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Flow Injection Immunoanalysis Based on a Magnetoimmunosensor System

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A new immunosensor integrated to a flow system has been developed. It is based on magnetic immunoparticles immobilized on a solid-state transducer using a magnetic field. The described technique renews the immunoparticles reproducibly for each analysis allowing a good measurement precision. The developed experimental approach permits the implementation of an automated immunoassay that is quick (analytical cycle <30 min) and sensitive in the micromolar concentration range. The system was applied to the determination of rabbit immunoglobulin G as an analyte model.

There is a clear difficulty when traditional analytical methods are used for measuring organic molecules in trace quantities. This has motivated the development of immunoassay techniques using antibodies as the analytical reagents. Antibodies recognize the analyte by following specific interactions.[1] Although there is a high number of immunoassay techniques, only a few are widely used. Among these are indirect techniques where the analyte is measured through a label species conjugated with one of the immunoreagents. If one of the components of the immunologic reaction is immobilized on a solid phase (heterogeneous assay), the experimental procedure is simplified.[2−4]

There is a wide range of labels (radioactive isotopes, chromogenic or luminescent groups, etc.). However, enzymes offer an acceptable sensitivity and achievable working conditions.[4]

Immunosensors are a specific case of heterogeneous immunologic techniques. Here, the immunologic material is immobilized on a transducer. In this way the experimental procedure is simplified and sensitivity is raised. The main problem affecting immunosensors is the reproducible regeneration of the sensing surface.[5] The need for renewal of the sensing surface arises from the high affinity constants derived from the strong antigen−antibody reaction. This renewal is a difficult task since the drastic procedures required alter the immunoreagent bound to the surface of the transducer. This drawback make immunosensors difficult to integrate in automatic systems.

With flow injection analysis (FIA), analytical procedures can be automated easily and the analyses performed quickly and continuously[8,14,15]. Flow injection immunoassay (FIIA) techniques have been reported extensively.[6−13] They are based mainly on immunoreactors filled by immunologically modified solid particles, gels, CPGs, etc.

On the other hand, magnetic particles with immobilized immunoreagents are used with increasing frequency in immunoassay manual methods.[16,17] This approach simplifies the renewal of the solid phase. Several reports have appeared in recent years where biologically modified magnetic particles are used in conjunction with analytical flow systems.[6,18−20] These particles are handled easily and they make the regeneration procedures of the biological material unnecessary. It is possible

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to build a new and reproducible bioreactor simply by changing the magnetic particles with the immobilized biological reagent. This change of the modified solid phase is done by handling the flow and the external magnetic field.

Kindervater et al.[18] and later Günter et al.[19] used magnetic particles with acetylcholinesterase to detect pesticides using FIA systems. The only contributions in the field of immunoassay using magnetic particles in flow systems have been proposed by Ruzicka et al.[20] and Stöcklein and Schmid.[6] They developed a sequential injection immunoassay (SIIA) and a FIIA system, respectively. Both systems are not immunosensors as such but bioreactors working independently from the detector. The magnetic particles are fixed to the inner walls of the main flow channel using a magnet placed upstream from the sensor, forming an open tube reactor.

A novel immunosensor using magnetic particles and integrated to a flow injection system is described here for the first time. The fact that the immunologic phase is on the transducer, forming an immunosensor, implies a simpler setup. It also produces a higher sensitivity since the product of the enzyme−label reaction is detected directly without the dispersion associated to the flow system. A flow microcell (25 μL) has been designed *ex profeso* for this application. This cell integrates the immunologic reaction, the detection, and the magnetic field in a single point of the flow injection system. The detector is a miniaturized ISFET sensor.

The magnetoimmunosensor system reported here was applied to the measurement of rabbit immunoglobulin (RIgG) using a competitive technique with goat anti-rabbit immunoglobulin (GaRIgG) labeled with urease.

EXPERIMENTAL SECTION

Materials. The immunologic system was formed by rabbit immunoglobulin G (RIgG) (I-5006) and goat anti-rabbit immunoglobulin G (GaRIgG) conjugated with urease (U-1379). Both were obtained from Sigma (St. Louis, MO).

Dynabeads M-280 magnetic particles (2.8-μm diameter) activated with tosyl groups were obtained from Dynal (Dynal, Unipath, Barcelona, Spain).

Bovine serum albumin (BSA) came from BDH. Ammonium chloride was obtained from Panreac (Barcelona, Spain). Tris(hydroxymethyl)aminomethane (Tris) and EDTA was supplied by Merck and urea by Fluka.

The rest of the reagents used were of proanalysi quality or similar. All standard urea solutions and all buffer solutions were prepared using bidistilled water.

Apparatus. The peristaltic pumps (Gilson, Minipuls 3) were used to transport the carrier solution, the sample, the immunoconjugate, the substrate, the magnetic particles, and air through the Teflon tubes (0.5-mm i.d.).

A 14-way valve (Rheodyne, Cotati, CA) permitted the consecutive injection of the analyte (RIgG) and the urease−GaRIgG to the flow system. The four-way and the six-way valves were obtained from Omnifit.

The detector was a pH-sensitive ISFET supplied by the Centre Nacional de Microelectrònica (CNM, Bellaterra, Spain). The reference element was a double-junction Ag/AgCl reference electrode from Orion (Orion 900200). The sensing system was integrated to the flow system using a 2.5 × 4.0 × 3.0 cm^3 methacrylate module (see Figure 1) formed by two blocks. The

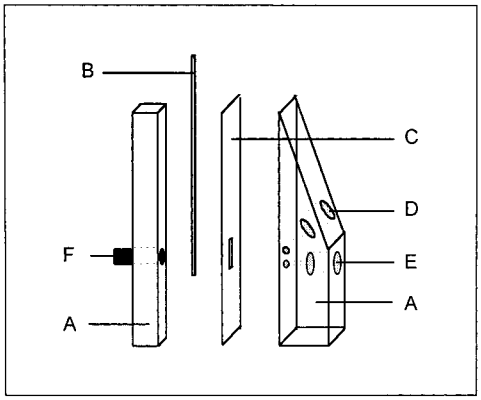

Figure 1. Schematic representation of the magnetoimmunosensor. The dimensions of the module are 2.5 × 4 × 3 cm^3. Two channels enable flow entry and exit. The entry channel is perpendicular to the sensor surface. The exit channel forms a 45° angle with the sensor surface: (A) methacrylate block; (B) ISFET transducer mounted on a IC board; (C) perforated silicone sheet; (D) flow exit; (E) flow entry; (F) cylindrical neodymium magnet placed in a cavity of the methacrylate block.

ISFET was fixed between these two methacrylate blocks. One of these blocks had the entry and exit ports and the other contained a cylindrical magnet. A silicone sheet was placed between the two blocks to achieve an airtight coupling. The silicone sheet formed a 25-μL hollow coinciding with the dimensions of the sensitive area of the ISFET (130 × 610 μm), thus forming a flow cell. The magnet is placed in a cavity situated underneath the sensing area.

Potential measurements were performed by an ISFETmeter (Centre Nacional de Microelectrònica, Bellaterra, Spain). This apparatus measured the feedback voltage that is applied to the reference electrode to compensate for any potential variations at the pH-sensitive gate. These potential variations are caused by pH changes produced by the products of the enzyme reaction. A constant drain voltage (V_{DS}) of 500 mV was used in conjunction with a fixed drain current (I_{DS}) of 100 μA.[21]

Immobilization of the Magnetic Particles with RIgG. A suspension of magnetic particles (20 mg/mL) in 0.1 M phosphate buffer at pH 7.5 was placed in an Eppendorf-type tube. A solution of RIgG in the same phosphate buffer was added until a concentration of 80 mg/mL was achieved. This mixture was incubated at 37 °C using gentle stirring for 24 h. After this period, the supernatant was removed and the particles were washed three times with PBS/BSA (0.15 M NaCl in 0.01 M NaH_2PO_4 at pH 7.5, and 1% (w/v) BSA) and one time using Tris/BSA (0.2 M Tris at pH 8.5 with 0.1% (w/v) BSA). This washing eliminated all nonspecific adsorptions from the surface of the magnetic particles. A 10 mg/mL magnetic RIgG−particle stock solution was used. This solution may be kept as a suspension in PBS/BSA solution at 4 °C for several months.

Description of the FIA System. The flow system is shown in Figure 2, and the main experimental procedure steps are schematized in Figure 3. Its analytical principle is based on a competitive immunoassay.

First, 50 μL of magnetic RIgG−particles (1.5 mg/mL) was injected in a 0.1 M NH_4Cl carrier solution at a 0.2 mL/min flow

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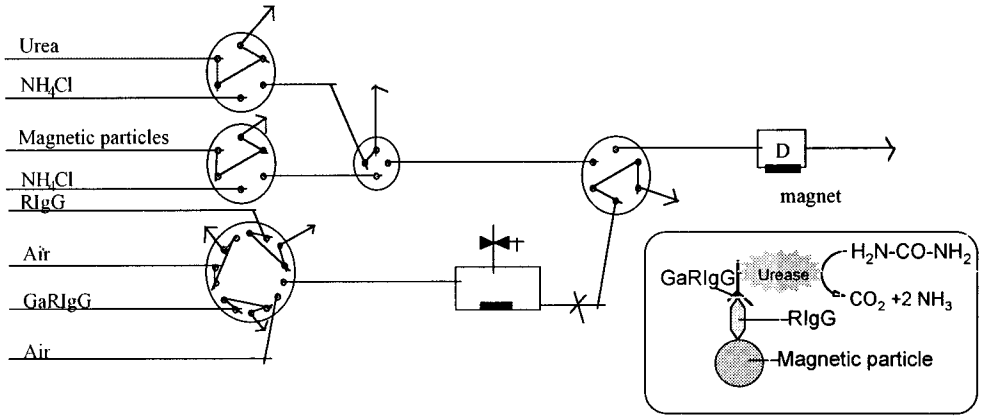

Figure 2. Flow system for enzyme immunoanalysis using magnetoimmunosensor detection. The immunologic and enzyme reactions taking place on the magnetic particles retained on the ISFET gate are shown in the inset.

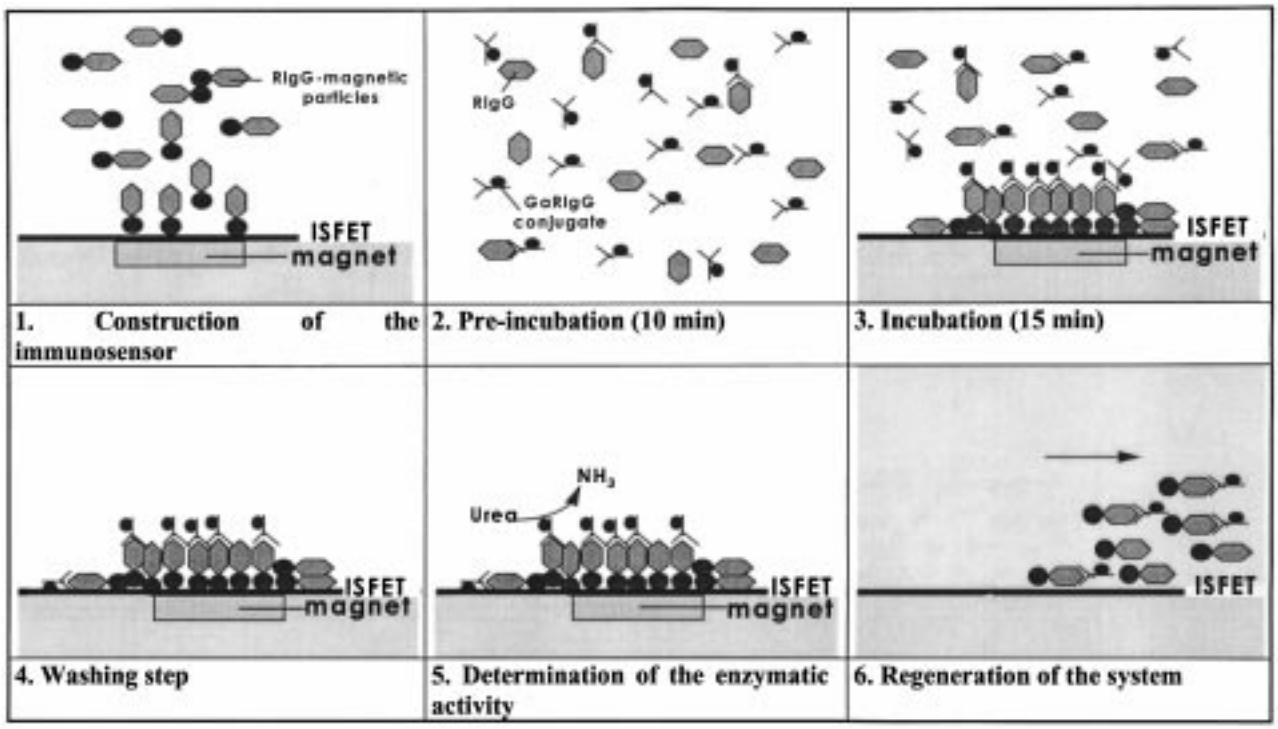

Figure 3. Schematic representation of the steps followed in the FIIA assay: (1) injection of the magnetic particles and insertion of the magnet to immobilize the beads; (2) preincubation stage of the GaRIgG conjugate and the RIgG; (3) incubation stage of the preincubation solution and the RIgG−particles; (4) washing of the surface of the immunosensor; (5) determination of the enzymatic activity by the injection of the enzymatic substrate; (6) release and discharge the spent beads to be regenerated off line.

rate. The particles were transported to the ISFET gate by the flow. They were retained in the gate by the action of a magnetic field produced by a magnet. The resulting assembly formed the immunosensor.

The competitive assay was performed in two stages.[22] First, there was a preincubation stage for 10 min where 200 μL of 1.2% (v/v) GaRIgG labeled with urease were injected in succession with 200 μL of analyte sample through a 14-way valve. The immunoconjugate was dissolved in 0.1 M Tris, 0.001 M EDTA at pH 7.5, and 0.6% (w/v) BSA and the analyte was RIgG in 0.1 M Tris, 0.001 M EDTA at pH 7.5, and 0.1% (w/v) BSA. Air was used as the carrier.[23] These solutions were impelled to a reaction chamber where the first incubation takes place using constant stirring for 10 min. Then, 200 μL of the incubated solution was injected in the 0.1 M NH_4Cl carrier channel (0.02 mL/min) and transported through the sensor where the second incubation takes place with the magnetic particles fixed to the gate of the ISFET.

After 15 min, the flow rate of the carrier solution was raised to 0.2 mL/min and the enzymatic activity retained by the magnetic RIgG−particles was assessed by injecting 50 μL of 0.1 M urea in the 0.1 M NH_4Cl carrier solution and recording the ISFET peak signal. The inset in Figure 2 shows the enzymatic reaction taking place on the surface of the magnetoimmunosensor.

Sensing Surface Regeneration. When the analysis was finished, the magnet was removed and the flow rate was raised to 3 mL/min washing down the magnetic particles. At this

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moment, the system was ready for a new injection of magnetic RIgG−particles for a new analytical cycle.

RESULTS AND DISCUSSION

The developed flow injection immunoanalysis based on a magnetoimmunosensor permits the implementation of an automated immunoassay procedure. The procedure implemented is a variation of the classic competitive method since it has a preincubation step between the analyte (RIgG) and an excess of enzyme immunoconjugate (urease−GaRIgG). This solution is injected to the main channel where the modified solid magnetic phase is immobilized on the sensor. The excess of immunoconjugate that had not reacted with the analyte in the preincubation step reacts with the RIgG present in the modified solid magnetic phase. The enzymatic activity retained by the magnetic particles is appraised by injecting substrate (urea) to the main channel. Urea is hydrolyzed by the enzyme according to the reaction[24]

$$H_2NCONH_2 + 2H_2O \rightarrow 2NH_3 + H_2CO_3$$

and, if a buffering system formed by NH_3 and NH_4^+ is considered, the pH in the injection zone will vary according to $pH = pK_a + \log([NH_3]/[NH_4^+])$. If $[NH_4^+]$ is kept constant in the carrier channel and at a value higher than $[NH_3]$, then the enzyme reaction will produce a rise of pH that will be a linear function of $\log[NH_3]$. Therefore, since the concentration of NH_3 generated by a determinated enzymatic activity during a fixed time is proportional to the concentration of urea, pH will rise linearly with the logarithm of the concentration of urea. See inset in Figure 2 for the reaction taking place at the detector.

In the system developed, urea concentration and reaction time are fixed and urease activity is a limiting factor. Therefore, the quantity of ammonia generated by the enzyme reaction depends on the enzyme immunoconjugate (urease−GaRIgG) bound to the magnetic RIgG−particles. Hence, enzyme activity is inversely proportional to the concentration of the analyte (RIgG) in the original sample. If the sample contains little analyte, the quantity of free immunoconjugates will be high and the decrease of the detector signal peak will be minimal. On the other hand, if there is much analyte in the sample, the free immunoconjugate will be low and the signal peak in the flow detector will fall significantly.

Optimization of the FIIA System. Definitively, the quantity of detectable immunoconjugate will depend on the RIgG available in the solid phase. Therefore, the signal and the sensitivity of the technique will be affected directly by the number of modified magnetic particles in the immunosensor.

On the one hand, too many magnetic particles renders the method too expensive since more reagent is needed. Additionally, there is a higher dispersion of the sample and the sensor response and the time consumed in the analysis are higher as well. On the other hand, too few magnetic particles will confer a very low sensitivity to the system. Figure 4 shows the results obtained with different quantities of magnetic particles. A 50-μL sample of a suspension containing 1.5 mg/mL magnetic RIgG−particles provides an acceptable sensitivity and a short response time (t_{95} <60 s for 0.1 M urea) at a reasonable price.

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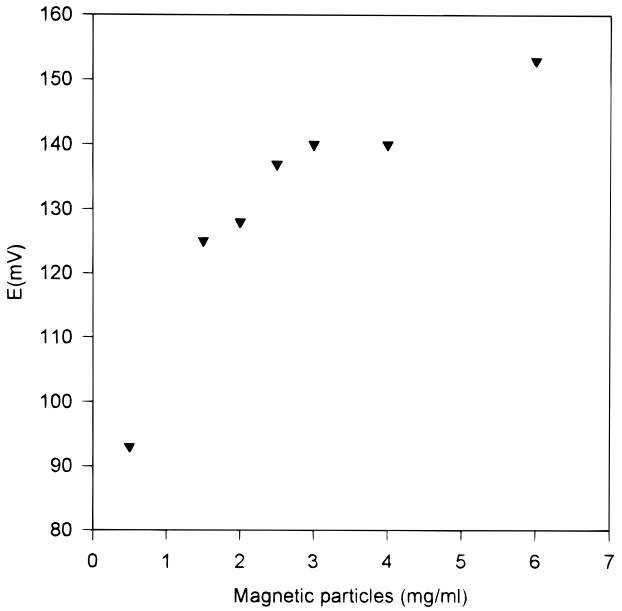

Figure 4. Signal peaks obtained with different magnetic immunosensor surfaces. The microcell was filled by pumping 50 μL of magnetic RIgG−particle suspension of different compositions for each assay. A 200-μL sample of a solution containing equal volumes of 1.96% (v/v) GaRIgG−urease conjugate and buffer solutions was injected. The incubation flow rate was 0.02 mL/min. Carrier, 0.1 M NH_4Cl (0.2 mL/min); enzyme substrate, 0.1 M urea; and injection volume, 50 μL.

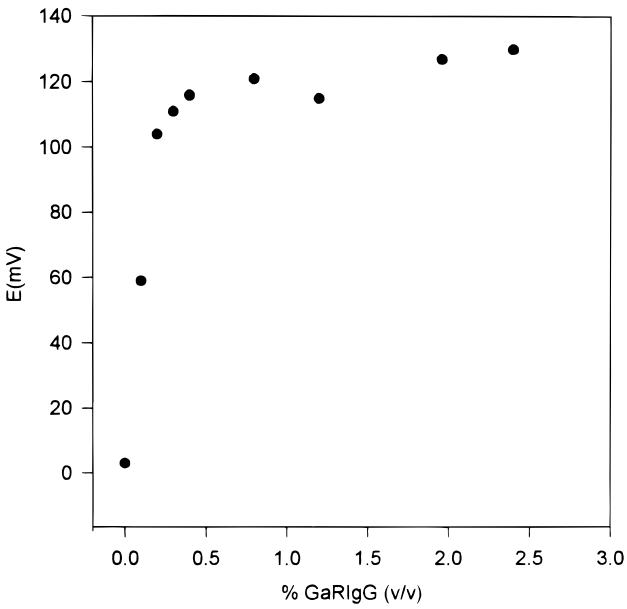

Figure 5. Signal peaks obtained with differents quantities of GaRIgG−urease conjugate. The immunosensor was filled by pumping 50 μL of magnetic RIgG−particle suspension (1.5 mg/mL) for each assay. A 200-μL sample of a solution containing equal volumes of immunoconjugate and buffer solutions was injected. For incubation flow rate and other flow system parameters, see Figure 4.

A competitive assay can be optimized using a slight excess of immunoconjugate. Figure 5 shows the signal resulting from using different concentrations of labeled antibody (urease−GaRIgG) after a 0.1 M urea solution is injected to the system. These results show that it is possible to detect different urease−GaRIgG activities using magnetic RIgG−particles as the solid phase

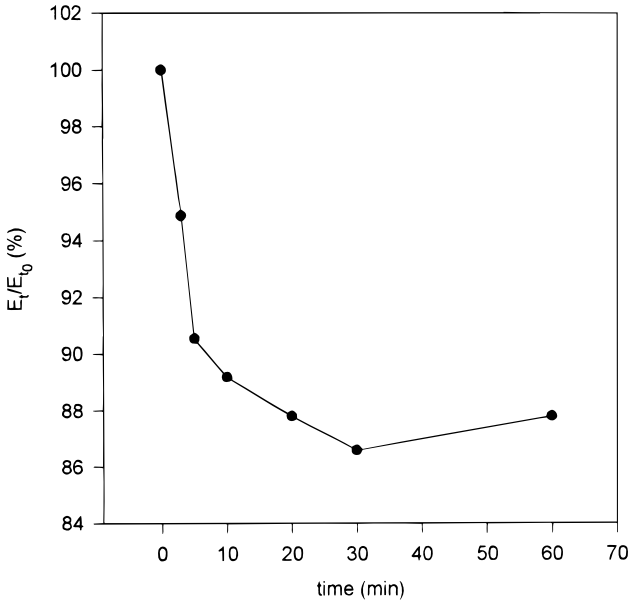

Figure 6. Relative signal peak obtained with different preincubation times. The immunosensor was filled by pumping 50 μL of magnetic RIgG−particle supension (1.5 mg/mL) for each assay. A 200-μL sample of a solution containing equal volumes of 1.26 μM RIgG and 1.2% (v/v) GaRIgG−urease conjugate incubated solutions was injected. For incubation flow rate and other flow system parameters, see Figure 4.

working under determinated conditions. The optimal working zone is between 0.0 and 0.4% (v/v) GaRIgG, since a very high sensitivity is present in this range. A higher initial concentration of 1.2% (v/v) GaRIgG is used so that the optimal working range is attained after of the initial preincubation period.

Figure 6 shows the influence of the preincubation period on the competitive technique. An increase of the preincubation time favors competition. Consequently, the amount of free labeled antibody in excess decreases, causing a diminution of the signal. For a urease−GaRIgG concentration of 1.2% (v/v) and a concentration of 1.26 μM RIgG, a diminution of 38% of the available signal is noticed after the antibody and the antigen have been in contact for 5 min. If the preincubation period is raised to 10 min, the response diminishes 77%. The equilibrium state is reached after 30 min of contact between the antibody and the antigen.

The extent of the binding between the free immunoconjugate and the RIgG bound to the magnetic particles depends on the flow rate used. The flow rate determines the residence time of the free urease−GaRIgG in the immunoreactor formed by the magnetic RIgG−particles[15] and corresponds to the incubation time in a static immunoassay. With a flow rate higher than 0.2 mL/min, the magnetic particles are carried by the carrier solution even if the magnetic field is present. On the other hand, if the flow rate is too low, the time consumed in the analysis will be very high. A flow rate of 0.02 mL/min optimizes the ratio between sensitivity and analytical time.

Good reproductibility was found as shown by the 2% relative standard deviation for series of six assays (see Table 1). The mechanic exchange of the immunomagnetic phase in a reproducible fashion eliminates the problems of instability of the immunosensing surfaces and also eliminates the need for additional time traditionally required for chemical regeneration processes, thus saving time and increasing sampling frequency.

Table 1. Reproducibility of the Construction of Magnetic Immunosensor Surfaces[a]

n	[RIgG] (μM)	E (mV)	RSD (%)
6	0	72.0	1.9
6	1.20	62.5	2.0
6	1.66	57.2	2.2

[a] The microcell was filled by pumping 50 μL of magnetic RIgG−particles (1.5 mg/mL) for each assay; 200 μL of a solution containing equal volumes of 1.2 % (v/v) GaRIgG urease conjugate and differents RIgG incubated solutions were injected. Preincubation time, 10 min. n stands for the number of replicated assays. For incubation flow rate and other flow system parameters, see Figure 4.

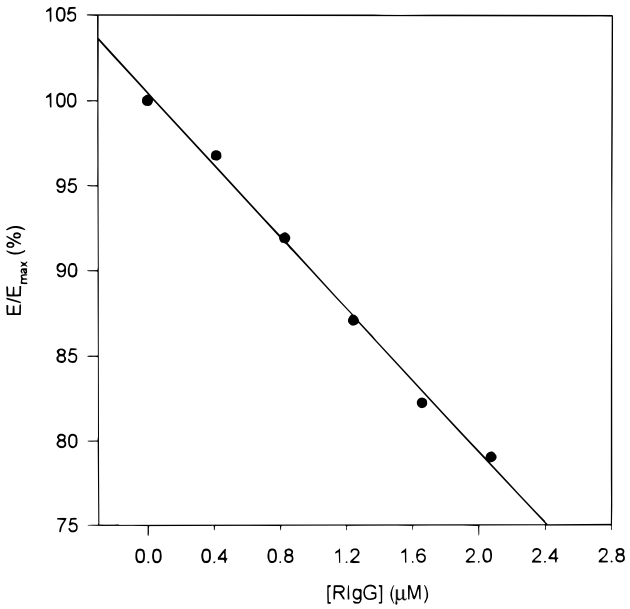

Figure 7. Calibration curve for the measurement of RIgG using a competitive technique and a flow system featuring a magnetoimmunosensor. The microcell was filled by pumping 50 μL of magnetic RIgG−particle suspension (1.5 mg/mL) for each assay. A 200-μL sample of a solution containing equal volumes of 1.2% (v/v) GaRIgG and RIgG incubated solutions was injected. Preincubation time, 10 min. For incubation flow rate and other flow system parameters, see Figure 4. E_{max} stands for signal where no analyte is present in the sample Regression: slope, -10.5 ± 1 L·μmol^{-1}; Y-intercept, 100 ± 1; $r = 0.998$.

The calibration curve for the measurement of RIgG under the optimized conditions described is depicted in Figure 7. The system implemented, using a <30-min analytical cycle, produces a linear range between 0 and 2.07 μM. The detection limit, defined as the concentration yielding a signal three times the background noise, is 8 nM.

The analytical cycle is relatively long because the necessity to develop two incubations. However, it has been noticed that is possible to significantly reduce incubation times without affecting the sensitivity of the system by using other detection systems.

CONCLUSIONS

The described technique offers several advantages over traditional flow injection immunoassays since it is based on a compact immunosensor with a renewable surface. This approach implies a higher sensitivity and shorter response time due to the

proximity between the immunoreactor and the electrochemical transducer. The use of magnetic immunoparticles as a renewable phase of the immunoreactor obviates the need for the chemical regeneration of the solid phase. This produces a better reproducibility and increases sampling frequency, and the assay can be automated easily.

Finally, the method is versatile and flexible and may therefore be adapted to many different applications.

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