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Research paper

Silver nanoparticle enhanced immunoassays: one step real time kinetic assay for insulin in serum

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Abstract

Silver nanoparticle enhanced fluorescence is introduced as an alternative method to surface plasmon resonance techniques for real time monitoring of biorecognitive interactions or immunoassays. This method relies on the phenomenon that an electromagnetic near field is generated upon illumination on the surface of silver nanoparticles. The interaction of this field with nearby fluorophores results in fluorescence enhancement. Thus, fluorophores in the bulk solution can be discriminated from surface bound fluorophores. Anti-insulin-antibodies were immobilized on the surface of silver colloids in the following order: A ready to use microplate was prepared by bottom up coating with layers of aminosilane, silver nanoparticles, Fc-recognizing $F(ab)_2$-fragments and anti-insulin-antibodies. At equilibrium conditions fluorescein-labeled insulin could only be detected in the presence of the colloid; the detection limit was 250 nM, and a fourfold increase in fluorescence was observed upon real time monitoring. The competitive assay of labeled and unlabeled insulin revealed a working range of 10–200 nM insulin in serum. The rapid single step immunoassay is easy to perform even in microplate format, its sensitivity is comparable to ELISA techniques, and offers broad application for real time monitoring of molecular recognitive processes.

Keywords: Silver enhanced fluorescence; Immunoassay; Insulin; Real time monitoring; Surface plasmon resonance

1. Introduction

The development of simple, fast and inexpensive methods to study molecular recognition processes like binding kinetics, affinity and specificity of bioactive compounds to a certain target structure, is most important in drug research. Especially, high throughput screening of new chemical entities requires simple and fast detection without washing steps or addition of signal generating substances. Up to now, the most advanced method to study real time binding kinetics of biological processes such as hormone/receptor-, antigen/antibody- or enzyme/substrate-interactions predominantly relies on surface plasmon resonance [1,2]. Surface plasmon resonance biosensing with colloidal gold enhancement is already commercially used (e.g. BIACORE™-system), where excitation with coherent light in attenuated total reflection geometry allows to discriminate surface bound from free molecules in solution. Despite the advantage that specific labeling is not necessary, the major drawbacks of these systems are the expensive equipment and maintenance due to the complexity of the set up of the measurement as well as the incompatibility of the sensor chips to standard microplates.

Another approach to follow the interaction between fluorescent labeled ligands and their receptors in real time relies on silver nanoparticle enhanced fluorescence [3]. The principles of silver nanoparticle enhanced fluorescence are not yet known in detail, but a well known phenomenon is observed (Fig. 1) [4,5]: Upon bottom up or top down excitation of fluorescent labeled molecules in the supernatant of surface bound noble metal nanoparticles,

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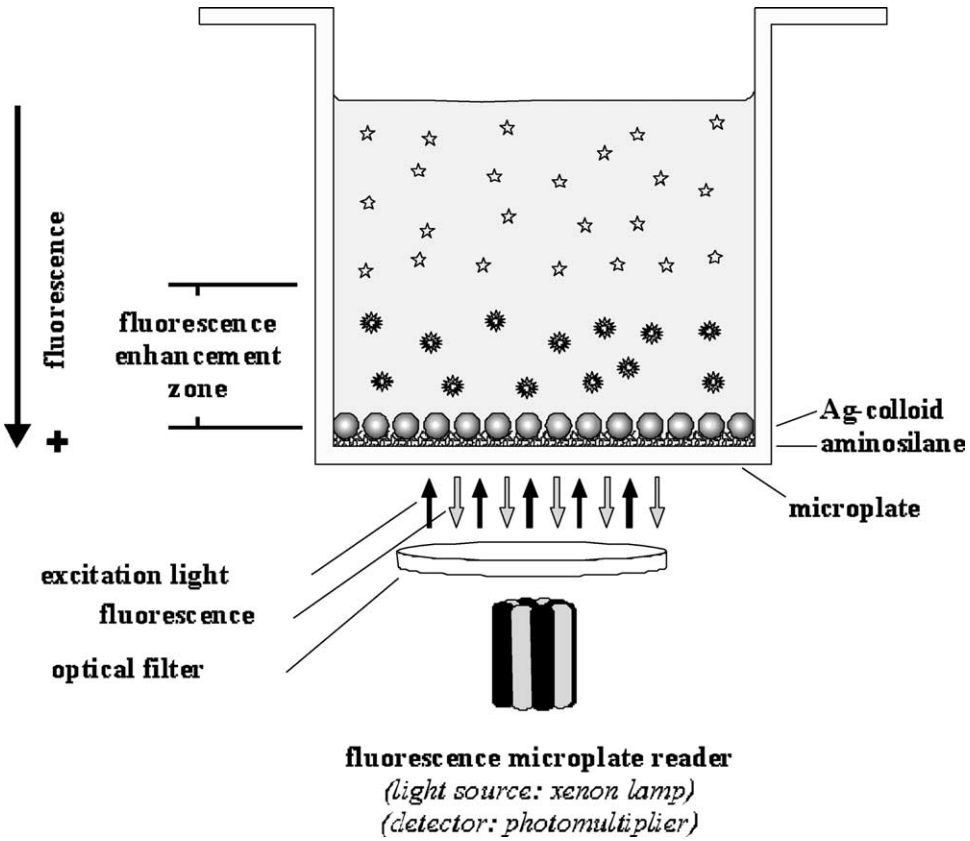

Fig. 1. Principle of silver colloid enhanced fluorescence.

the interaction between electromagnetic radiation and fluorophores close to this surface is strongly enhanced. Thus a fluorescence enhancement zone is generated, which allows the discrimination between fluorophores in close vicinity to the silver colloid and fluorophores in the bulk solution. Consequently the binding of a fluorophore labeled antigen to its antibody, immobilized on the colloid surface should result in an increase in fluorescence, which enables direct real time monitoring of binding kinetics.

According to the literature immunoassays utilizing colloidal gold enhanced surface plasmon resonance result in picomolar detection of human IgG [6]. To estimate the utility of silver enhanced fluorescence for immunoassays, a new assay, designed for quantitation of human insulin with commercially available fluorescein-labeled insulin, is presented. The real time monitoring of insulin binding to its antibody, and the steady state detection of the antigen–antibody complex as well as the enhancement effect provided by the silver colloid are elucidated. In addition, a competitive assay for detection of unlabeled insulin in serum is described.

The proposed assay is fully compatible to the microplate format, and a standard fluorescence microplate reader is used as a measuring device. Due to discrimination between bound and free ligand, washing steps are not required, resulting in a "one step" assay lasting 3–10 min per measurement.

2. Materials and methods

2.1. Chemicals

Sodium citrate, 3-aminopropyltrimethoxysilane, silver nitrate, skim milk powder and all buffer chemicals were purchased from Fluka (Vienna, Austria). Insulin from bovine pancreas, FITC-labeled insulin (F-insulin, fluorescein/protein = 0.8), $F(ab)_2$ anti-mouse IgG (Fc-specific) fragment of affinity isolated antigen specific antibody developed in goat were purchased from Sigma (Vienna, Austria). Murine monoclonal anti-insulin-antibodies were obtained from Biogenesis (Berlin, Germany), 96-well polystyrene microplates from Greiner (Kremsmünster, Austria).

2.2. Silanisation of microplates

Two hundred and fifty microliters of 0.5% (v/v) aqueous solution of 3-aminopropyltrimethoxysilane was filled in each well of a 96-well flat bottom microplate, covered with a lid and incubated for 1 h at 50 °C. After discarding the supernatant, the microplate was dried for 1 h at 50 °C. Excessive aminosilane was removed by three subsequent washing steps in a microplate washer with 400 μl deionized water per each well, and incubated for 15 min at room temperature.

2.3. Colloid coating of the microplates

The silver colloid solution (7 mM) was prepared by dissolving 0.30 g silver nitrate in 250 ml double distilled water at 100 °C followed by addition of 30 ml of 1% (w/v) sodium citrate in double distilled water. This solution was kept boiling for 5 min. The quality of the silver colloid solution was controlled by recording the UV/Vis-spectrum from 350 to 800 nm against deionized water [3].

The aminosilanized microplate-wells were filled with 200 μl chilled silver colloid solution and incubated for 2 h at room temperature. The colloid-coated microplates were washed three times with 400 μl deionized water in a microplate washer and dried with compressed air.

The colloid density at the surface was estimated by reading the absorption at 405 nm against air using a microplate reader (2001 Anthos labtec, Fresenius, Austria).

2.4. Coating of silver colloid microplates with $F(ab)_2$ fragments of anti-mouse IgG and anti-insulin-antibodies

Prior to coating, the wells of the silver colloid-coated microplate were incubated with 100 μl PBS (25 mM phosphate buffered saline, 100 nM NaCl pH 7.3) for 15 min at room temperature. After incubation for 1 h with 100 μl 1:100 diluted $F(ab)_2$ fragment of murine IgG-specific antibody the wells were washed three times with 250 μl PBS each in a microplate washer. Subsequently, the anti-insulin-antibody was immobilized by incubation with 100 μl of antibody-solution (4.9 μg/ml) for 1 h. The wells were washed three times with 250 μl PBS each, and non-specific binding sites were blocked with 200 μl blocking buffer (3% (w/v) solution of skim milk powder in PBS) for 30 min. The microplate was washed again with 250 μl PBS.

2.5. Real time monitoring of binding F-insulin to anti-insulin-antibody

Each well of the colloid- and antibody-coated microplate was filled with 40 μl of a dilution series of F-insulin (0–0.5 μM in PBS or human serum), and was immediately inserted into a fluorescence microplate reader (SpectraFluor SLT, Tecan, Austria). Binding kinetics were monitored by excitation and fluorescence detection at the bottom of the plate (excitation 485 nm, emission 535 nm) for 10 min at 30 s intervals at room temperature.

2.6. Equilibrium detection of F-insulin bound to anti-insulin-antibody

After determining the binding kinetics of F-insulin to its antibody the microplates were incubated for another 30 min, washed three times with 250 μl PBS in the microplate washer, and were filled with 100 μl PBS. The fluorescence intensities of the bound insulin were measured in the fluorescence microplate reader using the same geometry and filters as for the kinetic measurements.

2.7. Competitive immunoassay for insulin

The antibodies were adsorbed on the colloid-coated microplate, and non-specific sites were blocked as described above. To set up a calibration curve, a mixture of unlabeled insulin at various concentrations (0–0.2 μM) and 0.3 μM F-insulin was prepared in PBS and human serum, respectively. Immediately after adding 40 μl of the mixture, the microplate was inserted into the reader and the binding kinetics of fluorescein-labeled and unlabeled insulin to the antibody were observed as described above. After real time monitoring of the binding of insulin to anti-insulin-antibody, the microplate was incubated for another 30 min in the dark to achieve equilibrium setting. Unbound insulin was removed by three subsequent washing steps with 250 μl PBS in a microplate washer. Prior to endpoint detection, the wells were filled with 100 μl PBS each and the fluorescence intensities of bound insulin were detected in the microplate reader.

3. Results

3.1. Preparation of silver colloid-coated microplates

Colloidal silver was prepared by reduction of silver nitrate with citric acid and yielded metal particles in the nanometer range. The silver colloid-solution was characterized by UV/Vis-spectroscopy showing maximum absorption at 470 nm with an OD of 1.4. According to preliminary experiments, the shift of maximum absorption to higher wavelengths reflects the increase in particle size. Additionally, broadening of the peak indicates particle agglomeration or loss of spherical shape [3].

As fixation of colloidal silver particles on the hydrophobic surface of polystyrene microplates was insufficient, the wells were precoated with a thick-film of aminosilane (Fig. 2). The interaction between the amino-groups of aminosilane and the noble metal particles enabled the stable and reproducible anchoring of the silver colloid on the wells. The density of the colloid layer on amino-microplates was estimated by reading the absorption of the empty colloid-coated microplate at 405 nm against air. The absorbance at 405 nm reflects the silver colloid density at the surface, but also the size and the shape of the colloidal particles [3]. Best reproducibility of the biorecognition assays was observed when the absorption was in the range of 0.8–1.1.

3.2. Coating of the silver colloid microplates with antibodies

The amount of anti-insulin-antibody adsorbed to the polar silver colloid surface was very low probably due to

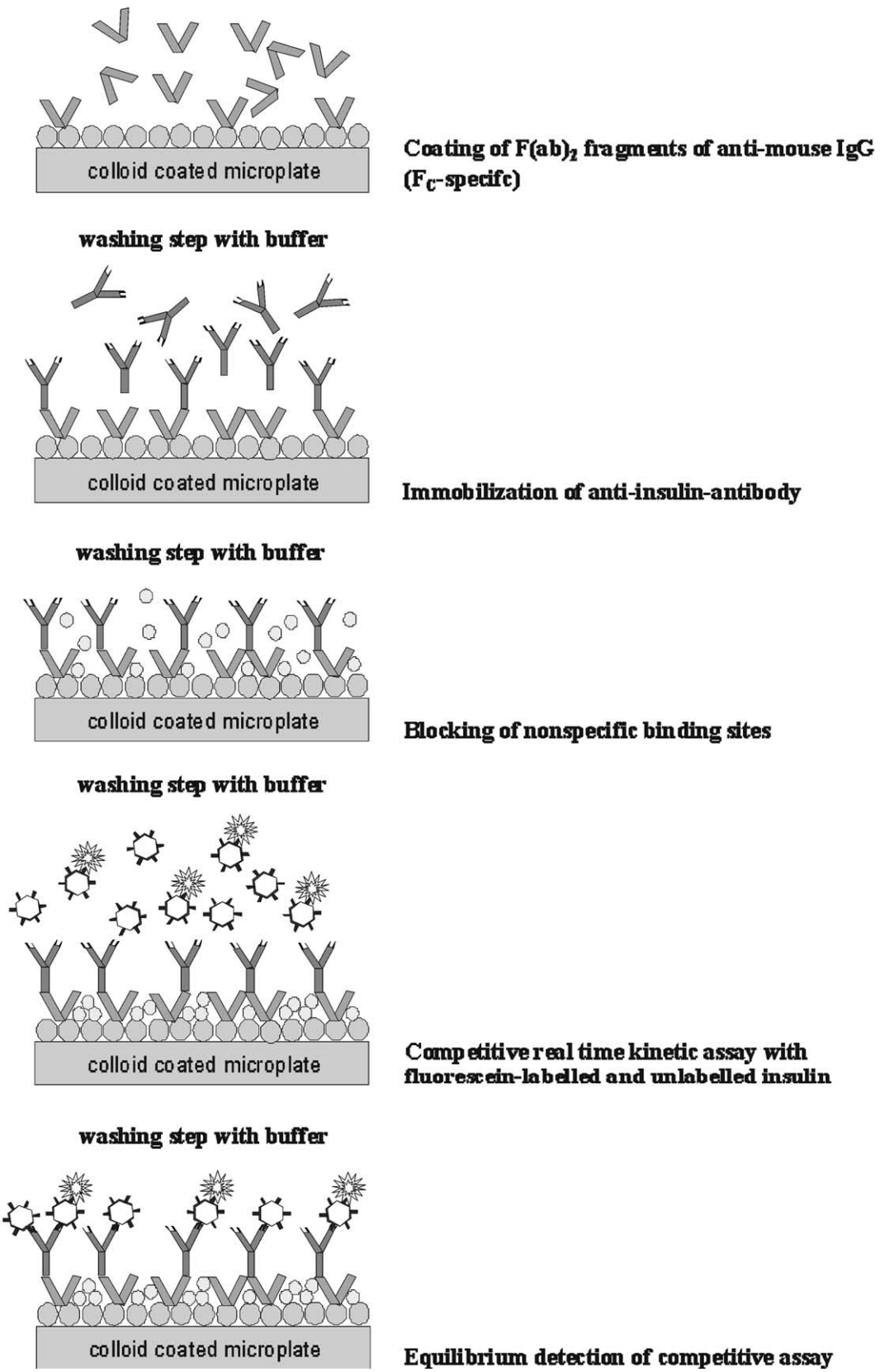

Fig. 2. Scheme representing the preparation of colloid coated microplates and the competitive immunoassay of unlabeled insulin pursuing real time monitoring or endpoint detection (Fc specific $F(ab)_2$-fragment of anti-mouse IgG, anti-insulin-antibody, ○ blocking agent, unlabeled insulin, fluorescein-labeled insulin).

the hydrophobicity of the Fc-proportion of IgG, which yielded poor results. To overcome this obstacle, the Fc-specific $F(ab)_2$ fragment of anti-mouse IgG was used as a linker for the anti-insulin-antibody. The polar disulphide-bridges at the hinge region of the $F(ab)_2$ fragment are readily accessible, and promoted the interaction with the silver colloid-coated microplates. Finally, the Fc-proportion of the anti-insulin-antibody was bound to the immobilized $F(ab)_2$ fragment allowing optimum steric orientation of the insulin binding sites (Fig. 2).

To obtain optimum results, different concentrations of the $F(ab)_2$ fragment of anti-mouse antibody and the anti-insulin-antibody as well as different ratios of both ligands were examined by monitoring the binding kinetics of F-insulin. Optimum binding kinetics were observed when 1:100 diluted $F(ab)_2$ fragment of the anti-mouse antibody and 4.9 μg/ml anti-insulin-antibody were used to coat the wells (results not shown). These concentrations have been retained in all further experiments.

Prior to the assay, non-specific binding sites were blocked with skim milk proteins to reduce the background. According to preliminary assays, the number of non-specific binding sites was more efficiently reduced with skim milk proteins than with bovine serum albumin (data not shown).

3.3. Real time monitoring and endpoint detection of F-insulin binding to anti-insulin-antibody (in PBS or serum)

By use of antibody-coated colloid microplates prepared as described above, the kinetics of the interaction between F-insulin and its corresponding antibody in buffer were determined. The time required to reach the steady state decreased with the concentration of the analyte as to about 250 s in case of 125 nM F-insulin or 150 s in case of 500 nM F-insulin (Fig. 3). For quantification of the analyte, the mean velocity of interaction was calculated from the first 10 reading points using the Biolise®-software. The obtained calibration curve was strictly depending on the concentration with rather low standard deviation and exhibited a detection limit in the nanomolar range.

For endpoint detection, the same microplate used for the kinetic measurement was washed after 30 min incubation to remove the unbound F-insulin (Fig. 4). In the kinetic the blank was low as 48 ± 47, in case of endpoint detection it was 1674 ± 49. The same background was observed when the colloid plate was coated with only the $F(ab)_2$ fragment of anti-mouse IgG, but omitting the anti-insulin-antibody. In the latter case contribution of non-specific F-insulin binding increased up to 1959 ± 51 compared to the specific binding

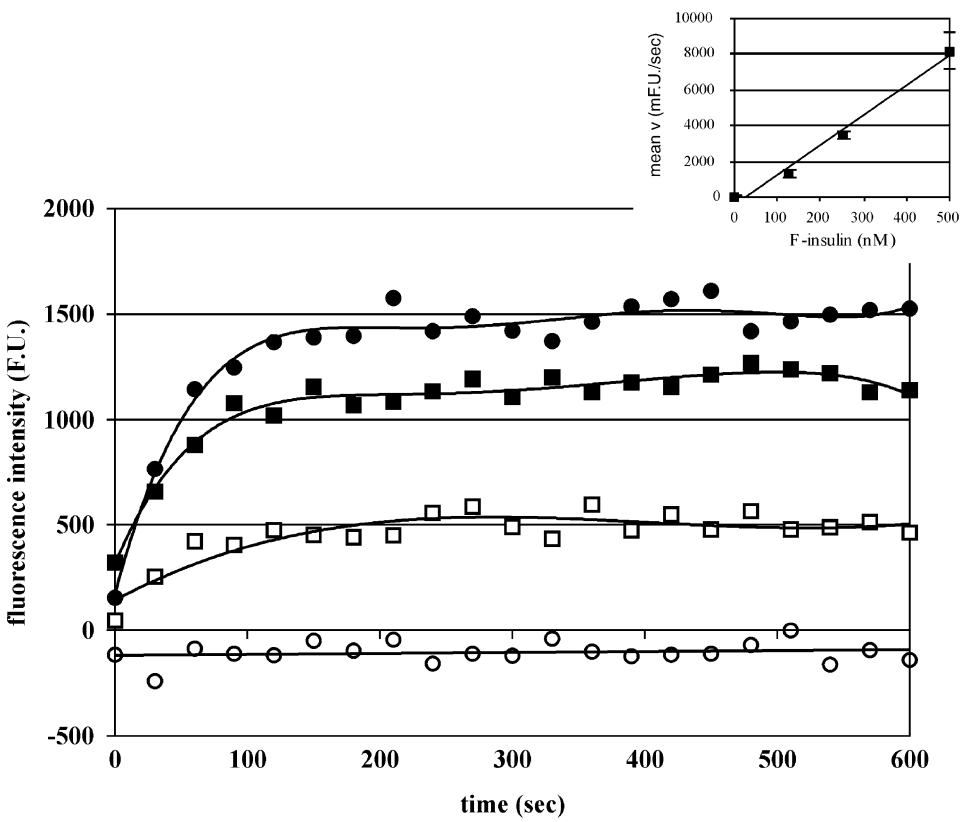

Fig. 3. Real time binding kinetics of F-insulin to anti-insulin-antibodies in buffer (● 500 nM F-Insulin, ■ 250 nM F-Insulin, □ 125 nM F-Insulin, ○ 0 nM F-Insulin). The insert shows the mean velocity of interaction calculated thereof ($n = 4$).

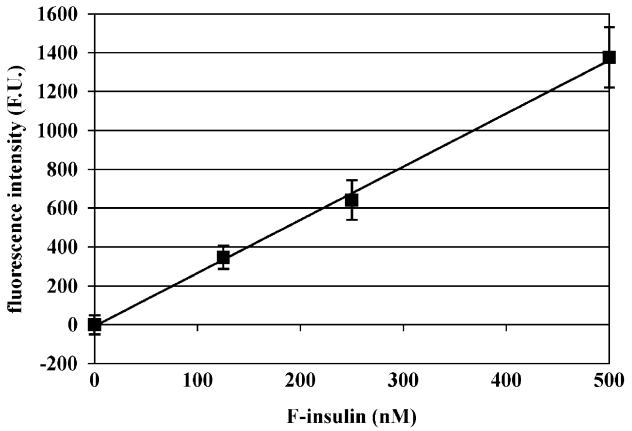

Fig. 4. Endpoint detection of fluorescein-labeled insulin binding to anti-insulin-antibodies in buffer. The data derive from the same microplate, which had been used for kinetic measurement and exhibited comparable detection limits ($n = 3$).

of 1375 ± 155 in the presence of the anti-insulin-antibody. Whereas the net signal intensities of the kinetic assay corresponded to those of the endpoint detection at high F-insulin concentrations, a lower signal intensity was observed in the endpoint assay; especially, at F-insulin <250 nM decreasing concurrently with concentration. Nevertheless, the endpoint detection yielded a linear calibration curve and a detection limit comparable to that of the kinetic assay, but with higher standard deviations.

As reducing compounds present in human serum are known to interfere with immunoassays, the kinetic assay was performed as above in serum. The concentration-dependent kinetics of F-insulin binding exhibited a sigmoid run, which is typical for complex media such as human serum (Fig. 5). Nevertheless, a concentration-dependent calibration curve with a detection limit comparable to that in buffer was obtained. According to these results, no additional protective layer is necessary to prevent reducing compounds from getting into contact with the silver colloid particles.

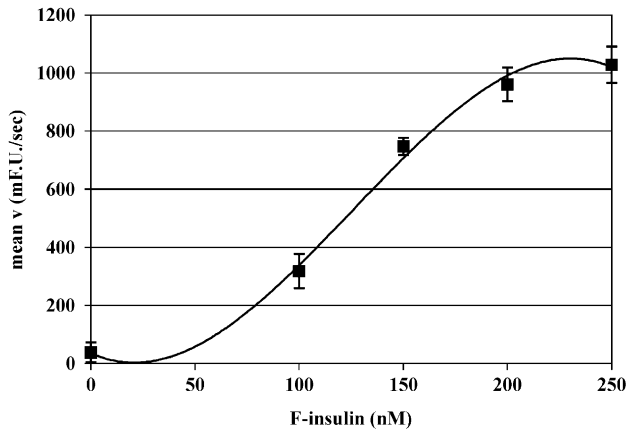

Fig. 5. Real time monitoring of the binding of fluorescein-labeled insulin to anti-insulin-antibodies in human serum ($n = 3$).

3.4. Fluorescence amplification of antibody bound F-insulin by the silver colloid coat

In order to estimate the gaining of the fluorescence provided by the silver colloid coat, the kinetic and endpoint assay were performed on microplates with the layers arranged as mentioned above, but in the presence and the absence of the silver colloid coat. Using coated plates without silver colloid layer, the endpoint assay yielded signal intensities with high standard deviations similar to the blank and such quantitative determination of F-insulin was impossible (Fig. 6). In the presence of the silver colloid layer, the linear calibration curve of the endpoint determination exhibited a slope value of 2.49 ± 0.45 ($r = 0.96$) and a detection limit of 250 nM F-insulin. The detection limit of the kinetic assay in the absence of the silver colloid coat was comparable to that of the endpoint assay in the presence of the colloid coat, but the slope value was quite high being 6.15 ± 0.84 ($r = 0.98$). The highest increase in fluorescence was observed during the kinetic assay in the presence of the silver colloid yielding a linear calibration curve with a slope value of 25.96 ± 2.84 ($r = 0.98$) and a detection limit of about 100 nM F-insulin. Thus, the relative fluorescence intensity of the kinetic assay on silver colloid-coated plates is about fourfold higher than that without the coat.

However, the increase in fluorescence does not derive from a higher antibody-density on silver colloid-coated microplates. As determined by an ELISA using a second alkaline phosphatase labeled antibody for detection of the first antibody, the protein coverage of silver colloid-coated microplates was found to be only 87 ± 8% ($n = 8$) compared to that of microplates without colloid [3].

3.5. Competitive immunoassay for insulin

For the determination of unlabeled insulin in serum, a competitive design was chosen: unknown amounts of unlabeled insulin were allowed to compete with known amounts of F-insulin for binding to the anti-insulin-antibody. According to preliminary assays, a 300 nM solution of F-insulin in buffer or human serum was most appropriate, yielding adequate signal intensity for detection of insulin at the lower nanomolar level.

The quantification of unlabeled insulin in buffer by endpoint detection was not reliable due to high standard deviations and moderate ascent of the calibration curve (Fig. 7). Moreover, when the same competitive immunoassay was carried out in serum, the correlation between signal intensities measured and the standard solutions was higher, and a somewhat steeper calibration curve was obtained. Although excess unbound F-insulin was removed and sufficient time for equilibrium setting was offered by endpoint detection of the competitive assay, measurement of real time kinetics yielded more reliable results. Probably due to rather rapid competition of insulin and F-insulin for

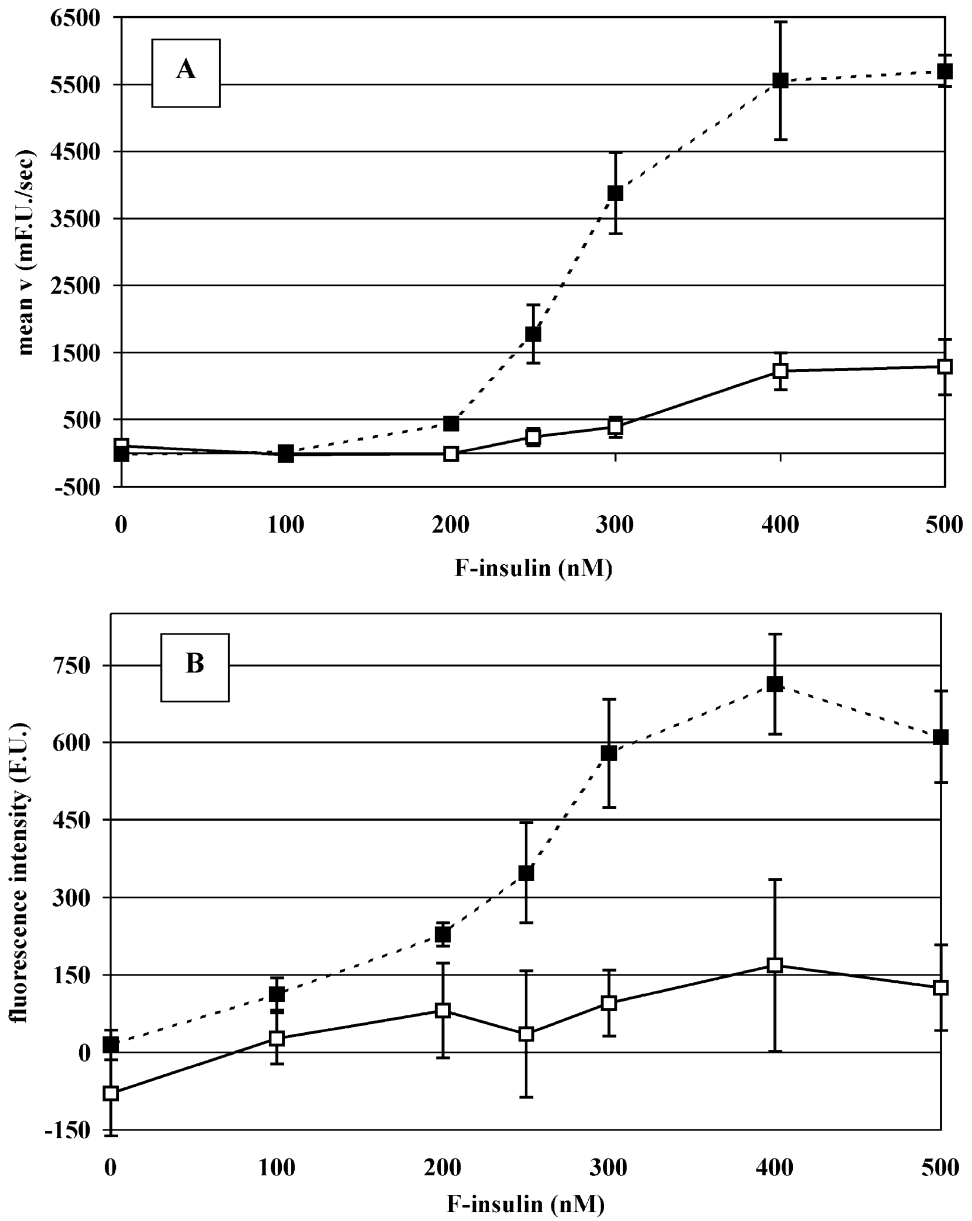

Fig. 6. Real time binding kinetics (A) and endpoint detection (B) of antibody-bound fluorescein-labeled insulin in presence (■) and in absence (□) of the silver colloid layer ($n = 6$).

binding to the anti-insulin-antibody, the amount of antibody bound F-insulin was detected more reliably by the kinetic assay. Although the amount of F-insulin added was constant in all samples and the unbound F-insulin was still present during measurement in this one step kinetic assays, the mean velocity of interaction was still depending on the concentration of unlabeled insulin. This indicated an enhancement effect provided by the silver colloid coat. When the assay was carried out in serum, a steeper calibration curve was observed as compared to the assay in buffer. Consequently, the working range proposed is 50–150 nM insulin in buffer and 10–200 nM insulin in serum.

4. Discussion

At present, the phenomenon of silver nanoparticle enhanced fluorescence is not fully understood, but according to the literature it might be explained as follows [7–9]: Due to the small diameter and the resonant polarization of noble metal nanoparticles, the silver colloid particles absorb more light than the fluorescent molecules. The absorbed light provokes resonant electron plasma oscillations at the surface of the noble metal particles, and generates an electromagnetic field near the surface, which might be more intensive than the light for excitation. Thus, fluorescent molecules near the colloid are more likely to be excited than

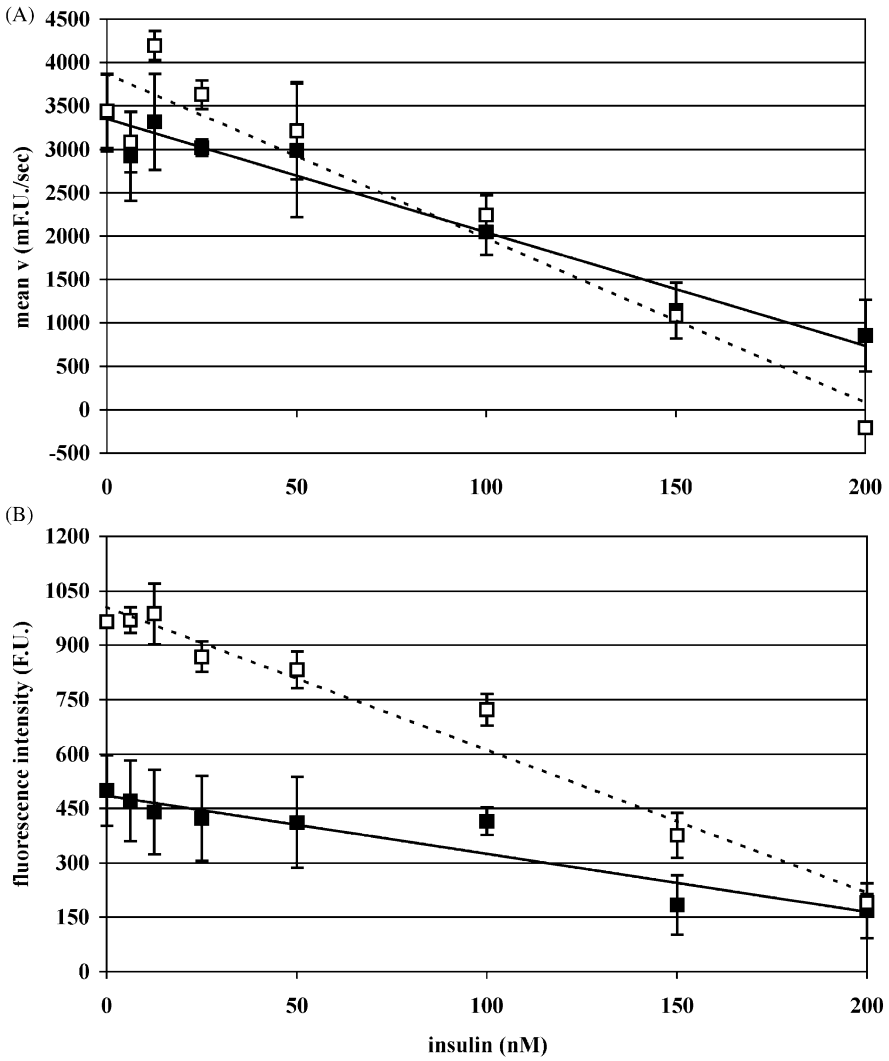

Fig. 7. Real time monitoring (A) and endpoint detection (B) of the competitive assay of unlabeled insulin in human serum (□) and in buffer (■) ($n = 6$).

molecules farther away. This higher probability of excitation seems to act like an exciting light field with higher intensity.

Not only during excitation, but also during emission a gain in fluorescence is possible. When a weak fluorophor is in the excited state and interacts with the silver colloid, it is more likely to emit photons than to return into the ground state without emission. Thus the electronic plasma resonance of the silver colloid helps to get rid of energy by radiation.

Overall, the fluorescence enhancement strongly depends on the distance between the fluorophor and the noble metal-nanoparticles. The probability of interaction decreases by distance and also by immediate vicinity to the particles, which causes radiationless return of the fluorophor to the ground state. Consequently, there seems to be an optimum distance between the silver colloid particles and the fluorophor forming the fluorescence enhancement zone.

An additional factor for fluorescence enhancement is an overlap of the absorption spectrum of the silver colloid particles and the excitation or emission spectrum of the fluorophor. The fluorescence increases with higher overlap.

In order to prove the theory of silver enhanced fluorescence as a tool for rapid single step immunoassays, required for high throughput screening of biologically active proteins, an immunoassay for insulin was established. Due to different polarities of the layers required for stable binding of silver nanoparticles and the anti-insulin-antibodies on the hydrophobic surface of microplates, the layers had to be arranged according to the hydrophobic- or hydrophilic interactions (Fig. 2). Despite the fact that the silver colloid particles were coated with an antibody sandwich of about 20 nm in height, an overlap of the electromagnetic near field at the surface of the silver particles and antibody-bound F-insulin was observed, which resulted in fluorescence enhancement. Because endpoint detection of F-insulin by the conventional fluorescence immunoassay was not possible, the protein could be determined quantitatively in the presence of the silver colloid. Not only the increase in fluorescence intensity is

further supported by the kinetic curves, but also the discrimination between surface bound- and bulk-fluorescence was achieved, which results in single step assays without washing steps. Because the protein binding of both colloid and non-colloid coated surfaces is comparable, the slope value in the presence of silver colloid is fourfold higher and real metal particle fluorescence enhancement is observed [4]. Altogether the combination of silver colloid coating and kinetic assay makes quantification of F-insulin possible, and yields a calibration curve with a 10-fold higher slope value as compared to endpoint detection in the presence of silver colloid. Furthermore, as required for the detection of molecules in biological matrices, the assay works in serum as well as in buffer. Thus no protective layer for the silver colloid is needed.

The prerequisite for silver enhanced fluorescence immunoassays is a labeled analyte. This drawback can be surmounted by a competitive assay design. By careful selection of the concentration of the F-analogue, regarding the expected level of the analyte and the signal intensity required, quantitative determination of the analyte by a competitive design is possible. We tried to prove the feasibility of the silver enhanced kinetic assay for determination of insulin in serum since insulin has a half life of about 5 min in plasma requiring a kinetic assay with a short readout time. The detection limit observed in serum was about 10 nM insulin, but the serum level in humans is 21–243 pM, which can only be estimated by skillful RIA-techniques up to now [10]. As the signal intensity of competitive immunoassays is inversely proportional to the concentration of the analyte, the detection limit is exclusively determined by the signal intensity of the labeled analyte. The labeling ratio of the insulin commercially available is rather low, equivalent to 0.8 mol fluorescein per molecule. Thus, larger molecules with a higher degree of labeling are expected to yield lower detection limits. The detection limit of insulin might be improved by the usage of more efficient fluorescent labels or other signal amplification techniques such as the avidin–biotin system.

5. Conclusions

The silver nanoparticle enhanced immunoassay represents a simple method to gain information about molecular recognition processes e.g. binding kinetics, specificity and affinity of new developed drugs. Due to discrimination between surface- and bulk-fluorescence, the method requires no washing steps and direct monitoring of the reaction is possible. This results in short readout times necessary for high throughput screening. The ease of colloid-surface preparation, the simultaneous analysis of 96 samples in one microplate, the screening of a wide spectrum of drugs and detection limits comparable to ELISA techniques seems to make the described method, relying on silver enhanced fluorescence, a valuable tool to exclude less efficacious new chemical entities at early stages of drug development. Consequently, the amount of new developed drugs and formulations to be tested in animals might be reduced. All in all, the method is a promising tool for different fields of research in natural sciences.

Acknowledgements

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