1 - b^*) - K_{DD}b^*]_{y^*=0}$$

(15)

where $Da_D = b_{max}k_{off}\delta/D$ is the kinetic Damköhler number that represents the balance between the reactive and transport flux in systems operating at extremely fast flow rates, i.e., at $Pe_d \gg 1$. Under these conditions, analyte–receptor reaction time is much longer than the time taken for the analytes to be transported across $\delta$ when $Da_D \ll 1$ and vice versa when $Da_D \gg 1$. It should be emphasized that both $Da_H$ and $Da_D$ describe the balance between reaction and transport times. However, we stress that each Damköhler number has physical significance and implications only in the appropriate operating conditions.

These dimensionless parameters, i.e., $Pe_H$, $Pe_d$, $Da_H$, and $Da_D$ (Table 2), collectively allow us to estimate the extreme physical limits acting on the microfluidic sensor but also enable us to intuitively predict how and when different characteristic variables have the most dominant impact on the analyte detection efficiency.

4. DEFINING THE EFFICIENCY OF THE MICROFLUIDIC SENSOR

As described in the previous sections, the derived dimensionless ratios, i.e., $Pe_H$, $Pe_d$, $Da_H$, and $Da_D$ collectively allow us to estimate if the analyte detection in a given microfluidic sensor is dependent on the rate of analyte transport (transport-limited detection when $c_i \approx 0$) or on the rate of analyte–receptor reaction (reaction-limited detection, when $c_i \approx C_0$), but how can we exploit this knowledge to develop highly efficient microfluidic sensors?

In a typical microfluidic sensor, a signal is generated when analytes are captured by the receptors in the device. The intensity of this signal is directly proportional to the concentration of analytes that bind to the receptors. By tracking the evolution of these signals over time, we are able to monitor the reaction kinetics between the analytes and receptors. For any given initial analyte concentration ($C_0$), the largest and most stable signal can be obtained when the receptor–analyte reaction reaches equilibrium. As this equilibrium signal is highly dependent on $C_0$, it enables us to reliably and reproducibly quantify analytes from a sample fluid. As a consequence, this equilibrium signal is used as a quantitative marker for analyte detection in most microfluidic sensors.

| Table 2. Dimensionless Parameters Characterizing the Microfluidic Bioassay System |
|---------------------------------------------------------------|
| **dimensionless parameter** | **definition** | **description** |
| Re = $\hat{U}H/\mu$ | Reynolds number | inertial force |
| $\beta = H/d$ | dimensionless reaction site size | microchannel height |
| $Pe_H = Q/WD$ | bulk Peclet number | convection rate |
| $Pe_d = \gamma d^2/D$ | local Peclet number | shear rate |
| $Da_H = C_0k_{off}H^2/D$ | diffusive Damköhler number | diffusion rate |
| $Da_D = b_{max}k_{off}\delta/D$ | kinetic Damköhler number | mass transport rate |
| $K_D = k_{off}/k_{on}C_0$ | dimensionless dissociation constant | analyte dissociation rate |
| $e^* = C_dH/b_{max}$ | dimensionless binding fraction | bulk analyte concentration |

4.1. Reaction Equilibrium Time: The Detection Speed Limit

In the best case scenario, there is a surplus of analytes that are transported through the channels such that the time taken for the signal to equilibrate for a given $C_0$ is purely determined by the time taken for the kinetics of the analyte–receptor reaction to reach equilibrium. In this reaction-limited regime, the analyte concentration close to the reaction site is nearly equal to the bulk analyte concentration, i.e., $c_i \approx C_0$. Correspondingly, the fraction of receptors bound with analytes ($b/b_{max}$) at any given time ($t$) can be estimated by solving the exponential kinetic receptor–analyte equation (eq 8).

$$\frac{b(t)}{b_{max}} = \frac{k_{off}C_0}{k_{on}C_0 + k_{off}}(1 - e^{-(k_{on}C_0 + k_{off})t})$$

(16)

When the receptor–analyte reaction reaches equilibrium, the fraction of receptors bound with analytes can be estimated as $b_{eq}/b_{max} = C_0/(C_0 + K_D)$ given that $\partial b/\partial t = 0$, based on eq 8. Here, the equilibrium dissociation constant $K_D = k_{off}/k_{on}$ helps us quantify the affinity of the receptors toward the analytes. The lower the value of $K_D$, the higher the affinity of the receptors. Furthermore, the time taken to reach the state of equilibrium is termed as the reaction equilibrium time, $t_R$:

$$t_R = (k_{on}C_0 + k_{off})^{-1}$$

(17)
to determine the equilibrium signal speed that can be reached but not exceeded. Depending on the system under investigation, there have been different ways proposed to estimate the LoD. Although each method has subtle differences, all formulas effectively allow us to estimate the lowest concentration of analytes that specifically bind to the receptors by statistically differentiating the specific signal from the noise, i.e., nonspecific analyte adsorption. The most widely used set of formulas to estimate the LoD of assay systems was first proposed by Armbruster et al.,36 where they accounted for the effect of matrix interferences due to chemical components in blank buffer samples by means of the limit of blank, or LoB:

$$\text{LoB} = \text{mean}_{\text{blank}} + 1.645(\text{SD}_{\text{blank}}) \quad (18)$$

where mean_{blank} and SD_{blank} are the mean and standard deviation of the blank signals. Subsequently, the LoD can be estimated from the LoB and the standard deviation of the signal obtained from the specific analyte−receptor reaction for the lowest concentration sample (SD_{lo}):

$$\text{LoD} = \text{LoB} + 1.645(\text{SD}_{\text{lo}}) \quad (19)$$

The LoD serves as a vital tool to quantify the experimentally feasible limit of analytical sensitivity that can be achieved in any microfluidic sensor, where the smaller the LoD, the higher the sensitivity of the assay system. By putting everything into perspective, we now see that an ideal microfluidic sensor should enable sensitive and rapid analyte detection with a low LoD, and analyte detection at the equilibrium signal speed limit, i.e., at $\tau_R$.

In order to develop such an ideal system, several characteristic variables influencing the analyte transport and receptor functionality must operate like clockwork. However, accurately identifying how to tune these characteristic variables is no small task. This is where the derived dimensionless parameters, $P_e$, $P_{\text{e0}}D_{\text{a0}}$, and $D_{\text{aq}}$ come into play. In the following subsections, we will demonstrate how these dimensionless parameters can be used as handy tools to intuitively quantify how and when different characteristic variables have the strongest impact on the sensitivity and analyte detection speed of the microfluidic sensor.

### 5. Reaching the Detection Speed Limit

Of the various factors influencing the analyte detection speed of a microfluidic sensor, analyte transport and replenishment have the strongest influence. Theoretically, the ideal and purely $\tau_R$-dependent equilibrium signal speed can be achieved only if the analytes are supplied to the receptors at an extremely fast rate, i.e., at a rate surpassing the rate at which analytes are captured by the receptors. However, in most experimental scenarios, the analytes are supplied to the receptors at a rate much slower than the capture rate, thereby drastically reducing the equilibrium signal speed.

In order to be able to picture these scenarios, we draw your attention to four well-studied analyte model systems listed in Table 3: (i) an inflammatory protein named c-reactive protein (CRP), whose elevated levels in patient blood indicate disorders ranging from infectious diseases to cardiovascular disease and organ damage; (ii) immunoglobulin G (IgG) antibodies indicative of past pathogenic infections and, most recently, past COVID-19 infections; (iii) ovarian cancer biomarker (CA125); and (iv) a cytokine interleukin-6 (IL6), indicative of inflammatory disorders and cancers.

Of these analytes, CRP and IgG are relatively large molecules with low diffusivity ($D \approx 10^{-11}$ m$^2$/s), CA125 is a moderately sized molecule ($D \approx 10^{-10}$ m$^2$/s), and IL6 is a small molecule with relatively high diffusivity ($D \approx 10^{-8}$ m$^2$/s). All of these analytes are typically detected by their reactions with specific antibodies employed as receptors, with moderate to high “on rates” ($10^4 \leq k_{\text{on}} \leq 10^7$ M$^{-1}$ s$^{-1}$) and a moderate range of “off-rates” ($10^{-5} \leq k_{\text{off}} \leq 10^{-2}$ s$^{-1}$). Given these reaction rates, the detection speed limits set by $\tau_R$ (eq 17) are listed for three clinically relevant initial concentrations, i.e., $C_0 = 1 \mu$M, 1 nM, and 1 pM.

To begin with, we focus on the relationship between initial analyte concentration $C_0$ and the detection speed limit ($\tau_R$) for the receptor–analyte model systems listed in Table 3. At high analyte concentrations, i.e., $C_0 \gg K_D$, the receptor–analyte reaction reaches equilibrium when all receptor sites are saturated with analytes ($b_{\text{eq}} \approx b_{\text{max}}$). Here, $\tau_R$ is predominantly dependent on $(k_{\text{off}}C_0)^{-1}$, the “on-rate” of the receptor–analyte kinetic reaction. As all the receptor–analyte model systems listed in Table 3 rely on the use of moderate to high affinity receptor antibodies, the low $\tau_R$ values for $C_0 \geq 1 \mu$M indicate that the receptor–analyte reactions reach equilibrium almost instantaneously. On the other hand, at extremely low analyte concentrations, i.e., $C_0 \leq K_D$, only a fraction of receptors can be saturated with analytes at equilibrium ($b_{\text{eq}} \approx b_{\text{max}}C_0/K_D$). As a result, the reaction reaches equilibrium only when there is a self-sustaining feedback loop created by the bound analytes.
dissociating from the receptors and these dissociated analytes re-binding with the free receptors. In this scenario, \( \tau_R \) is estimated from \((k_{oE})^{-1}\), the “off-rate”, where the larger the \( k_{oE} \) the shorter the time it takes to reach equilibrium, i.e., the smaller the \( \tau_R \). For instance, reaction equilibrium can be reached within \( \tau_R = 0.64 \) min for \( C_0 = 1 \) pM of CRP, owing to the fast “off-rate” of \( k_{oE} \approx 10^{-5} \) s\(^{-1}\). On the contrary, as a result of the slow \( k_{oE} \approx 10^{-6} \) s\(^{-1}\), it takes \( \tau_R \approx 278 \) min for the reaction of the same concentration of IL6 to reach equilibrium.

In order to build a microfluidic device that enables us to obtain the equilibrium signal at \( t = \tau_R \), we must ensure optimal operation conditions in this device. To understand what these optimal conditions are, let us first build a relatively moderate-sized microfluidic device with a channel height of \( H = 100 \) \( \mu \)m, operating at low flow rates, i.e., \( Pe_H \ll 1 \) and \( Pe_S \ll 1 \). Under these conditions, only those analytes close to the reaction site surface are steadily captured by the receptors. The time it takes for an analyte molecule to reach the receptors and replenish the consumed analytes close to the reaction site is defined by the diffusion time, \( \tau_D = H^2/D \). In the case of smaller analyte molecules with high \( D \) such as IL6, a low \( \tau_D \approx 0.1 \) min ensures fast analyte replenishment, such that \( Da_E = (C_0 k_{oE} H^2/D) \approx 1 \) for \( C_0 = 1 \) \( \mu \)M, in a microchannel with \( H = 100 \) \( \mu \)m (Table 4).

However, in the same channel, larger analytes such as CRP and IgG take longer (\( \tau_D \approx 3-8 \) min) to replenish the consumed analytes such that \( Da_E \gg 1 \) for \( C_0 = 1 \) \( \mu \)M. In both cases, the equilibrium signal time is now dependent on \( \tau_D \). Intuitively, in these cases, the equilibrium signal time can be pushed closer to \( \tau_R \) by simply reducing the channel height to \( H = 1 \) \( \mu \)m, such that \( Da_E \ll 1 \). Consequently, we observe that channel height \( H \) is a critical operational variable that can be tuned to achieve the equilibrium signal at \( \tau_R \) for any analyte–receptor system analyzed in microfluidic devices operating at \( Pe_H \ll 1 \) and \( Pe_S \ll 1 \). This principle has been successfully exploited by researchers to improve the speed of microfluidic sensors.78-84

In these systems, an important thing to note is that the equilibrium signal speed is already limited by \( \tau_R \) for \( C_0 = 1 \) pM for all four model systems reacting in a microchannel of \( H = 100 \) \( \mu \)m. This is mainly because \( \tau_R \approx (k_{oE})^{-1} \gg \tau_D \) in this low concentration scenario, where \( C_0 \ll K_{D_0} \). Under these conditions, the equilibrium signal time is purely dependent on \((k_{oE})^{-1}\) and does not get affected by the reduction of \( H \). Thus, it appears that the effect of \( H \)-dependent detection of equilibrium signal at \( \tau_R \) is only observed at \( C_0 \approx K_{D_0} \), for low flow rate systems. As a general rule, the optimal channel dimensions can be computed by identifying that \( H \) at which \( Da_E \ll 1 \) for \( C_0 = K_{D_0} \) for any analyte–receptor reactions carried out in microfluidic devices operating at \( Pe_H \ll 1 \) and \( Pe_S \ll 1 \).

Going one step further, it is also possible to design perfect collection microfluidic systems where every analyte molecule that is delivered by the flow is collected by the reaction site. These perfect collection systems can be developed by fine-tuning the flow rates such that the convective analyte flux matches the analyte diffusive flux reaching the reaction site.29 Experimentally, this has been shown to be possible in submicron channels with channel heights of a few hundred nanometers.65-67 Collectively, these low flow rate systems have been shown to be immensely valuable for single molecule detection68,69 and in cases where detection of low concentration of analyte from a limited sample volume is the major requirement.70-72

While diffusion-driven micro/nanofluidic devices have several benefits, the development of these systems is highly reliant on the micro/nanofabrication73-75 facilities available to the users. Consequently, an increasing number of microfluidic systems with larger channel dimensions are being designed to operate at high flow rates, i.e., \( Pe_H \gg 1 \) and \( Pe_S \gg 1 \). Under these conditions, the analyte transport through the channels is predominantly controlled by convection. In systems operating at extremely high flow rates, the equilibrium signal time is influenced by the time taken for a convected analyte to diffuse across the steady depletion zone \( \delta \) formed just above the reaction site (Figure 3). In other words, the dominant time scale in this scenario is now, \( \tau_D \), i.e., the time taken for the diffusive flux \( (J_D = D(C_0 - C_\delta)/\delta) \) to feed the receptor–analyte reaction on the reaction site:

\[
\tau_D \approx \frac{h_{eq}}{J_D} \approx \frac{1}{(1-c_\delta/C_0)}Da_E \tau_R
\]

As seen in eq 20, the kinetic Damköhler number \((Da_E = k_{oE} h_{eq} \delta/D)\) holds the key to push the convection-driven reaction equilibrium time \( (\tau_D) \) to reach the detection speed limit \( (\tau_R) \). Specifically, \( \tau_D \approx \tau_R \) when \( Da_E \ll 1 \) and \( c_\delta \approx C_0 \). This can be easily achieved by simply enhancing the flow rates to specifically shrink the depleting zone thickness \( \delta \) to subsequently enhance the convective replenishment of analyte close to the reaction site for a given set of molecular parameters \((k_{oE}, h_{max}, \delta)\).77 In circumstances where sample volume is a constraint, microfluidic systems have been designed to allow recirculation of analyte samples through the microchannels to enable the use of high flow rates for rapid analyte detection while preserving sample volumes.78,79

In addition to simple convection-controlled systems, microfluidic systems are being developed to employ hydrodynamic flow focusing via the use of sheath flows80-82 or obstacles.83 By focusing the analyte fluid to a thin layer above the reaction site, the depletion layer is minimized, subsequently allowing for enhanced analyte transport. These initial investigations are paving ways for further experimental research to develop rapid microfluidic sensors applicable to a wide range of analytes. As these systems are independent of channel geometry, i.e., the

| \( H = 100 \) \( \mu \)m | \( H = 1 \) \( \mu \)m |
|---|---|
| \( Da_E \) | \( Da_E \) |
| \( C_0 = 1 \) \( \mu \)M | 4.55 \( \times 10^{-3} \) | 4.55 \( \times 10^{-3} \) |
| \( C_0 = 1 \) pM | 4.55 \( \times 10^{-5} \) | 4.55 \( \times 10^{-7} \) |
| \( \tau_D \) (min) | 5.68 \( \times 10^{-1} \) | 5.68 \( \times 10^{-1} \) |
| \( \tau_D \) (min) | 4.55 \( \times 10^{-8} \) | 4.55 \( \times 10^{-8} \) |
| \( \tau_D \) (min) | 7.58 \( \times 10^{-4} \) | 7.58 \( \times 10^{-4} \) |
| \( \tau_D \) (min) | 3.78 \( \times 10^{-4} \) | 3.78 \( \times 10^{-4} \) |
| \( \tau_D \) (min) | 8.77 \( \times 10^{-4} \) | 8.77 \( \times 10^{-4} \) |
| \( \tau_D \) (min) | 1.96 \( \times 10^{-3} \) | 1.96 \( \times 10^{-3} \) |

Table 4. List of Estimated Diffusion Times (\( \tau_D \)) and Diffusive Damköhler Numbers (\( Da_E \)) for Two Initial Analyte Concentrations (\( C_0 \)) for the Four Receptor–Analyte Model Reactions Carried out in Microchannels with Two Different Heights (\( H \))
channel height $H$ no longer has an influence on the analyte detection speed, micron-scale channels can be comfortably fabricated using conventional microfabrication techniques.\textsuperscript{84−87}

With simple integration of robust microfluidic channels and well-controlled pressure-driven fluid delivery systems, these design rules help us to tune the device geometry and operational variables to achieve rapid analyte detection according to reagent−volume requirements. Recently, more complex sample preconcentration strategies are being exploited to increase the local analyte concentrations close to the receptors (i.e., to achieve $c_i \approx C_i$). These strategies include the use of magnetic bead-based immuno-affinity techniques, electrostatic interaction-based capture and dielectrophoretic techniques.\textsuperscript{88−91}

However, these operational variables can only be tuned up to a certain extent, as defined by the detection speed limits set by the functionalities of the receptors. Therefore, it is important to understand how and what molecular variables can be enhanced and up to what extent in order to further improve the efficiency of the microfluidic sensors.

6. ENHANCING ANALYTICAL SENSITIVITY AND SPECIFICITY

In most cases, the most standard features that are used to quantify the “efficiency” of microfluidic sensors are the “analyte detection speed” and the “analytical sensitivity”.\textsuperscript{9,10} In addition to these key features, there is another important feature that determines the feasibility of these systems in real medical applications; the “analytical specificity”.\textsuperscript{92} Here, the analytical specificity of the device is determined by its ability to differentiate positive signals arising from specific analyte−receptor binding reactions from the negative signals originating from the noise in the system. Owing to the complexity of these systems, there are multiple factors, ranging from receptor specificity to sample fluid contaminants, that cumulatively add to the noise in these systems. The higher the noise, the less reliable it becomes to use the information obtained from the positive signals.

In order to be able to reliably employ these systems in real applications, it is therefore of utmost importance to not only develop devices with high analyte detection speed and analytical sensitivity, but also, with the highest achievable analytical specificity. As discussed in the previous section, the analyte detection speed can be enhanced by ensuring continuous and rapid analyte transport through the microchannels so as to push the analyte detection time to $\tau_R$. While operating at this detection speed limit, the efficiency of the device is now purely dependent on the molecular characteristics of the respective receptor−analyte reaction. In what follows, we discuss how these molecular parameters, i.e., $K_D$, $k_{off}$ and $k_{on}$ can be specifically tailored to enhance analytical specificity and sensitivity, while ensuring rapid analyte detection.

6.1. Tailoring Receptor Affinity

Of the several factors that influence the analytical specificity of any assay system, the molecular characteristics of the receptors top the list. Irrespective of the type of receptor chosen for the assay, the receptors must be able to selectively distinguish the desired analytes from a sea of molecules with similar structures and functionalities. For example, antibodies, particularly immunoglobulin G (IgG), have been exploited to serve as effective receptors for a plethora of protein analytes such as those listed in Table 3. Owing to the simple, fast, and low cost technologies needed for production, polyclonal antibodies are

the most commonly employed receptors for protein-based analytes. As different polyclonal antibodies are able to recognize multiple parts or epitopes of an analyte molecule, more antibodies are likely to be able to efficiently capture the same analytes as they reach the reaction site, thereby generating larger signals.\textsuperscript{93−95} However, there are a number of drawbacks to using such antibodies for quantitative analyte assays. These antibodies have low affinities to analytes with relatively high $K_D$ values ranging from $10^{-9}$ to $10^{-6}$ M.\textsuperscript{99,101} In addition, these antibodies have high cross-reactivity with analytes with similar structures since they are able to recognize multiple epitopes, thereby increasing the nonspecific signals obtained in any given system. As a result, systems employing polyclonal antibodies as receptors tend to have very low analytical sensitivity.\textsuperscript{96,97}

On the contrary, a monoclonal antibody is able to recognize only one epitope on the analyte, making the analyte recognition and capture highly specific. These antibodies typically have high affinities to the analytes, with $10^{-12} \leq K_D \leq 10^{-9}$ M.\textsuperscript{41,43} Due to the high specificity and analyte selectivity, monoclonal antibodies have low cross-reactivity to other analytes, thereby lowering the probability of nonspecific signals in the systems. These high-affinity antibodies in turn significantly increase the analytical specificity of microfluidic sensors. However, a larger concentration of monoclonal antibodies is required to generate the same signal that can be obtained by using polyclonal antibodies. Additionally, these antibodies require complex technology for production, thereby significantly increasing their production time and cost. As these monoclonal antibodies are able to recognize only one epitope of the analyte, any changes in analyte structure due to changes in temperature, ionic strength or pH of the analyte fluid, significantly affects the ability of these antibodies to effectively capture analytes.\textsuperscript{96,97}

Owing to recent progress in genetic engineering, protein-based receptors are being slowly replaced by high affinity nucleic acid-based receptors such as DNA and RNA aptamers. These aptamers are serving as synthetic antibodies that can be easily tailored to bind to specific analytes with extremely high affinity ($K_D \ll 10^{-12}$ M).\textsuperscript{99} Furthermore, these single-stranded molecules are stable at a wide range of temperatures and for long periods of time, with low susceptibility of conformation changes.\textsuperscript{99} Owing to these advantages, these molecules are being increasingly exploited in a wide variety of analyte detection systems.\textsuperscript{99−101}

While increasing the receptor affinity leads to enhancement of the analytical specificity of microfluidic sensors, it has a negative impact on the speed of reaching the equilibrium signal for low analyte concentrations. Typically, the affinity ($K_D = k_{off}/k_{on}$) of the receptors is enhanced by increasing $k_{on}$ and decreasing $k_{off}$. As elaborated in previous sections, the time taken to obtain the equilibrium signal is dependent on $\tau_R \approx (k_{off}/k_{on})^{-1}$ for high analyte concentrations where $C_0 \gg K_D$ or $K_D \ll 1$. At these high analyte concentrations, the higher the $k_{on}$ the lower the $\tau_R$ and the faster the reaction equilibrium. On the contrary, at low analyte concentrations where $C_0 \ll K_D$ or $K_D \gg 1$, the equilibrium signal time is now dependent on $\tau_R \approx (k_{off})^{-1}$. In this situation, the higher the receptor affinity, the lower the $k_{off}$ the longer the $\tau_R$ and, consequently, the longer it takes to obtain the equilibrium signal. This dilemma forces us to sacrifice analyte detection speed in an attempt to increase analytical specificity of the system. In systems where speed of analyte detection is a priority, choosing moderate affinity receptors with large $k_{on}$ and $k_{off}$ values is beneficial to be able to detect low analyte concentrations rapidly and with high analytical specificity.
6.2. Tailoring Surface Density

As seen in the previous sections, we observe that $k_{on}$ and $k_{off}$ have a significant influence on the analytical specificity of the system. In addition, according to Zimmermann et al.,

$$
\text{fl}_{\text{eff}} = \frac{C_0}{K_{D,r}}
$$

and $k_{off}$ irrespective of the surface density of receptors on the reaction site ($b_{\text{max}}$). They elaborate that irrespective of $b_{\text{max}}$, it will always take $t_{R}$ for the reaction to reach equilibrium for a given $C_0$. This is because, at high $b_{\text{max}}$, the quantity of analytes binding per unit time is large, and vice versa for low $b_{\text{max}}$. As a result, the way the reaction reaches equilibrium may be quantitatively different, where $b_{\text{eq}}$ varies with $b_{\text{max}}$ but $t_{R}$ remains unchanged. Intuitively, we see that $b_{\text{max}}$ does not affect speed of analyte detection, but has a significant effect on the maximum equilibrium signal that we can obtain from positive analyte binding. Ultimately, $b_{\text{max}}$ has a direct effect on the analytical sensitivity of the device, or in other words, the LoD.

In systems operating at diffusion-dominant conditions ($P_{\text{eqi}} \ll 1$, $b_{\text{max}}$ can be tailored according to $C_0$ and $H$ using the dimensionless ratio $e^* = C_0 H/b_{\text{max}}$. Ensuring $e^* < 1$ entails that there are sufficiently large number of receptor molecules in the reaction site to generate a large enough signal at equilibrium. In the convection-dominant systems ($P_{\text{eqi}} \gg 1$), although the rule states that $D_{\text{eq}} = (b_{\text{eq}} K_{\text{eq}} \delta/D) \ll 1$ to detect analytes at $t_{R}$, we must be careful in choosing those variables that allow us to reach $D_{\text{eq}} \ll 1$. Intuitively, it seems that decreasing $k_{on}$ or $b_{\text{max}}$ would help us reach the detection speed limit. However, by decreasing $k_{off}$ we would be consequently decreasing analytical specificity and at the same time increasing $t_{R}$, ultimately increasing equilibrium signal time, thereby significantly decreasing the analyte detection speed.

Similarly, decreasing $b_{\text{max}}$ has a significant impact on the analytical sensitivity or the LoD. In cases where $C_0 \gg K_{D,r}$, $b_{\text{eq}} \approx b_{\text{max}}$ at equilibrium, such that all reaction sites are bound at equilibrium. In this case, a large equilibrium signal can be generated if $b_{\text{max}}$ is sufficiently large. However, at low analyte concentrations when $C_0 \ll K_{D,r}$, only a fraction of reaction sites are bound at equilibrium, where $b_{\text{eq}} \approx C_0 b_{\text{eq}}/K_{D,r}$. Therefore, at the same $b_{\text{max}}$ used to detect $C_0 \gg K_{D,r}$, the equilibrium signal obtained for low analyte concentrations is significantly smaller. This ultimately decreases the LoD and the analytical sensitivity of the system.

To address this issue, it is important to have a sufficiently large $b_{\text{max}}$ so as to be able to obtain high equilibrium signals for the range of $C_0$ required to be analyzed by the system. In order to identify the appropriate $b_{\text{max}}$ and reaction site length ($d$) for a given system, another characteristic parameter comes in handy. Squires et al.,

$$
\text{fl}_{\text{eff}} = \frac{C_0}{K_{D,r}}
$$

define this parameter as a critical analyte concentration, $C_{\text{crit}}$, that allows us to estimate the lowest $C_0$ at which only 1 molecule binds at equilibrium, for a given $d$ and $b_{\text{max}}$:

$$
C_{\text{crit}} = \frac{K_{D,r}}{d H b_{\text{max}}} \times 1 \text{ molecule}
$$

At this theoretical limit where $C_0 = C_{\text{crit}}$, an equilibrium signal is obtained from the binding of one molecule. The detection of single molecule binding events are realistically almost impossible to detect using conventional fluorescence or absorbance measurement techniques. On the other hand, more sensitive optical and electronic measurement techniques could potentially allow for detection of such binding events. Nevertheless, based on the desired application, we can ensure that our systems are not operating in this single molecule binding regime by simply lowering $C_{\text{crit}}$ while ensuring that $d^2 b_{\text{max}} \approx K_{D,r}$. Consequently, we can tailor the size of the reaction site ($d$) and $b_{\text{max}}$ using the lowest $C_0$ employed in the system, by simply ensuring that the lowest $C_0 \gg C_{\text{crit}}$. At these conditions, we can ensure that the fraction of bound sites ($C_{\text{eq}}/b_{\text{max}}$) is detectable for $C_0 \ll K_{D,r}$ using conventional detection methods. To achieve this goal, $d$ can be easily tailored by simply coating a larger area of the device substrate with receptors, depending on the application.

On the other hand, tailoring $b_{\text{max}}$ is more challenging yet has the most significance. In the journey to optimizing $b_{\text{max}}$, the first key concept to keep in mind is that $b_{\text{max}}$ must remain constant throughout the analyte detection experiments in order to ensure reliable signal evolution and signal saturation at reaction equilibrium. The most straightforward way to fix $b_{\text{max}}$ is by covalent immobilizing the receptors on the device substrates. This can be achieved with appropriate surface chemistry techniques based on the type of device substrate employed to develop the microfluidic sensor. Of the different materials used to fabricate microfluidic sensors, silicon-based materials such as glass and polydimethylsiloxane (PDMS) are the most commonly employed materials. Linker molecules called silanes have been shown to effectively modify the surfaces of glass and PDMS so as to generate functional groups that are amenable to further receptor coupling. For instance, aminosilanes such as (3-aminopropyl)triethoxysilane (APTES) and (3-aminopropyl)trimethoxysilane (APTMS) have been used to generate amine (NH$_2$) functional groups on the surfaces of glass and PDMS for subsequent coupling with the carboxyl (COOH) moieties on the receptor surfaces using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) or sulfo-NHS by forming covalent amide bonds.

Although organosilanes allow effective receptor immobilization, it must be noted that they are highly susceptible to hydrolysis when exposed to moisture. As we showed in a previous study, APTES-coated glass substrates are functional up to 3 months of storage at room temperature or 4 °C, after which hydrolysis significantly reduces the receptor immobilization efficiency. Similarly, thiols such as 11-mercaptoundecanoic acid (MUA) have been used to generate COOH functional groups on gold-based substrates. On the other hand, acid/base treatments and highly reactive ionized gas plasmas have been used to generate NH$_3$ or COOH functional groups on thermoplastic substrates such as poly(methyl methacrylate) (PMMA), polytetrafluoroethylene (PTFE), and polycarbonate (PC).

Using covalent immobilization strategies, typical desirable values ranging from $10^{-9}$ to $10^{-7}$ mol/m$^2$ can be obtained, whose values mimic the distribution of receptors on surfaces of cells.

While the quantity of covalently immobilized receptors on chemically modified substrates reflect the total concentration of receptors on the reaction site, $b_{\text{max}}$ is truly represented by the fraction of “active” receptors that are able to bind to analytes. Here, the “active” receptors are those receptors that are (1) structurally and functionally preserved after the immobilization process and (2) oriented in a direction favorable for analyte capture. The higher the quantity of active receptors, the larger the $b_{\text{max}}$ In order to ensure that the receptors remain biofunctional after being covalently coupled to the substrates, it is important to choose the appropriate immobilization technique. For instance, drop-casting has been the most widely used method to deliver receptors to chemically modified
substrates, owing to availability of a wide array of automated droplet dispensing systems. These droplet dispensing systems allow users to precisely control the volume of receptor solutions that can be sequentially dispensed on a wide range of substrates. The dispersed droplet solutions are allowed to evaporate over time, leaving behind precise arrays of nanoscale reaction sites. However, significant conformational changes are observed as these receptors are exposed to dehydrating conditions. To address these issues, additives such as ethylene glycol are commonly added to the receptor solutions to prevent droplet evaporation and to improve the homogeneity of receptor coating. On the other hand, recent developments in colloidal lithography have allowed for selective immobilization of functional receptors in well-defined patterns on silica-based substrates.

Microcontact printing is another widely used method to create well-defined patterns of receptor-sites on different substrates. Using this technique, a liquid solution containing receptor molecules is inked on micropatterned elastomeric PDMS stamps. After incubation, the stamps are washed, dried, and stamped onto a chemically modified substrate with higher surface energy than that of the stamp. This causes the transfer of the inked molecules from the stamp to the substrate, where the receptors are now covalently immobilized. However, since partial dehydration of receptors is a prerequisite to the microcontact printing technique, the probability of receptor denaturation and impaired analyte binding is high. To address this issue, we previously demonstrated a two-step patterning approach that involves microcontact printing of APTES on glass substrates to preserve the functionalities of immobilized receptors, followed by covalent coupling of receptors in solution using microfluidic networks. As these receptors are never exposed to dehydrating conditions, their functionalities are preserved, thereby ensuring high $b_{\text{max}}$. Covalent immobilization of receptors in solution has also been frequently employed to coat nano/microbeads that function as reaction sites. Owing to the increased surface to volume ratios of these bead-based reaction sites, the density of active receptors is much higher than those with planar surfaces. It is worth noting that bead-based bioassay systems that incorporate packed bed or porous media rely on different transport mechanisms than those explained in this perspective. However, the assumptions made in this perspective are still applicable in systems where the receptor-coated beads are immobilized in well-defined, closely packed patterns onto microchannel surfaces, with sufficient distance from the microchannel roofs. Assuming that the beads are tightly packed, such that there is minimal fluid flow through the interstitial spaces, the total surface area occupied by the immobilized beads that is exposed to the analyte solution can be considered as the overall reaction site size. By tuning the sizes of the immobilized microbeads, we can not only optimize the receptor surface density $b_{\text{max}}$ but also tune the microchannel height $H$ by increasing the proximity of the beads to the microchannel roofs. Therefore, in addition to allowing us to enhance the number of available receptor sites, bead-based surface bioassays could provide an alternate method to induce confinement in microfluidic bioassay systems.

In addition to preserving functionality of the immobilized receptors, several techniques are being employed to improve receptor orientation after immobilization. For instance, linker proteins such as protein A or protein G are commonly employed to bind to the Fc-component of antibodies to ensure that the analyte binding domain, i.e., the Fab-component of the antibodies is accessible to the analytes reaching the reaction sites. More recently, owing to the high binding strength between streptavidin and biotin, receptors are being genetically engineered to consist of biotin domains on their tail ends that are subsequently able to bind to streptavidin-coated substrates.

Collectively, these different techniques allow us to enhance $b_{\text{max}}$ by increasing the concentration of structurally preserved and oriented active receptors. At this stage, we are now experimentally equipped to enhance $b_{\text{max}}$ while controlling the size of the reaction site ($d$), thereby allowing us to improve the maximum equilibrium signal that can be obtained for low concentrations of analytes ($C_0 \ll K_D$) by ensuring $C_0 \gg C_{\text{crit}}$. The advantage of tailoring $b_{\text{max}}$ and $d$ according to the lowest $C_0$ employed in the system not only allows us to increase the LoD but also ensures that high $C_0$ can also be effectively quantified using the same conditions. Being able to detect a wide range of analyte concentrations is of utmost importance when trying to use these systems in a clinical setting. For example, any elevations in CRP concentrations above 80 nM indicate high risk of coronary heart disease in patients. Therefore, a microfluidic sensor should enable the detection of 1 nM $\leq C_0 \leq 100$ nM of CRP to be used to effectively diagnose these patients.

Thus, by collectively employing all of these rules elaborated in these sections, we are now able to design a microfluidic sensor that is able to rapidly detect a wide range of analyte concentrations, with high analytical sensitivity and specificity. While these sections paint a picture on how to selectively increase the positive signals that can be obtained in response to analyte binding, the next section is focused on how to identify and minimize the negative signals obtained in these systems.

### 6.3. Reducing Nonspecific Adsorption

While the LoD and analytical specificity can be significantly enhanced by enhancing receptor affinity and surface density, the overall efficiency of signal detection can be further improved by decreasing the noise in the system. Depending on the type of analyte sample fluid employed in the system, there are different factors that contribute to the noise. In most laboratory-scale experiments, the microfluidic sensors are first designed to detect analytes from buffer solutions. In most cases PBS (phosphate buffered saline) solutions are doped with varying concentrations of analytes, filtered using standard microporous filter membranes to remove any aggregates and debris, and delivered into the microchannels. In these systems, the noise that arises during operation is most typically a result of nonspecific adsorption of analytes and/or detection molecules on the walls of the microchannels. This is most commonly observed in devices fabricated with hydrophobic materials such as PDMS or PMMA that have high affinity toward hydrophobic residues in the analyte proteins. This nonspecific protein adsorption can be significantly reduced by increasing the hydrophilicity of the microchannel walls by means of chemical modification or by using blocking agents that repel proteins and other molecules. The most common blocking agents used to reduce nonspecific protein absorption range from protein-based agents such as bovine serum albumin (BSA) and casein from dry milk to chemical agents such as Tween-20 and omniphobic fluorinated silanes.
On the contrary, in systems operating with real biological fluids such as whole blood, blood plasma, or serum, there are a plethora of interferences that add to the noise in the system. These factors range from heterophilic antibodies, molecules with structural similarities with the analytes, fibrinogen, and cell debris.\textsuperscript{155} Collectively, these factors termed as matrix interferences compete with the analytes to bind to the receptors thereby increasing both the noise and analyte detection time. Sample dilution with buffers and filtration of samples prior to assays are the most commonly used methods to counteract the influence of these interferences. In addition to these strategies, the nonspecific binding of these molecules to the receptors can be reduced by increasing the specificity and affinity of the receptors, as described in the previous sections. As recently suggested by Barbosa et al., apart from the affinity and specificity of the receptors, the enhancement of binding capacity, i.e., \(b_{\text{max}}\), was also shown to reduce matrix interferences in blood-based assay systems.\textsuperscript{156} By coupling these strategies with the use of appropriate wash buffers, the remaining nonspecifically adsorbed proteins can be flushed out from the channels prior to signal detection, thereby minimizing the noise arising from sample contaminants.\textsuperscript{157}

The above sections collectively allow us to optimize the device geometry and flow rates to enhance analyte detection speed. In parallel, we elucidated how the molecular parameters can be altered to further improve the analytical specificity and sensitivity of the microfluidic sensors by increasing positive analyte signals and decreasing the noise arising from nonspecific signals. However, no matter how much we push the experimental limits of these systems, the device efficiency is still limited by the ability of the detectors to detect and differentiate the equilibrium signals from the detector noise.

7. DETECTION OF RECEPTOR–ANALYTE REACTIONS

Most microfluidic sensors rely on quantification of analyte concentrations when the receptor–analyte reaction reaches equilibrium. In a typical single-use device, a sample containing a single concentration of analytes is delivered through a microchannel and made to react with the receptors coated on the reaction sites. Once equilibrium is reached, the channel is first washed by a buffer solution to remove nonspecifically bound molecules, after which the equilibrium signal is measured. This signal is compared with a standard concentration calibration curve to correlate the obtained equilibrium signal with the analyte concentration. This strategy, also commonly employed in most ELISA-based systems, does not allow real-time analysis and continuous measurements. In addition, the extended reaction equilibrium times (\(t_{\text{eq}} \approx \text{hours}\)) for low analyte concentrations (\(C_{\text{A}} \ll K_{\text{D,eq}}\)) in systems employing low affinity receptors impose practical speed limitations while employing equilibrium measurements for analyte detection.

To address these issues, microfluidic systems can be tailored to perform continuous monitoring of real-time analyte–receptor reactions in parallel. This can be achieved by stopping the reactions as soon as a detectable signal is obtained for the lowest \(C_{\text{A}}\) delivered to the system, without having to wait for the reaction to reach equilibrium. Under these conditions, the analytical sensitivity of the device is primarily dependent on the detector’s ability to respond to low intensity signals. Depending on the type of signal detection mechanisms employed to detect analytes, the analytical sensitivity of the system can be improved using different strategies.

7.1. Label-Based Detection

In most laboratory-scale microfluidic sensors, the analyte–receptor reaction is analyzed using label-based detection methods, such as colorimetric detection\textsuperscript{158–160} and fluorescence detection.\textsuperscript{161,162} These conventional methods rely on the use of secondary detection molecules that are labeled with (1) enzymes such as horserasish peroxidase (HRP), capable of catalyzing chromogenic or chemiluminescent substrates to generate visible signals, or (2) fluorophores such as fluorescein isothiocyanate (FITC), cyanine derivatives (e.g., Cy2, Cy5, etc.) and other fluorescent dyes. The signals obtained from these detection molecules can be easily captured by common imaging instruments such as absorbance readers and microscopes.

Colorimetric detection is most commonly employed in paper-based microfluidic systems,\textsuperscript{163–165} owing to the ease of signal detection using commonly available absorbance readers for quantitative measurements and by naked eye for qualitative analyte detection. Absorbance-based measurements are less commonly used in flow-based microfluidic systems owing to the short optical path lengths of sub-millimeter to microscale microchannels.\textsuperscript{166,167} On the other hand, fluorescence-based measurement is the more preferred detection strategy, in both the laboratory and industrial settings. In a typical fluorescence-based assay, the fluorescently labeled detection molecules are either mixed with the analyte sample fluid or delivered through the channels after the receptor–analyte reaction reaches equilibrium. Subsequently, the changes in fluorescence intensity are monitored via fluorescence microscopy. Here, the fluorescence intensity is proportional to the concentration of analytes captured in the microfluidic sensor, based on the Beer–Lambert law: \textsuperscript{168}

\[
F = k[I_0\phi(1 - 10^{-bC_{\text{A}}})]
\]  \hspace{1cm} (22)

where \(F\) is the fluorescence intensity, \(k\) is the proportionality constant related to the instrument, \(I_0\) is the intensity of incident light, \(\phi\) is the fluorescence quantum yield, \(b\) is the molar absorptivity of the molecule (L/mol-cm), \(b\) is the path length, and \(C_{\text{A}}\) is the concentration of analytes (mol/L). In most cases, even at high analyte concentrations (\(C_{\text{A}} \gg K_{\text{D,eq}}\)), dilute solutions of fluorescently labeled detection molecules are employed. As a consequence, it is assumed that only <2% of the excitation energy is absorbed, resulting in the simplified form:

\[
F = k[I_0\phi bC_{\text{A}}]
\]  \hspace{1cm} (23)

This relationship has been serving as the foundation of quantitative analyses in fluorescence-based bioassays. In these systems, the analytical sensitivity or LoD is directly influenced by the sensitivity of the camera employed to detect the fluorescence signals. Of the available cameras, charge-coupled device cameras are the most popular as a result of their affordable costs and compatibility with a wide range of microscopes. Using these conventional instruments, moderate LoDs in the sub-nanomolar concentration range can be achieved.\textsuperscript{114,115} By efficiently tuning the excitation light intensity and exposure time, fluorescence-based microfluidic sensors can be developed to detect different types of analytes with large dynamic concentration ranges, i.e., \(C_{\text{A}} \in [\text{nM}, \text{M}]\).

Recently, different signal enhancement strategies are being employed to further improve the LoD of these fluorescence-based systems. Strategies that involve the use of highly stable quantum dots,\textsuperscript{169–172} plasmonic nanomaterials for metal-enhanced fluorescence,\textsuperscript{173–174} fluorescence resonance energy

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transfer techniques$^{179,180}$ and total internal reflection fluorescence microscopy$^{181,182}$ have enabled the development of highly sensitive microfluidic sensors (LoD ≤ aM) that have the capability to detect single molecule binding events ($C_0 \approx C_{eq}$). However, by using such sensitive detection systems, we lose the ability to detect high analyte concentrations ($C_0 \gg K_D$), thereby drastically shortening the detectable dynamic concentration ranges, i.e., $K_0 \in [pM, nM]$. Therefore, the choice of detection mechanism is purely dependent on the specific applications of these systems.

7.2. Label-Free Detection

Recently, label-free strategies are increasingly being employed in analyte detection systems to eliminate the use of secondary detection molecules and to achieve high analytical sensitivity. These strategies rely on the direct detection of receptor–analyte reactions occurring on recognition elements such as metallic thin films, nanostructures, or field-effect transistors by monitoring localized changes in absorbance wavelengths or electronic transduction proportional to the changes in surface mass adsorption.$^{183}$ The most commonly used label-free detection strategies rely on optical sensing of mass dependent surface electron resonance energy changes using surface-enhanced Raman spectroscopy,$^{184−186}$ surface plasmon resonance,$^{187−189}$ and localized surface plasmon resonance sensors,$^{190−196}$ or direct detection of mass variation using quartz crystal microbalance.$^{197−200}$ In addition to optical detection techniques, high sensitivity field-effect transistors have enabled the development of ultrasensitive electrochemical analyte detection systems with LoDs in the low attomolar to zeptomolar ranges.$^{202,203}$

Despite the several benefits offered by these label-free detection techniques, they suffer from several drawbacks. Owing to the highly specific structural requirements needed for the efficient functioning of these sensor substrates, they require complex fabrication techniques and instrumentation that are not commonly available to all users. In addition, it proves to be very challenging to create an integrated microfluidic device with these optical sensors, due to the intricate instrumentation required for signal detection and analysis. As these systems heavily rely on optical absorbance measurements, any changes in local pH and ionic strength of the sample fluid, significantly affects the stability of optical signals obtained from the sensors. This in turn significantly increases the noise in the system, thereby affecting the analytical specificity of the system. Nevertheless, these issues can be addressed by efficiently controlling the ambient fluid environments and by appropriate engineering of the sensing instrumentation to reduce analytical noise of the systems.

As these sensors rely on localized surface effects, their ability to detect molecular adsorption heavily relies on the concentration of molecules on their surfaces.$^{201}$ Thus, they are most sensitive to analytes at low concentration ranges ($C_0 \ll K_D$), but due to saturation of sensor response at high analyte concentrations, these sensors are unable to detect large dynamic concentration ranges of analytes. As a result, these systems allow detection of short dynamic concentration ranges, i.e., $C_0 \in [pM, nM]$. Nonetheless, the dynamic ranges of optical microfluidic sensors can be significantly extended by appropriately diluting concentrated analyte solutions with compatible buffer solutions.$^7$

Recently, Barulin et al.$^{202}$ elucidated a novel label-free ultraviolet (UV) fluorescence spectroscopy technique for the potential detection of single protein molecules. This technique exploits the combination of oxygen scavenging enzymes, antioxidants and triplet state quenchers to enhance the photostability of natural tryptophan autofluorescence in proteins when exposed to UV light. With the help of fluorescence correlation spectroscopy, the concentrations and diffusion coefficients of the protein molecules can be extracted without the need for external fluorescent markers. While this approach was shown to be immensely sensitive to extremely low protein concentrations, the simplicity of the technique and instrumentation could also allow for the detection of wide dynamic concentration ranges. These collective features make this approach a promising alternative to commonly used label-free detection techniques.

Based on the available technologies, we observe that both label-free and label-based detection methods allow us to develop microfluidic sensors with enhanced analytical sensitivity and large dynamic ranges. Hence, the choice of detection method is purely based on user discretion, where the decision should be based on geometrical constraints such as optical path length, accessibility to instrumentation for both fabrication, and signal acquisition and processing.

Finally, depending on the method of detection, appropriate chemometric methods must be chosen to ensure reliable signal processing and data interpretation. Simple statistical methods such as linear and nonlinear regression analysis have been the primary means to interpret laboratory-scale analyte sensing data. However, these conventional analysis and interpretation methods become tedious and less robust when it comes to handling complex data in high-throughput and multiplexing applications. The advent of machine learning and deep learning methods has paved way for the reliable discrimination of complex overlapping signals for different analytes, while also allowing quantitative prediction of low analyte concentrations.$^{203}$ By doing so, the analytical sensitivity and specificity of complex microfluidic bioassay systems can be significantly enhanced in point-of-care and high-throughput screening applications.

8. THE MICROFLUIDIC SENSOR BLUEPRINT

At the beginning of this article, we defined an ideal microfluidic sensor as a system that is capable of detecting analytes fast, with high sensitivity and specificity, and with minimal sample volume requirements. In order to quantify the feasibility of reaching this goal, we employed key dimensionless parameters (Table 2) to estimate the physical limits, and key quantifiable parameters (Table 1) to estimate the experimental limits that define the (1) analyte transport: bulk and local Peclet numbers ($Pe_B$ and $Pe_L$), (2) analyte detection speed: diffusive and kinetic Damköhler numbers ($Da_B$ and $Da_L$), and reaction equilibrium time ($\tau_E$); and finally, (3) analytical sensitivity of microfluidic systems: limit of detection (LoD). By entangling these physical and experimental limits, we see that creating an “ideal” microfluidic sensor is a mammoth task. By taking a top-down approach to design this ideal system for all receptor–analyte pairs, we are forced to sacrifice at least one feature to reap the benefits of the others. Instead, a more realistic way to approach microfluidic sensor development is to take a bottom-up path, where the system is designed according to the desired features needed for the specific application.

In Figure 4, we illustrate this bottom-up approach that is initially hinged on the sample volume requirements. In applications where minimized reagent consumption is the
primary desired feature, it is beneficial to design microfluidic devices operating at low flow rates, such that $P_{E_H} \ll 1$. In these diffusion-driven systems, the equilibrium signal speed can be pushed to reach $\tau_R$ by simply reducing the channel height $H$ such that $D_{A_H} \ll 1$ for analyte concentrations $C_0 = K_D$. This strategy is immensely powerful in single-cell analysis and to detect low-abundance analytes such as cancer biomarkers and exosomes from microliter volumes of blood plasma.

On the other hand, high flow rate microfluidic systems ($P_{E_H} \gg 1$, $P_{E_S} \gg 1$) can be designed to detect a wide range of analytes such as CRP, IgGs, etc. (Table 3) from milliliter sample volumes, e.g., bodily fluids such as blood plasma and urine. Under these conditions, the equilibrium signal speed can be pushed to $\tau_R$ by optimizing the flow rates ($Q$) to minimize the thickness of the depletion layer and achieve $D_{A_S} \ll 1$.

Under either of these operating conditions employed in the microfluidic devices, the equilibrium signal time can be pushed to reach $\tau_R$ by ensuring that the analytes are transported and replenished at rates much faster than the rates at which the analytes are captured by the receptors, i.e., $D_{A_H} \ll 1$ or $D_{A_S} \ll 1$ for low and high flow rate systems, respectively. As $\tau_R$ is purely dependent on the analyte concentration ($C_0$), kinetic analyte–receptor "on-rate" ($k_{o_{on}}$) and "off-rate" ($k_{e_{off}}$), i.e., $\tau_R = (k_{o_{on}} C_0 + k_{e_{off}})^{-1}$. At large analyte concentrations, $C_0 \gg K_D$, reaction equilibrium is almost instantaneous (on the order of milliseconds–seconds) as $\tau_R \approx (k_{o_{on}} C_0)^{-1}$ in most cases. However, at $C_0 \ll K_D$, equilibrium signal time is quite varied (on the order of minutes–hours) depending on the "off-rate" of the receptor–analyte reaction, as $\tau_R \approx (k_{e_{off}} C_0)^{-1}$. In these situations, it is recommended to use receptors with high $k_{o_{on}}$ to ensure rapid analyte detection. Consequently, the microfluidic systems should be designed according to the lowest $C_0$ employed in the systems in order to be able to achieve overall rapid analyte detection.

Irrespective of the operating flow conditions employed in the microfluidic devices, the analytical sensitivity of these devices can be enhanced by tailoring the reaction site size ($d$) and surface concentration of receptors ($b_{max}$), when operating at the detection speed limit. As a general rule, $b_{max}$ can be tailored to ensure that $C_0 \gg C_{crit}$ for the lowest $C_0$ employed in these systems. This rule ensures that the signals obtained from reaction equilibrium are not in the single molecule detection regime, i.e., when $C_0 \approx C_{crit} = K_D/(d b_{max})$. Here, $b_{max}$ is collectively influenced by the fraction of active receptors that are oriented in a direction favorable for analyte capture. This fraction of active receptors can be significantly increased by (1) covalently immobilizing them on device substrates, (2) choosing the appropriate immobilization strategy that allows preservation of their structural and functional integrity, and (3) employing linker molecules that aid in the controlled orientation of immobilized receptors.

Finally, by employing high affinity receptors ($K_D \ll 10^{-12}$ M), effective blocking agents, specific biofluid processing techniques, and appropriately sensitive signal detectors, the analytical specificity and sensitivity of the device can be enhanced to achieve significantly low LoDs and large dynamic concentration ranges.

9. CONCLUSION

In summary, we have attempted to connect the well-known physical limits imposed on surface-based microfluidic systems with the experimentally achievable limits in these systems, with the aim of presenting a comprehensive microfluidic sensor blueprint. This blueprint will allow users to carefully tailor their microfluidic sensors to achieve fast, sensitive and specific analyte detection, based on the final application. Specifically, with reference to model biomarker systems, we provided explicit examples on how and when to optimize various operational and molecular variables in the microfluidic systems. In addition, we reviewed recent progress made in different areas to support our discussions and provide concrete evidence to show the impact of these variables on the device efficiency. Although these rules were defined using model biological receptor–analyte systems, these principles can be easily extended to a wide range of analyte–receptor systems, given that the users are equipped with the knowledge of the kinetic constants. We are hopeful that the collective information detailed in this article will serve as a building block for scientists to easily design and create high-efficiency microfluidic sensors with potential applications in both point-of-care and large-scale clinical settings in the near future.

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Notes

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