Gung Biotechnology. The authors have filed patent applications related to the preparation and use of medicinal mushrooms.

Acknowledgments

The authors' work is supported by Primordia Institute of New Sciences and Medicine, grant MOST103-2320-B-182-027-MY3 from the Ministry of Science and Technology of Taiwan, and grants CMRPD190303, BMRPA04, and QZRPD120 from Chang Gung Memorial Hospital.

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http://dx.doi.org/10.1016/j.tibtech.2017.06.011

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Forum

Nanoenvironmental Effects Dramatically Influence the Sensitivity of Immunoassays

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It is possible to improve the sensitivity of immunoassays by several orders of magnitude by exploiting nanoenvironmental effects. This approach can detect trace amounts of compounds and will better illuminate the presence of signal substances in biological systems. Here we describe a method for ultrasensitive immunoassays using 'normal' antibodies (Abs).

Ab–Antigen (Ag) Binding

The binding efficiency between Abs and Ags is influenced by the nanoenvironment around them. Immobilizing Abs on a sensor surface achieves a high concentration of macromolecules in the close vicinity of the transducer surface. Abs on solid surfaces show different binding constants compared with what is measured during conventional assays (e.g., ELISA in a microtiter plate) [1–4]. If the reading of a binding assay is localized to the very close vicinity of the immobilized Abs, it should be possible to exploit the apparent differences in binding constants.

The binding of Ags to Abs is normally described by Equation 1.

$$[Ag] \times [Ab] = [AgAb] \quad [1]$$

If [Ab] is increased dramatically, the equilibrium will shift towards Ab–Ag complex formation. Therefore, an Ab preparation that under normal conditions might be regarded as a relatively weak binder might be very efficient.

$$[Ag]_{Tot} = [AgAb] + [Ag]_{free} \quad [2]$$

The kinetics of the binding is described by Equation 3.

$$K = [AgAb]/[Ag][Ab] \quad [3]$$

If [Ag] is very low and all or most of the Ag binds to the Ab, [AgAb] = [Ag], giving $K = 1/[Ab]$ (see Equation 2). This simplification is based on the assumption that the binding between Ab and Ag is very strong. Under conditions of excess Ab, improved binding strength becomes apparent since dissociating Ag can be captured by the surplus Abs in the vicinity of this small environment and rebind, thus forming firm binding.

Some literature reports cite very high sensitivities for certain immunoassays [5–8]. Exploiting Ab–Ag interactions at a surface where a high concentration of Abs is located is less efficient by conventional spectrophotometric assays, which involve thick layers of liquid being assayed. However, the use of assay procedures that measure only the binding

close to the surface would give information that is more appropriate. Examples of such methods are immunoassays based on biosensor technology, where Abs are immobilized on an electrode surface and direct interaction with the Ag is measured. These include, for example, surface plasmon resonance and capacitive measurements. Capacitive measurements are orders of magnitude more sensitive and open a world of opportunities to exploit Ab–Ag interactions as the

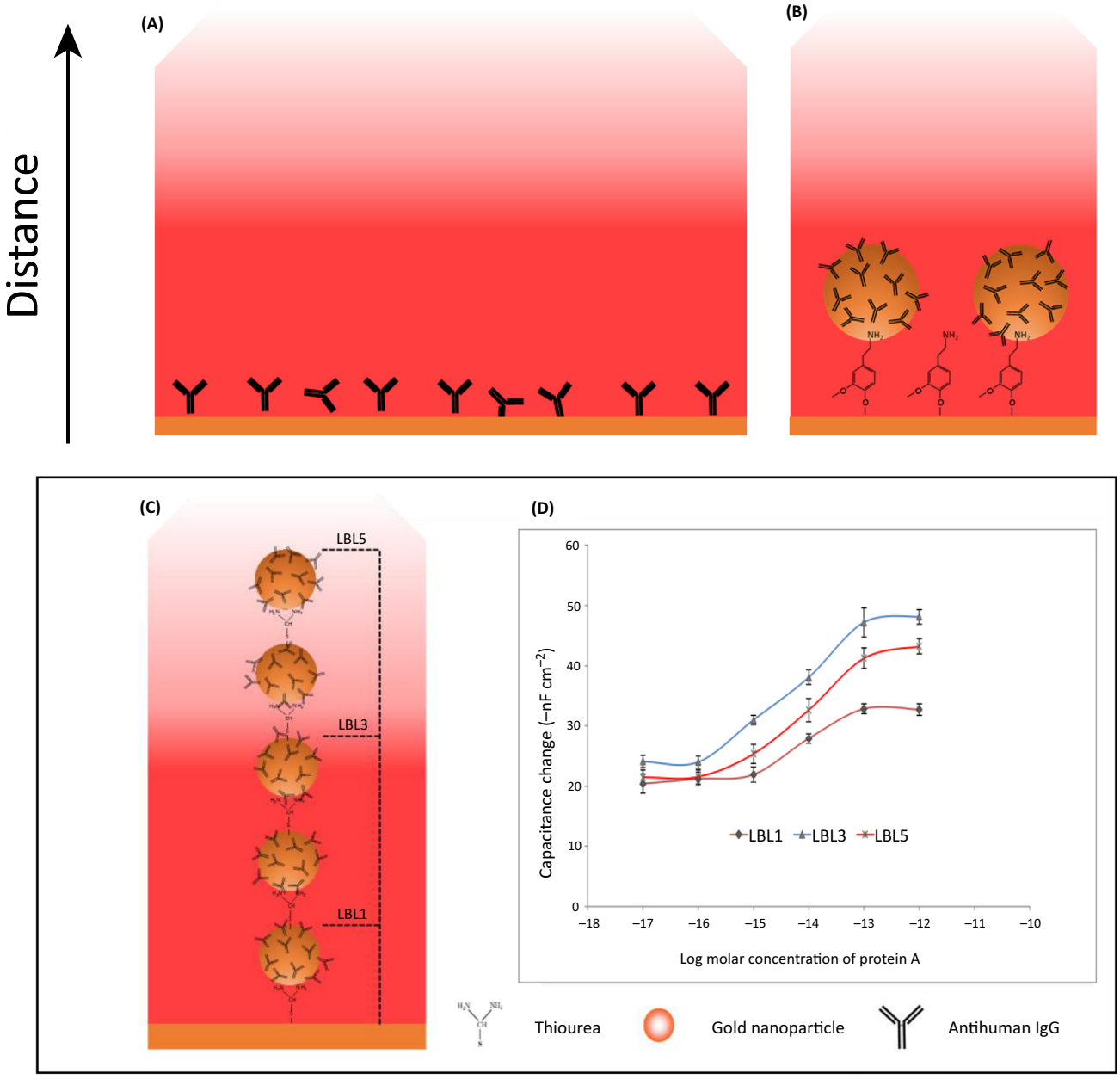

Figure 1. Schematic Presentation of the Capacitive Assay and Its Dependence on the Design of the Surface of the Sensor Chip. (A) Schematic illustration of the special operational range of the sensor. The gradient of red color illustrates the space where capacitance is registered. (B) Surface expansion via the introduction of gold nanoparticles with immobilized antibodies on the nanoparticles. (C) The stepwise construction, layer by layer, of gold nanoparticles with antibodies and the effect on the registered capacitance signals. (D) Analytical outcome when using sensors with different numbers of gold nanoparticles. As can be seen, three layers is beneficial while five is too many, as the operating range ends outside the optimal zone of the sensor. Image reproduced with permission from K. Teeparuksapun, PhD thesis, Lund University, 2013.

measurements are based on displacement of counter ions, which requires only a minimal nanospace.

Nanoenvironmental Conditions

Nanoenvironmental effects may look unrealistic at first sight, but the extreme concentrations of crucial reagents open a new field for binding assays. The concept of concentration is usually not applied to extremely small volumes, but to get a better understanding of the immunochemical binding reactions the following calculation might be useful.

For a general surface-based binding application, the concentration of Abs on a sensor surface with a monolayer of Abs can be calculated theoretically. The hydrodynamic diameter of an Ab is on the order of 40 Å. Assuming Abs to be cubes with each side of 40 Å, there would be 0.6×10^{14} molecules per cm^2. A layer with a thickness of 40 Å has a volume per cm^2 of 4×10^{-7} cm^3. With that number of molecules, there will be a local concentration of approximately 25 mM (Figure 1).

Ultrasensitive Assays

An example where the nanoenvironmental concept plays a role is a capacitive immunosensor for cholera toxin detection where the use of gold nanoparticles considerably improved sensitivity by increasing the amount of Ab immobilized on the transducer surface (Figure 1) and thus contributed to the ultrasensitivity of the capacitive sensor. In this work the sensor could detect the target cholera toxin at subattomolar levels [9].

Other examples are the comparative study of capacitive immunosensors versus ELISA for the detection of microcystins (L. Lebogang, PhD thesis, Lund University, 2014) and the HIV-1 p24 Ag [10]. The sensitivity of the capacitive immunosensor was 1000 times better than that of ELISA. A conventional ELISA may have the same density of Abs immobilized on the bottom of a microtiter plate. However, the readout of such an analysis is spectrophotometric, and the usual light path length is 10 mm, where the Ab layer is sitting on the very bottom. It is therefore impossible to differentiate what is in the bulk and what is sitting at the bottom of the well, and thereby much of the assay's sensitivity is lost.

Capacitive Biosensors

By contrast, capacitive assays are based on registering the capacitance at the electrode–solution interface. The binding of Ag to Ab on the sensor surface displaces ions in the diffused layer to the bulk solution [11]. The more Ag bound, the further the layer of counter ions is displaced with a concomitant decrease in the capacitive signal. At a certain distance from the electrode surface, further decreases in signal cannot be detected; that is, the range suitable for measurements has been surpassed. This behavior was observed when capacitance measurement was performed with gold nanoparticles added layer by layer, as illustrated in Figure 1 (K. Teeparuksapun, PhD thesis, Lund University, 2013).

The arrangement of ions on the electrode surface is based on long-range electrostatic interaction, so if the measuring layer is too thick, the electrostatic force will be weak. Thus, the ionic arrangement in the electrolyte solution might be very weak: most of the ions may be present in the diffused layer and the electrode–solution interface would not resemble a capacitor.

Considering the sensor as a capacitor comprising two plates separated by a dielectric layer, the capacitance can be calculated using Equation 4.

$$C = \varepsilon\varepsilon_0 A/d \quad [4]$$

where ε is the dielectric constant of the medium between the plates, ε_0 is the permittivity of free space (8.85419 pF/m), A is the area of the plates, and d is the distance between them. Hence, a change in the distance (d) from the interaction of the analyte to the biological sensing element will result in decreased capacitance. If the layer over the electrode is too thick, the change in capacitance will be very small. Therefore, preparing the immobilization for a capacitive biosensor requires a thin and sufficiently insulated layer.

That the distance from the surface for the reading has a strong influence is clearly seen in the capacitance change on binding of bacteria (1–2 μm), which are detectable, but when the size of the organism increases towards that of yeast cells (5 μm) the resolution is lost.

The influence of the nanoenvironmental conditions on the binding properties of the affinity binder leads to the conclusion that weaker-affinity binders can also be used due to their apparent stronger binding under the conditions studied. This phenomenon was furthermore observed when studying hydrophobic interactions in chromatography [12].

By concentrating an affinity binder in a small volume where the measurement occurs, it is possible to substantially improve the sensitivity. In addition, it is possible to utilize affinity binders that have been regarded as of no interest due to weak binding in free solution. It would therefore be advantageous to use preparations with small volumes of immobilized Abs that are concentrated in a small space where the assay can be conducted.

Concluding Remarks

A high concentration of affinity binder on the sensor surface and detection with a method that operates only in close proximity to that layer results in binding assays with sensitivities far beyond what has been reported before. The high sensitivity makes it possible to perform analyses using very small sample volumes. It is

furthermore possible to detect, for example, infections at an earlier stage than that achieved with conventional assays.

Acknowledgment

Support from the Swedish Research Council is gratefully acknowledged.

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http://dx.doi.org/10.1016/j.tibtech.2017.06.001

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