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Recent developments for SPIE-IA, a new sandwich immunoassay format for very small molecules

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Abstract

Recent publications describing new elegant approaches to assay small analytes using noncompetitive format were briefly reviewed. Among these methods, we have developed a new protocol, named SPIE-IA, which involves a cross-linking step achieved using chemical hombifunctional reagents, UV irradiation or free radicals. This new method proved to be useful to detect naturally occurring analyte/antibody complexes or to protect the analytes against degradation by peptidases. On the other hand, SPIE-IA could allow to study the adverse biological effects of UV and some aspects of free radical chemistry or to evaluate the antioxidant activity of molecules.

Keywords: Noncompetitive assays; Small analytes; Oxidant stress; Free radicals; Irradiation; Cell culture; Immune complex

1. Introduction

Since the first description of immunoassay in the 1960s, wide analytical applications of these methods have been described in various biological fields. The success of this technology is mainly due to the remarkable properties of antibodies to recognize and bind molecules with high affinity and specificity in very complex media, such as biological fluids (blood, urine, etc.) and cellular or tissular extracts. Since the pioneering work of Yalow and Berson, these immunological methods have been subject to perpetual evolution to analyze different types of molecules (hapten or antigen) by using various kinds of (i) tracer (analyte or specific antibody), (ii) antibody specific to the analyte (monoclonal or polyclonal antibodies), (iii) type of label (radioactive, enzymatic, fluorescent, luminescent, etc.), and (iv) format (homogeneous or heterogeneous assay including or not a separation step between the bound and free forms of the analyte). Immunoassays fall into two broad types, competitive or two-site assays, essentially differing in the concentration of the components in the immunological reaction. The competitive method involves the use of limited

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concentrations of specific antibody and labeled (or immobilized) analyte. The two-site immunometric assay is based on two different antibodies, present in excess, able to bind the antigen simultaneously. The theoretical study of Jackson and Ekins has demonstrated that noncompetitive immunoassays are superior to competitive immunoassays in terms of sensitivity, precision, kinetics and working range of analyte [1].

However, two-site immunometric assays appear unsuitable for the measurement of too small analytes that cannot be simultaneously bound by two antibodies due to steric hindrance. So far, we have reported that angiotensin II (AII) (1048 Da, eight amino acids) is the smallest molecule for which a two-site immunometric assay has been developed [2]. To overcome this difficulty, different groups have proposed various elegant solutions to develop new noncompetitive formats based on several approaches using chemical modification, unusual antibodies (anti-idiotype antibodies, anti-metatype antibodies or single chain antibodies), or special separation steps (capillary electrophoresis, affinity column) that we shall briefly describe.

In the laboratory, we have developed and patented a new noncompetitive format for various molecules, including some very small ones, and we report here some applications of this technology in terms of immunoanalysis and also some unexpected applications.

2. New formats for small molecules

Since the early 1990s, different groups have developed several original approaches to measurement of small molecules using noncompetitive methods. Each time the authors have found an elegant trick to preserve the advantage linked to the use of a reagent in excess.

2.1. Ishikawa's method

A first approach, developed by Ishikawa's group, involves the introduction of a molecule of biotin into the analyte prior to the assay. This prederivatization enables a sandwich assay using an anti-analyte antibody and a molecule of avidin. The trick thus relies on the use of a strong affinity system, i.e. the avidin–biotin complex, covalently linked to the analyte in order to obtain a two-site immunometric assay. This method

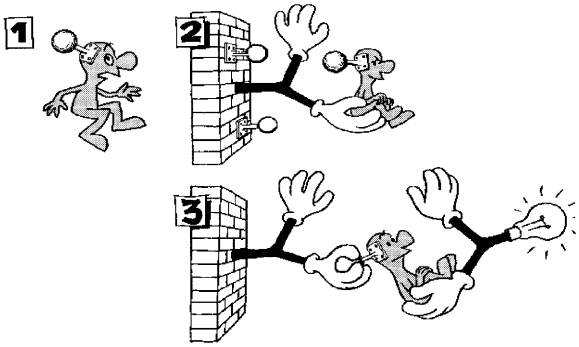

Fig. 1. Principle of Ishikawa's method. Several procedures were described by Ishikawa's group and the simplest one is presented here [3,6]. Step 1: biotin introduction into the analyte; step 2: Immunopurification of biotinylated analyte using a solid phase coated with an anti-analyte antibody; step 3: after analyte elution at pH 1 and neutralization, the analyte is mixed with labeled anti-analyte antibody and transferred onto a solid phase coated with avidin. The signal is measured after a washing step.

(Fig. 1) has been successfully applied to several analytes bearing a primary amino group, which is reacted with the biotin.

This technique presents very interesting properties and allows sensitive detection of 10 attomoles for small peptides like angiotensin I or arginine vasopressin [3,4]. However, the process is quite complicated due to the numerous solid phase transfer steps required to limit the interference induced by the labeling protocol (with biotin) of the biological samples. Indeed, since this reaction is not specific, it can lead to the labeling of various substances in the samples, possibly interfering during the assay via nonspecific adsorption to the solid phase (high background signal) or saturation of the avidin binding sites. The steps allowing the immunopurification of the biotinylated analyte involve dissociation treatment and transfers, previously described by the same group to develop highly sensitive immunometric assays [3–6].

2.2. Application of anti-idiotypic antibodies

In 1990, Kohen's group described an original method for immunometric assay of estradiol, based on the properties of anti-idiotypic antibodies [7,8].

In the first step (Fig. 2) the analyte reacts with an anti-analyte antibody (first antibody), bound to an anti-isotype enzyme-labeled tracer antibody. In

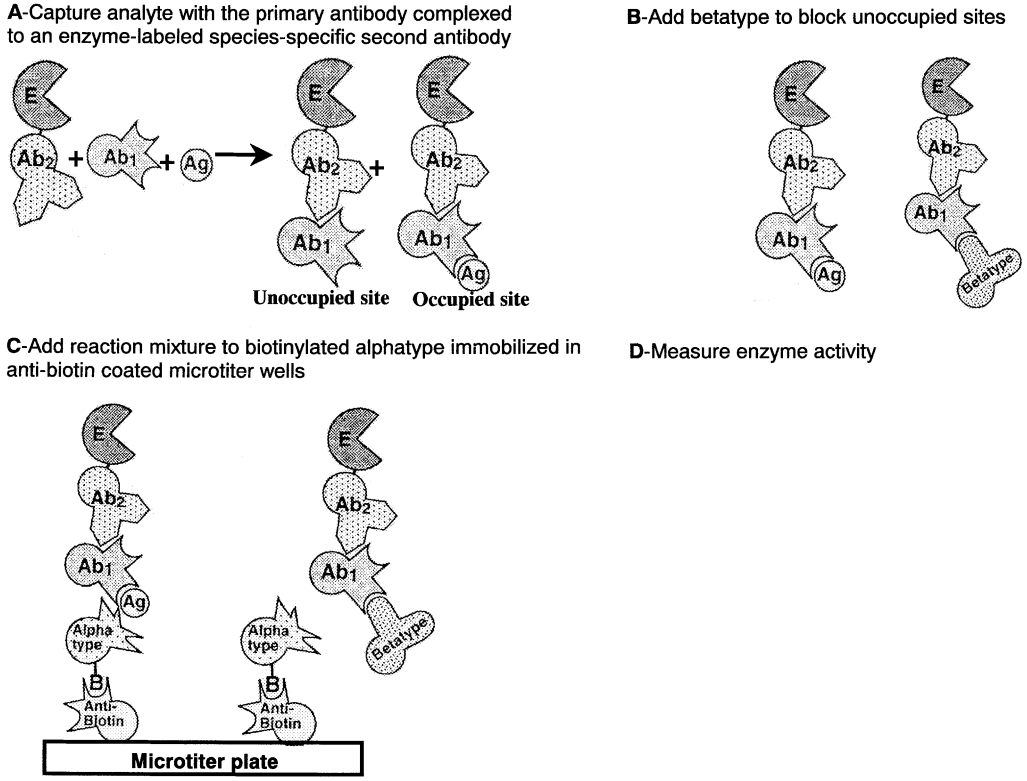

Fig. 2. Principle of immunometric assay based on anti-idiotypic antibodies. Scheme from Mares et al. [8].

the second step, a β-type anti-idiotype antibody, which only recognizes the free binding site of the anti-analyte antibody to the exclusion of the paratope bound to the analyte, is added. In the third step, antibody/analyte complexes are recognized by an α-type anti-idiotype antibody (directed against the first antibody), which cannot react due to steric hindrance with the first antibody/β-type anti-idiotype antibody complexes. Moreover, this α-type anti-idiotype biotin-labeled antibody, allows capture on the solid phase via an immobilized anti-biotin IgG. At the end of this complex protocol, the amount of first antibody on the solid phase, and thus the signal, is proportional to the amount of analyte. This process relies on the use of a hybrid monoclonal antibody produced by a rat-mouse hetero-hybridoma as first antibody, which can be complexed with a labeled anti-rat antibody.

In the initial description of this assay [9], the α-type anti-idiotype antibody was labeled while the first antibody was immobilized on the solid phase—a procedure also used by Kobayashi et al. [10].

In this case, the trick corresponds to the selective binding by the β-idiotype antibodies to free paratopes, and selective binding by the α-idiotype antibodies to the first antibody uncomplexed with β-idiotype antibody. The described assays have good sensitivity as illustrated by a 28 pg/ml limit of detection for estradiol [8]. However, the difficulty of producing and selecting convenient α-and β-idiotype antibodies considerably limits the development of this type of assay.

2.3. Application of anti-metatype antibodies

More recently a new approach based on anti-immune complex antibodies was proposed. This type of antibody is directed against a specific epitope generated after the formation of an antibody/antigen complex [11], leading to the name of “anti-metatype antibodies” initially proposed by Voss et al. [12].

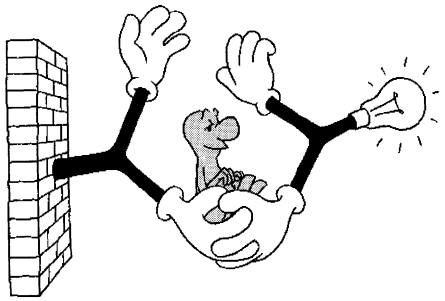

Fig. 3. Principle of immunometric assay using anti-metatype antibodies. In this format the capture antibody is directed against the analyte and the tracer antibody is directed against a capture antibody epitope generated after the formation of the antibody/antigen complex.

The anti-metatype antibodies recognize the antibody/antigen complex but exhibit very low or no affinity for the antibody or the antigen alone. This remarkable property was cleverly used by two groups [13,14] to develop two-site immunometric assays for small molecules. In this case, the first site corresponds to the analyte itself while the second epitope appears, after the analyte/first antibody complex formation recognized by an anti-metatype antibody, on the first antibody (Fig. 3). Self et al. have described a rapid and sensitive (30 pg/ml) assay for digoxin. The results obtained by Towbin et al. are also very interesting, with a 1 pg/ml limit of detection for the assay of angiotensin II.

However, this elegant approach is difficult to generalize because of the difficulties of obtaining these very special anti-metatype antibodies. Moreover, like the previous method, this approach requires monoclonal antibodies.

2.4. Using antibody fragments

A new kind of immunometric assay for small molecules, named Open Sandwich, has been described [15]. This assay is based on the observation that for some antibodies, the association of separated VH and VL chains from the variable domain of antibody is strongly favored in the presence of antigen. To realize their assay, the authors use the techniques of molecular biology to prepare two different fusion proteins containing the VH chain and the *Escherichia coli* alkaline phosphatase on the one hand and the VL chain and the protein G on the other hand. On a plate coated with human IgGs, the VL chain is first immobilized through the binding of protein G, before adding together the antigen and the VH-alkaline phosphatase fusion protein (Fig. 4). Since the binding of VH is dependent of the analyte concentration, the signal measured during the revelation step is proportional to the amount of analyte added. While this approach is elegant and shows a correct sensitivity (1 μM for 4-hydroxy-3-nitrophenacetyl in this format [15] and 40 nM in homogeneous format [16]), the preparation of the reagents is complex and time consuming (production and selection of monoclonal antibodies; preparation of DNA fragments encoding VH and VL; construction, production and purification of fusion proteins). Moreover, the principle of this assay, based on differential interactions between separated VH and VL chains in the presence or absence of the antigen, requires a strong antibody selection since only some antibodies meet these criteria [15]. On the other hand, the weak signal/background ratio observed (ratio of 3 for 250 μM) limits the sensitivity of the method. For these reasons, broad application of this method is difficult.

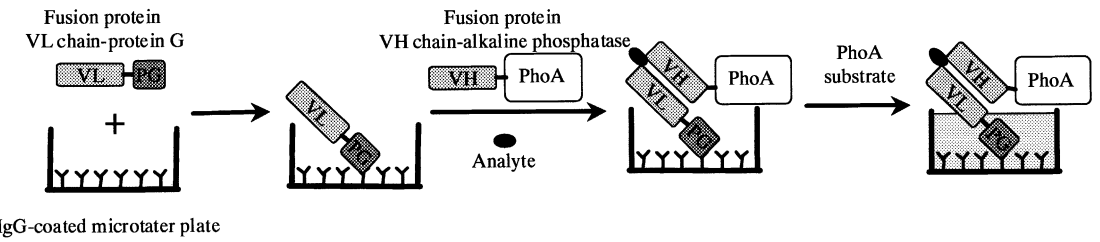

Fig. 4. Principle of open sandwich immunoassay. Step 1: immobilization of VL chain via protein G onto IgG-coated microtiter plate; step 2: incubation of VH-alkaline phosphatase fusion protein with different concentrations of analyte or sample; step 3: the signal is measured after washing and phosphatase substrate incubation.

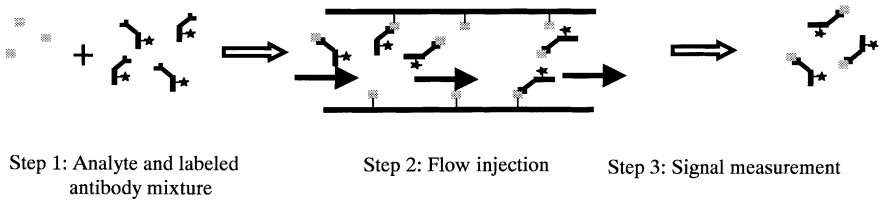

Fig. 5. Principle of flow injection immunoassay. Step 1: analyte is mixed with labeled antibody; step 2: separation of complex antibodies from free antibodies by immunochromatography; step 3: signal measurement.

2.5. Affinity probe capillary electrophoresis (APCE)

Capillary electrophoresis has recently emerged as an alternative to immunosorbent methods for immunoassays. Affinity probe capillary electrophoresis is a non-competitive assay, using a single antibody. Using capillary electrophoresis, the immune complex is separated from excess labeled antibody since the complexation of the antigen with the antibody induces small changes of electrophoretic behavior of the labeled antibody. This technique was first described by Shimura and Karger [17] using capillary isoelectric focusing for human growth hormone assay and further applied to small analytes by Hafner et al. [18]. The performances of this method are good, with a limit of detection of 5 pM for human growth hormone [17] and 10 pM for digoxin, respectively [18]. However, this technique requires electrophoretically homogeneous and monovalent probes, preventing the use of native monoclonal antibodies. Pure antibody fragments must be prepared using biochemistry protocols (proteolysis, reduction, purification steps) [17] or molecular biology [18]. Moreover, the correct separation appears difficult for some neutral analytes and the use of a charged analogue of the analyte is necessary to differentiate the complex from the unbound antibody [18]. Finally, this technique only analyzes one sample at a time, thus limiting its potential application to routine assay of numerous samples.

2.6. Flow injection immunoassays

In this technique, the sample containing the analyte is mixed with an excess of labeled antibody. After incubation, the unreacted antibody is removed by immunochromatography before the measurement. The eluting analyte/labeled antibody complex is then detected (Fig. 5). This method can be used with either affinity-purified polyclonal antibodies [19] or monoclonal antibodies. Monovalent antibodies appear more useful than divalent antibodies which can simultaneously bind both the analyte in solution and immobilized, even if Freytag et al. [20] reported only minor differences when using Fab′ or F(ab′)2. The sensitivity of this method is good: 10 pM for thyroxine [21]; 20 pM for α-(difluoromethyl)ornithine [19] and 40 pM for digoxigenin [22]. However, during the development of different assays, numerous parameters must be optimized: (I) the column capacity, related to the immobilized analyte density and column dimensions, must allow removal of all excess free labeled antibody; (II) the residence time of the sample in the column, related to the flow rate and column dimensions, should be suitable for total binding of free labeled antibody but not so long as to risk the dissociation of the antibody and immobilized analyte. This supposes a brief residence time and an affinity constant of the antibodies higher than 10^8 l/mol [19]. The latter condition is hardly met with polyclonal antibodies which are obviously heterogeneous, even after affinity purification [22]. Moreover, the column must be regenerated after several assays, 60 for Lovgren et al. [22], and once again this technique only allows measurement of a single sample at a time.

3. Solid phase immobilized epitope-immunoassay (SPIE-IA)

3.1. General procedure

A few years ago, we first described the SPIE-IA method for measuring small molecules in an immunometric format [23,24]. This multistep method, based

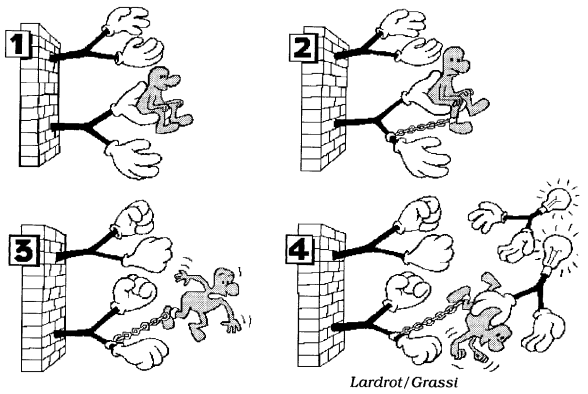

Fig. 6. Principle of SPIE-IA. Step 1: immobilization of different concentrations of analyte or sample on an anti-analyte antibody-coated plate; step 2: cross-linking of analyte with solid-phase protein (antibody and/or albumin used for saturation); step 3: dissociation of analyte/antibody complex (epitope release); step 4: analyte on the solid phase is revealed using enzyme-labeled anti-analyte antibody.

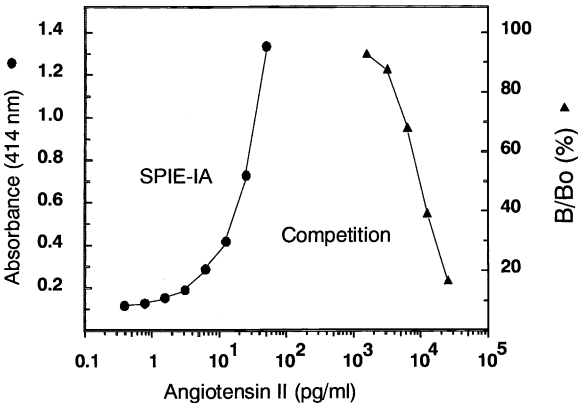

Fig. 7. Standard curves obtained for SPIE-IA and competitive assay of angiotensin II. SPIE-IA procedure: step 1: 4 h at 4 °C; step 2: cross-linking with 0.25% glutaraldehyde for 5 min, then sodium borohydride treatment (10 mg/ml, 5 min) to neutralize aldehyde function; step 3: epitope release using 10% formic acid, 2 min; step 4: antibody labeled with acetylcholinesterase, 18 h at 4 °C. The competitive assay is performed using the same monoclonal antibody using angiotensin II labeled with acetylcholinesterase as tracer. Revelation time: 1 h.

on the use of a single epitope recognized successively by the capture antibody and tracer antibody, involves covalent cross-linking of the analyte to the solid phase according to the scheme shown in Fig. 6. In step 1, the analyte is reacted with a solid phase immobilized capture antibody. In step 2, the bound analyte is covalently cross-linked with solid phase-bound antibody. In step 3, a dissociating treatment (acid, alkali, organic solvent) of the analyte/antibody complex releases the epitope from the antibody binding site. In step 4, the presence of analyte on the solid phase is revealed using an enzyme-labeled antibody (acetylcholinesterase enzyme activity detected using a colorimetric method). Washing of the solid phase is performed after each step to remove unreacted reagents or interfering substances. This procedure involves the use of excess reagents (capture and tracer antibodies) to insure the efficiency of analyte–antibody complexes formation and generates calibration curves in which the signal is directly proportional to the analyte concentration, as in conventional sandwich immunoassays.

3.2. Original SPIE-IA

Like Ishikawa's method, SPIE-IA was first applied to the measurement of analytes bearing a primary amino group excluded from the epitope site. The covalent cross-linking was achieved with bifunctional chemical amine reagent such as glutaraldehyde or disuccinimidyl suberate. This situation appears as optimal, due to the strong reactivity of primary amine functions which allows efficient immobilization. Moreover, a large number of anti-analyte antibodies are obtained by using immunogens corresponding to the covalent link of the analyte to carrier protein via its amino groups. This kind of protocol preserves the further chemical reactivity of the amine function of the analyte when engaged in the immune complex with the antibody. In terms of sensitivity, good results have been obtained using monoclonal antibody for various small analytes (<2500 Da) as substance P: 4.4 pM, [24]; endothelin: 8 pM, [24]; leukotriene C4: 3 pM, [25]; L-thyroxine:18 pM, [24]; angiotensin II: 0.5 pM, [26] or using polyclonal antibodies for an analog of sorbin (13 pM, [27]). In each case, the sensitivity was enhanced (10–300 fold) when compared to the corresponding competitive immunoassay performed using the same antibody. For example, limit of detection of angiotensin II is close to 0.5 and 45 pM with SPIE-IA and competitive assay, respectively (Fig. 7). Moreover, the precision of SPIE-IA is equivalent to that of conventional immunoassays.

On the other hand, SPIE-IA can also be applied to the determination of bigger molecules (antigens) as protooncogene p21 ras (21 kDa), interleukin 4 and interleukin 10 (20 kDa). In this case, the main advantage of the technique relies on the performance achieved with an immunometric format using a single monoclonal antibody. This could be highly valuable in many situations, particularly when a good pair of monoclonal antibodies is not available to set up a classical immunometric assay.

Since the six different washing steps used in the original protocol appeared as a limitation of the method, the procedure was further simplified and optimized during the development of the angiotensin II assay. For instance, we observed that epitope immobilization (using glutaraldehyde) was efficient directly in the capture medium without washing. Moreover, the borane trimethylamine complex was used as a reducing reagent (instead of sodium borohydride) dissolved in a methanol/HCL mixture to ensure simultaneously the reduction of the remaining aldehyde and Schiff base functions and the release of the epitope by dissociating the epitope–paratope interaction. This finally led to a simplified procedure including only 3 washing steps versus 6 for the initially described SPIE-IA procedure.

3.3. SPIE-IA also detects the presence of antigen–antibody complex

During the development of angiotensin II SPIE-IA, we observed that the non-specific binding (NSB), as the signal obtained in absence of analyte and determined by using buffer during the first incubation step, was abnormally high resulting in a reduced sensitivity for the total assay. To analyze better the origin of this phenomenon, we have performed various experiments in which each of the different SPIE-IA steps was successively omitted. We noted that a high NSB did not result from an interaction between the solid phase antibodies and the enzyme tracer (enzyme-labeled antibody), but was only observed when the entire protocol of SPIE-IA was run. At this stage, we hypothesized that the NSB signal corresponds, at least partly, to a specific signal due to angiotensin II (AII) or related molecules bound to the antibodies, copurified with them and finally assayed. To test this hypothesis, we started the SPIE-IA protocol directly

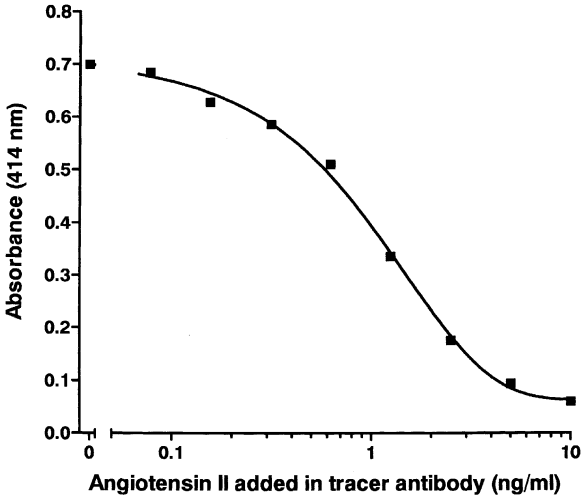

Fig. 8. Specific inhibition of apparent non-specific binding by adding angiotensin II to the tracer antibody. Step 1: no analyte incubation; step 2: cross-linking with 0.25% glutaraldehyde for 5 min, then sodium borohydride treatment (10 mg/ml, 5 min) to neutralize aldehyde function; step 3: epitope release using 10% formic acid, 2 min; step 4: antibody labeled with acetylcholinesterase containing different angiotensin II concentrations, 18 h at 4 °C. Revelation time: 1 h.

at the cross-linking step and pre-incubating the tracer used in the last step with known concentrations of angiotensin II. As seen in Fig. 8, this experiment resulted in a decrease in NSB signal proportional to the concentration of angiotensin II added to the tracer, demonstrating that a competition occurred for the recognition by the tracer between the angiotensin II-like immunoreactivity of the solid phase and the standard pre-incubated with the tracer. We could thus conclude that the NSB signal observed corresponds to specific interactions between the solid phase and the tracer. Further experiments [26] demonstrated that angiotensin II was indeed complexed with monoclonal anti-angiotensin II antibodies and thus we have developed an appropriate protocol for the purification of the monoclonal antibodies in order to eliminate these interactions and avoid any copurification. When antibodies purified by following this modified protocol were used as capture antibodies, the NSB for angiotensin II SPIE-IA was very low. This interference due to the presence of analyte/monoclonal antibody complexes in ascitic fluids was not general but almost frequently observed with SPIE-IA and characterized

Table 1
Angiotensin II concentrations in different culture media after different incubation times

Conditions	2 h	1 day	6 days	13 days
(1) 4 °C	109	112	124	117
(2) 37 °C	113 ± 5	131.5 ± 1.5	131.5 ± 4.5	183
(3) 37 °C + metanephric organs	108 ± 3	66 ± 7	1.65 ± 1.05	0.013 ± 0.007

Angiotensin II spiked at 100 ng/ml in different conditions

(1) Culture medium incubated at 4 °C

(2) Culture medium incubated at 37 °C

(3) Culture medium with metanephric organ incubated at 37 °C

Cultures of metanephric kidney were carried out on sterile permeable membranes of 3 μm pore size (Costar transwell) for up to 13 days. Two kidneys were deposited on each membrane, and the chamber was inserted in a well of a six-well tray filled with medium (2.5 ml) up to the level of the membrane. The medium used was composed of equal volumes of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 nutriment (Sigma), including 1.2 g/l sodium bicarbonate, and supplemented with selenium salt (10^{-8} M), transferrin (50 μg/ml), dexamethasone (5×10^{-8} M), and insulin (5 μg/ml).
After different incubation times, angiotensin II is quantified using the following SPIE-IA procedure:

Step 1: 4 h at 4 °C

Step 2: Cross-linking with 0.25% glutaraldehyde for 5 min, then sodium borohydride treatment (10 mg/ml, 5 min) to neutralize aldehyde function

Step 3: Epitope release using 10% formic acid, 2 min

Step 4: Antibody labeled with acetylcholinesterase, 18 h at 4 °C. Revelation time: 1 h.

several times using the above competition experiment, i.e. for anti-VMA antibodies [28], anti-substance P and anti-PrP (prion) antibodies (unpublished results). We think that this kind of immune complex may well exist in variable ratios, every time the antibodies are raised against a molecule similar or close to a molecule naturally present in the guest (mouse or rabbit). This "interference", which is not detected using a competitive format, could also be responsible for high NSB sometimes observed during the development of classical sandwich assay and should then be taken into account. The SPIE-IA protocol could thus be of help in characterizing the presence of endogenous molecules naturally bound to antibodies.

3.4. SPIE-IA is a tool to measure analyte production during cell culture

Using cultured metanephric organ or renal tubular cells (cell line RC.SV1 described by Vandewalle et al. [29]), we tried to measure the production of angiotensin II using SPIE-IA, but detected abnormally low concentration or even no angiotensin II in the first and the second biological medium, respectively. To explain this result, we postulated that metanephric organs or cells synthesize peptidases that metabolize the angiotensin II produced during the culture. To verify this assumption, the stability of a 100 ng/ml concentration of the angiotensin II peptide was first evaluated in culture medium at 4 °C, and at 37 °C in absence or presence of cultured metanephric organs. Angiotensin II was assayed after 2 h, 1 day, 6 days and 13 days of incubation. These experiments (Table 1) demonstrated that the molecule is stable in culture medium at 4 and 37 °C, but the assayed concentration rapidly decreased when metanephric organs were added, to reach only 1.5% of the initial value after 6 days.

To prevent the degradation of angiotensin II by peptidase, the use of classical inhibitor cocktail [30] was inopportune since these inhibitors would also affect the cellular enzymes. So, we developed a strategy based on different published observations, reporting the ability of monoclonal antibodies to protect the antigen region interacting with the antibody from proteolysis [31,32]. Since the SPIE-IA protocol uses the same epitope for both the capture on the solid phase antibodies and the binding by the tracer, we thus thought that, if angiotensin II was captured by monoclonal

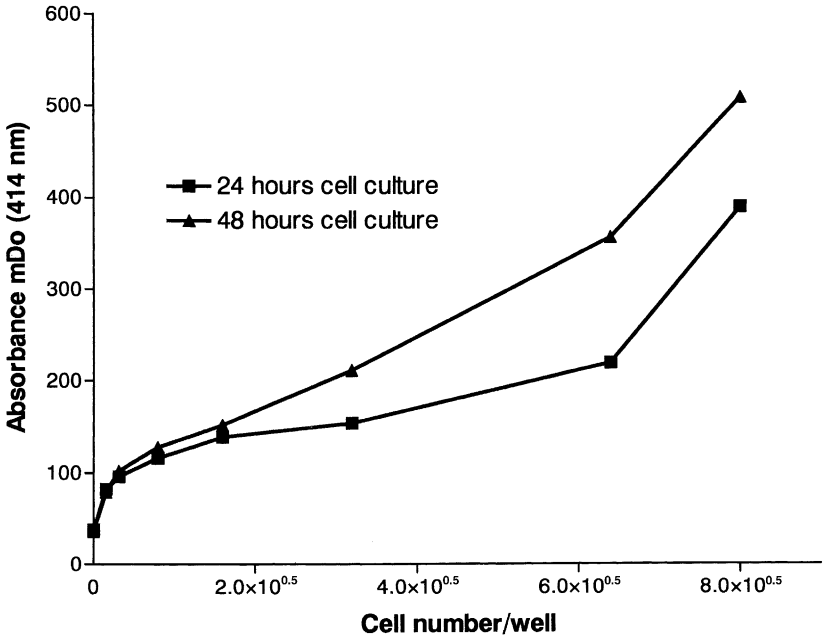

Fig. 9. Production of angiotensin II as a function of cell concentration and incubation time. Different concentrations of cells (in octuplicate) were cultivated for 24 and 48 h in 96-well plates coated with mAb anti-angiotensin II sterilized by filtration through sterile permeable membranes of 0.22 μm pore size. The plates are then washed and angiotensin II is quantified using the SPIE-IA procedure described in Fig. 8.

antibodies, immediately after its production by cultured cells, we should be able to assay this molecule.

For this experiment, we used renal tubular culture cells to establish a better correlation between angiotensin II production and the number of cells. We coated 96-well microtiter plates with monoclonal anti-angiotensin II antibodies, previously sterilized by filtration on sterile membrane (0.22 μm pore size). Cells at different concentrations were cultured in these coated plates for 24 and 48 h. As seen in Fig. 9, this protocol allowed the measurement of the production of angiotensin II with a good relationship with the culture time and the number of cells, as expressed with the intensity of the signal detected. The incubation, under the same conditions, of known concentrations of angiotensin II diluted in culture medium on the plates could allow quantification of this production. However, this quantification will remain unsatisfactory since the production of angiotensin II by the cells is probably continuous and thus the incubation time for capture of the molecule by the coated antibodies will be different for the standard and the molecule generated by the cells. However, SPIE-IA here demonstrates good ability to assay and monitor the production of small analytes during cell cultures, even when these molecules lack stability in the culture medium.

3.5. SPIE-IA as a method to study UV cross-linking

As previously discussed for Ishikawa's method, the original SPIE-IA, using a chemical reagent for cross-linking, can only be applied to analytes possessing an amino group. Sometimes the lack of this function can be overcome using a prederivatization step, before performing the SPIE-IA, to introduce this reactive function into the molecule as described for thyroliberin [33]. With the aim of extending the procedure to haptens devoid of an amine moiety, we have investigated new ways of cross-linking.

During the development of L-thyroxine SPIE-IA, we turned to advantage the capacity of L-thyroxine to be photoactivated directly by UV treatment, resulting in covalent cross-linking with its binding protein [34]. This technology was further successfully applied during the epitope immobilization step [35]

and SPIE-IA involving direct cross-linking of hapten by UV irradiation was then named Photo-SPIE-IA. However, the data collected during this work, using various thyroxine analogs, suggested the direct involvement of the amino group of the molecule during the covalent photo-cross-linking. We therefore investigated the feasibility of using direct UV irradiation in the development of Photo-SPIE-IA for other haptens specially devoid of amino groups. This was successfully achieved by developing the Photo-SPIE-IA of 17β-estradiol [36].

In order to understand this UV cross-linking better, we studied different parameters, taking angiotensin II as model molecule. Using different antibodies, we first tested the influence of energy and irradiation wavelength. As shown by the results (Fig. 10) expressed

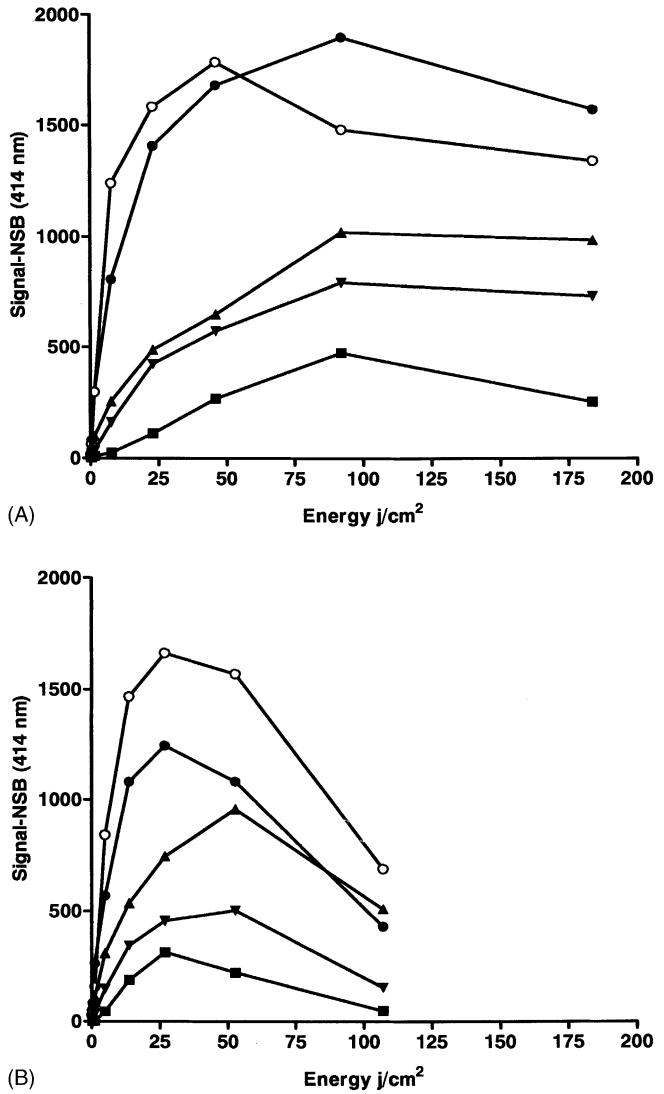

Fig. 10. Energy, wavelength and capture antibody influence on Photo-SPIE-IA signal: irradiation was performed at different wavelengths: 365 nm (A); 312 nm (B); 254 nm (C) and different capture antibodies: mAb 11 (■); mAb 110 (▲); mAb 116 (▼); mAb 131 (○); mAb 211 (●). Photo-SPIE-IA: Step1: 1 h at room temp; Step 2: in 0.1 M phosphate buffer pH 7.4; Step 3: 1N HCl 2 min; Step 4: mAb 31 as tracer, 1 h, room temperature.

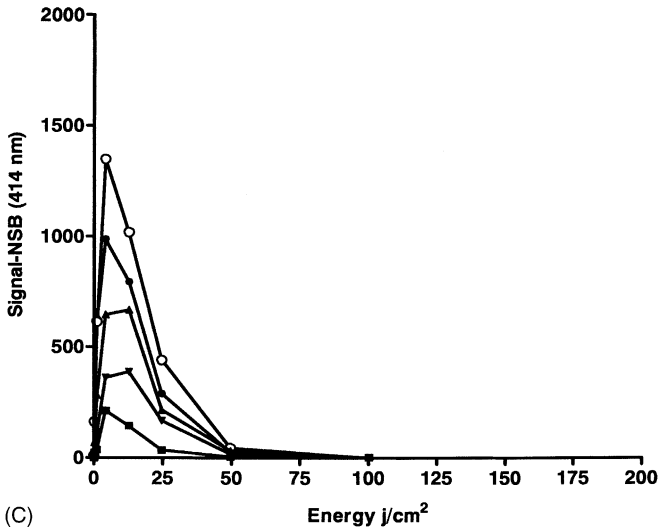

Fig. 10. (*Continued*).

in terms of energy (J/cm^2) versus specific signal (i.e. signal obtained for 1000 pg/ml of angiotensin II minus that obtained for buffer), we have obtained a signal for the different wavelengths and/or the antibodies. However, even if the curve shape changes with the wavelength, the signal intensity appeared to be correlated with the antibody affinity (affinities: mAb 211, mAb 131 > mAb 110, mAb 116 > mAb 11). Interestingly for the same wavelength, the maximal signal was not reached for the same energy, demonstrating that the antibody, particularly the nature of amino acids near or in the antibody paratope, affects cross-linking efficiency.

Moreover, for this experiment we expected to reach a plateau of saturation for the signal, but amazingly, after obtaining a maximum, the signal decreased in all cases. For the 365 nm irradiation, the different antibodies seemed to resist high energy treatment since we noted only a slow signal decrease. This phenomenon appeared dramatic for 254 nm irradiation and intermediary for 312 nm irradiation. The maximum signal at 365 nm was recovered for energy between 50 and 100 J/cm^2 while there was no more signal for these energies at 254 nm. To explain these results, we supposed that, during UV irradiation, two concomitant phenomena occur leading to opposite effects on the finally measured signal: covalent cross-linking between angiotensin II and the capture antibody (essential for the measurement) and strong damage of angiotensin II and/or antibody. In order to verify this hypothesis, we did Photo-SPIE-IA assay using: (i) angiotensin II pre-irradiated at 365 or 254 nm with 10 J/cm^2; (ii) antibody (mAb 131) pre-irradiated at 365 or 254 nm with 10 J/cm^2; as capture antibody (iii) native angiotensin II and antibody. As shown in Fig. 11, the

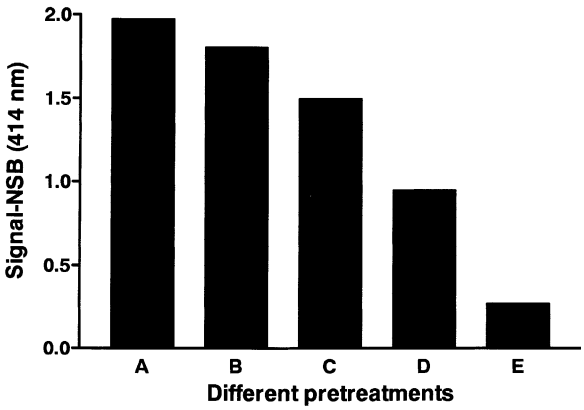

Fig. 11. Photo-SPIE-IA signal after different pretreatments of angiotensin II (AII) or solid phase. A: native AII and solid phase; B: AII irradiated at 365 nm, 10 J/cm^2; C: solid phase irradiated at 365 nm, 10 J/cm^2; D: AII irradiated at 254 nm, 10 J/cm^2; E: solid phase irradiated at 254 nm, 10 J/cm^2. Photo-SPIE-IA: step 1: 1 h at room temp; step 2: irradiation at 365 nm, 10 J/cm^2 in 0.1 M phosphate buffer pH 7.4; step 3: 10% formic acid 5 min; step 4: mAb 31 as tracer, 1 h, room temperature.

pre-irradiation of angiotensin II or the capture antibody exerted a negative effect on the signal. The decrease was more intense after the 254 nm irradiation with a loss of 50 and 75% with the pre-treatment of angiotensin II and mAb 131, respectively. These results strongly support our hypothesis of an initial favorable cross-linking of the two molecules followed by and/or concomitant with a degradation of the analyte and/or the antibody and probably of the complex during irradiation.

The cross-linking reaction obviously involves residues from both molecules, (i.e. the angiotensin II and the capture antibody) through an unknown mechanism to obtain a covalent bond. Although the characterization of the antibody residues involved is rather difficult, as it requires the preparation of recombinant antibodies which must retain the initial binding capacity, a large set of angiotensin II analogs is available to study their performances in the Photo-SPIE-IA protocol. These series of experiments could provide interesting information, at least from one of the partners of the reaction. The different analogs used are summarized in Table 2 and were initially characterized as fully immunoreactive in a competitive format using the monoclonal antibody used in further experiments as capture antibody. These results are in agreement with previous observations reporting a specificity of this antibody against the four carboxy-terminal residues of the angiotensin II molecule. Standard curves were then performed with these analogs using either SPIE-IA involving chemical cross-linking via glutaraldehyde or Photo-SPIE-IA, to compare the relative efficiency of these two procedures. The cross-reactivities obtained for the different molecules are shown in Table 2. The *N*-acetyl analog, although perfectly recognized by the antibody, was not detected by the two SPIE-IA protocols. This result unambiguously demonstrated the direct involvement of the primary amino function in the formation of the covalent bond, which is obvious for the chemical reaction with glutaraldehyde but more surprising via UV irradiation. This observation was partly confirmed using the analog presenting the substitution of the amino-terminal asparagine residue by a sarcosine. This molecule was correctly detected using Photo-SPIE-IA but hardly assayed via chemical cross-linking. This result shows that, as expected, a secondary amine function lacks reactivity towards glutaraldehyde but retains its reactivity during UV irradiation. The other truncated analogs indicated that, in general, the chemical cross-linking process is more sensitive to the modification of the environment of the amino function and/or its accessibility as compared with Photo-SPIE-IA.

Table 2
Cross-reactivity of different angiotensin II metabolites or analogs obtained using competitive assay, SPIE-IA and Photo-SPIE-IA

Analogs	SPIE-IA	Photo-SPIE-IA	Competition
Angiotensin II	100	100	100
N-acetyl-angiotensin II	3.56	1	103
[Sar^1]angiotensin II	5.3	71.9	94
Angiotensin III	36	130	79
[Val^4]angiotensin III	45.8	172	116
Angiotensin (3–8)	33	73.4	154
Angiotensin (4–8)	41.2	123	97.3

SPIE-IA procedure: step 1: 1 h, room temperature; step 2: 0.5% glutaraldehyde, 5 min, room temperature; step 3: 10 mg/ml borane–trimethylamine complex in 2N HCL/MeOH, 5 min, room temperature; step 4: 16 h at 4 °C.
Photo-SPIE-IA procedure: Step 1: 1 h, room temperature; step 2: U.V. at 365 nm 10 J/cm^2, room temperature; step 3: 1N NaOH, 2 min, room temperature; step 4: 16 h at 4 °C.
The competitive assay is performed using the same monoclonal antibody with acetylcholinesterase-labeled angiotensin II as tracer. Revelation time for the three methods: 1 h.

These different results show that Photo-SPIE-IA, taken as a model of ligand/receptor complexes, could help to understand the effect of UV irradiation on the interactions occurring in such complexes, using the receptor on the solid phase and an antibody anti-analyte as tracer. On the other hand, this method could also be used to study the photo-conjugation of proteins or amino acids to DNA which represents one of the major causes of UV-induced damage in biological systems [37].

3.6. SPIE-IA as a method to study free radical chemistry

The previously used mechanism of direct photocoupling is complex and may vary according to the analyte/antibody system used. However, it is generally believed to proceed through the generation of free radicals followed by fast reactions with its surrounding, thereby inducing a zero-length cross-linking. Among putative radical species, the hydroxyl radical

($HO^{\bullet}$) is well known to be a powerful agent for atom abstraction [38] and is involved in the damage caused by photolysis or radiolysis of biological samples. To support the hypothesis of an $HO^{\bullet}$-promoted cross-linking reaction and to investigate its involvement during photo-SPIE-IA procedure, we decided to produce in situ $HO^{\bullet}$ radicals via chemical reactions using Fenton-like reagents. Fenton described at the end of the 19th century [39] the powerful oxidizing properties of a mixture of H_2O_2 and ferrous salt (Fe^{2+}). Forty years later, Haber and Weiss established the oxidizing species as $HO^{\bullet}$ generated according to the following reaction [40]:

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + OH^{-} + Fe^{3+}$$

Others transition metals, like Cu^{2+}, were also used to generate radicals through Fenton-like chemistry [41], even if this is still matter of debate [42,43].

After optimization of the different reaction parameters (pH, nature and concentration of the buffer and the metal, H_2O_2 or urea hydrogen peroxide concentrations and reaction time), we have developed a new SPIE-IA procedure (named SPIE-Rad) using the free radical species produced by the Fenton-like reactions during the cross-linking step. This method was successfully applied to several analytes: 17β-estradiol [44], derivatized histamine and different peptides and proteins such as angiotensin II, substance P, endothelin, CGRP and interleukin-10 [45]. These results showed that Fenton-like chemistry could be useful in developing sandwich-like assays and advantageous due to its simplicity by comparison with the Photo-SPIE-IA protocol which requires a UV irradiation device.

To confirm the involvement of highly reactive radical species during the cross-linking reaction, the 17β-estradiol SPIE-Rad was performed in the presence of various $HO^{\bullet}$ scavengers (BSA, thiourea, trolox, cimetidine, tyrosine, ascorbic acid, mannitol and dimethylsulfoxide). The results (Fig. 12) show that the cross-linking reaction was indeed inhibited by several radical scavengers. However, while a strong decrease in cross-linking efficiency was observed by adding BSA and thiourea, mannitol and dimethylsulfoxide, both described as $HO^{\bullet}$ scavengers [46,47], failed to inhibit the SPIE-Rad signal. The discrepancies displayed by the different scavengers are

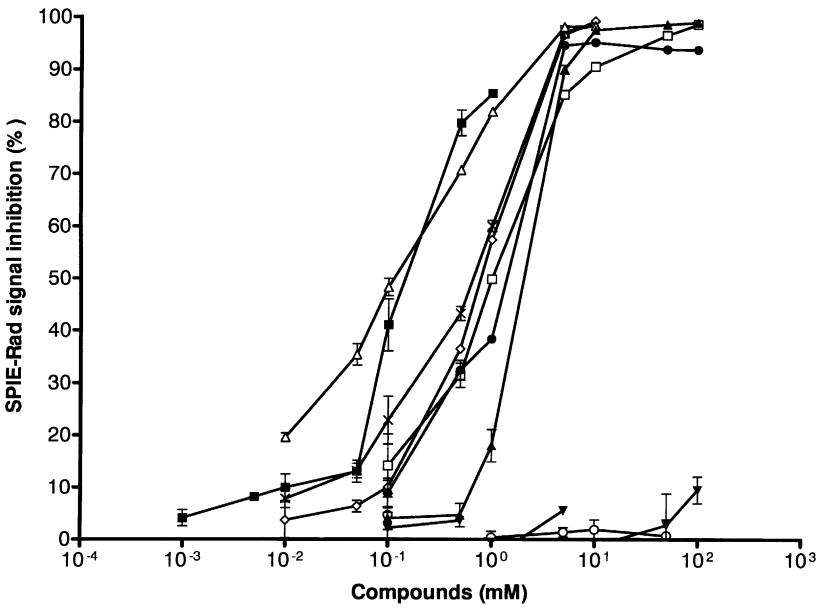

Fig. 12. Inhibition of SPIE-Rad signal by various compounds: BSA (■); thiourea (△); trolox (×); cimetidine (◇); tyrosine (□); uric acid (●); ascorbic acid (▲); mannitol (▼); dimethylsulfoxide (○). The Fenton-like reaction was performed by sequentially adding the compounds (anti-oxidants, $CuSO_4$ and urea hydrogen peroxide) into the wells. The reaction was allowed to proceed for 5 min. Inhibition (%) was calculated by reference to the SPIE-Rad signal obtained without addition of putative scavengers.

probably related to their capacity to trap the reactive oxygenated species.

These results led us to test the ability of the SPIE-Rad to screen for the antioxidant properties of different compounds. Since phenol derivatives like tannins, possess antioxidant activities [48,49] and are present in red wines [50] or other spirits [51], we have checked the possible inhibitor effect of different spirits on cross-linking using the 17β-estradiol SPIE-Rad [45]. The results (data not shown) showed that white wine or spirit (Chablis or Vodka) seem only to inhibit the cross-linking at a high concentration (1/10 dilution), which possibly results in a dissociation of the antibody/17β-estradiol complex rather than trapping of the free radicals. For the other spirits, the inhibition seems to be proportional to the tannin concentration since red wines (Saint Emilion, Santenay) appear more efficient than spirits of wine (Armagnac, Cognac), while whiskies exhibit only weak potency.

This last experiment demonstrated that SPIE-Rad, possibly using different antigen/antibody systems, may be useful in testing and comparing the putative antioxidant properties of natural or synthetic molecules. Moreover, the 96-well microtiter plate format and the rapidity of the test make it suitable for high-throughput screening applications. Finally, this assay does not require any purification step of the molecules, if the compound or the media tested are compatible with the analyte/antibody interaction.

To extend the application of SPI-RAD, we have also evaluated the ability of Nitric oxide radical to achieve the cross-linking step. Preliminary experiments fully demonstrated the efficiency of various $NO^{\bullet}$ donors to react with the immunological complex to ensure the generation of a covalent binding (data not shown). Moreover comparison of $NO^{\bullet}$ cross-linking with SPIE-IA, Photo-SPIE-IA and SPI-RAD using $HO^{\bullet}$ showed similar and promising results. Due to the important biological properties of this $NO^{\bullet}$ radical, this method could be thus envisaged to perform studies on the production and the effects of this radical in different situations.

4. Conclusions

Immunological assays for small analytes have considerably evolved during the past 15 years. Historically, small molecules were assayed in a competitive format, while immunometric assays were reserved for larger proteins. Due to the favorable sensitivity properties of sandwich assays linked to the use of reagent in excess and the continuous demand for greater efficiency, various research groups have made valuable efforts to break this “barrier”. Various elegant approaches have been successfully evaluated, involving nice tricks to develop such sandwich methods for small analytes. Assuming that the epitope recognized by a capture antibody should be further bound by the same antibody as tracer, once this molecule is covalently linked to the capture antibody, we developed a new immunoassay protocol, the SPIE-IA. Using chemical homobifunctional reagents, we demonstrated that this method is applicable to haptens bearing an amino group. During the course of this development, we encountered a problem due to the existence of naturally-occurring analyte/antibody complexes which were copurified with free antibody. This interesting observation could be helpful in explaining the important so-called non-specific background sometimes observed during the development of immunoassays and thus possibly characterized using SPIE-IA. On the other hand, the same protocol demonstrates how through analyte/antibody interactions it can protect against degradation by endogenous peptidases, as shown in the angiotensin II SPIE-IA applied to cultured cells.

However, the value of this SPIE-IA was initially limited to molecules bearing an amino function until we develop a derived protocol using UV irradiation for L-thyroxine. This physical treatment too proved possibly to involve the reactive amino group but was also successfully applied to 17β-estradiol, which has no such function. The analysis of our results with different molecules clearly shows that although it is efficient, UV irradiation has some deleterious effects by degrading the immunological complex more or less rapidly, depending on the wavelength and the energy used. However, this information could be helpful in studying the adverse biological effects of UV, using Photo-SPIE-IA as a model. Once again, this approach appears limited due to the need for an irradiation device and the limited number of irradiation wavelengths available. Since we supposed that UV irradiation generated free radicals probably responsible for the efficiency of the Photo-SPIE-IA,

we decided to use a well-known chemical reaction for cross-linking. The SPIE-Rad protocol works efficiently to assay different molecules. Moreover, this protocol was successfully used to test the efficiency of different free radical scavengers. SPIE-Rad should thus be of interest in studying some aspects of free radical chemistry or antioxidant activity, important parts of the oxidant stress research field.

This step illustrates that the development of immunoanalytical methods is constantly evolving to respond to the increasing need for sensitive methods for various applications in clinical research (drug monitoring, metabolism or toxicology), but also in the food, agricultural or environmental fields. Moreover, by taking advantage of the affinity and specificity properties of the analyte/antibody complex, some parallel useful applications, such as the development of screening methods to test the antioxidant potency of compounds, can also be developed. It is likely that new interesting applications and developments of immunoassays will continue to appear in the literature in the years ahead.

References

[1] T.M. Jackson, R.P. Ekins, J. Immunol. Methods 87 (1986) 13–20.
[2] J. Grassi, C. Creminon, Y. Frobert, E. Etienne, E. Ezan, H. Volland, P. Pradelles, Clin. Chem. 42 (1996) 1532–1536.
[3] E. Ishikawa, K. Tanaka, S. Hashida, Clin. Biochem. 23 (1990) 445–453.
[4] S. Hashida, K. Tanaka, N. Yamamoto, T. Uno, K. Yamaguchi, E. Ishikawa, Anal. Lett. 24 (1990) 1109–1123.
[5] E. Ishikawa, S. Hashida, T. Kohno, K. Hirota, Clin. Chim. Acta 194 (1990) 51–72.
[6] K. Tanaka, T. Kohno, S. Hashida, E. Ishikawa, J. Clin. Lab. Anal. 4 (1990) 208–212.
[7] G. Barnard, J. Osher, S. Lichter, B. Gayer, J. De Boever, R. Limor, D. Ayalon, F. Kohen, Steroids 60 (1995) 824–829.
[8] A. Mares, J. De Boever, J. Osher, S. Quiroga, G. Barnard, F. Kohen, J. Immunol. Methods 181 (1995) 83–90.
[9] G. Barnard, F. Kohen, Clin. Chem. 36 (1990) 1945–1950.
[10] N. Kobayashi, H. Oiwa, K. Kubota, S. Sakoda, J. Goto, J. Immunol. Methods 245 (2000) 95–108.
[11] E.J. Brown, J. Bekisz, J. Immunol. 132 (1984) 1346–1352.
[12] E.W. Voss Jr., S.D. Miklasz, A. Petrossian, M.A. Dombrink-Kurtzman, Mol. Immunol. 25 (1988) 751–759.
[13] C.H. Self, J.L. Dessi, L.A. Winger, Clin. Chem. 40 (1994) 2035–2041.
[14] H. Towbin, J. Motz, P. Oroszlan, O. Zingel, J. Immunol. Methods 181 (1995) 167–176.
[15] C. Suzuki, H. Ueda, W. Mahoney, T. Nagamune, Anal. Biochem. 286 (2000) 238–246.
[16] T. Yokozeki, H. Ueda, R. Arai, W. Mahoney, T. Nagamune, Anal. Chem. 74 (2002) 2500–2504.
[17] K. Shimura, B.L. Karger, Anal. Chem. 66 (1994) 9–15.
[18] F.T. Hafner, R.A. Kautz, B.L. Iverson, R.C. Tim, B.L. Karger, Anal. Chem. 72 (2000) 5779–5786.
[19] P.C. Gunaratna, G.S. Wilson, Anal. Chem. 65 (1993) 1152–1157.
[20] J.W. Freytag, H.P. Lau, J.J. Wadsley, Clin. Chem. 30 (1984) 1494–1498.
[21] A. Arefyev, S.B. Vlasenko, A.P. Eremin, A.P. Osipov, A.M. Egorov, Anal. Chim. Acta 237 (1990) 285.
[22] U. Lovgren, K. Kronkvist, B. Backstrom, L.E. Edholm, G. Johansson, J. Immunol. Methods 208 (1997) 159–168.
[23] P. Pradelles, 00869, 1993.
[24] P. Pradelles, J. Grassi, C. Creminon, B. Boutten, S. Mamas, Anal. Chem. 66 (1994) 16–22.
[25] H. Volland, L.N. Vulliez, S. Mamas, J. Grassi, C. Creminon, E. Ezan, P. Pradelles, J. Immunol. Methods 175 (1994) 97–105.
[26] H. Volland, P. Pradelles, P. Ronco, M. Azizi, D. Simon, C. Creminon, J. Grassi, J. Immunol. Methods 228 (1999) 37–47.
[27] E. Ezan, T. Tarrade, C. Cazenave, T. Ardouin, R. Genet, J. Grassi, J.M. Grognet, P. Pradelles, Peptides 16 (1995) 449–455.
[28] F. Taran, H. Bernard, A. Valleix, C. Creminon, J. Grassi, D. Olichon, J.R. Deverre, P. Pradelles, Clin. Chim. Acta 264 (1997) 177–192.
[29] A. Vandewalle, B. Lelongt, M. Geniteau-Legendre, B. Baudouin, M. Antoine, S. Estrade, F. Chatelet, P. Verroust, R. Cassingena, P. Ronco, J. Cell Physiol. 141 (1989) 203–221.
[30] K. Kohara, Y. Tabuchi, P. Senanayake, K.B. Brosnihan, C.M. Ferrario, Peptides 12 (1991) 1135–1141.
[31] R. Jemmerson, Y. Paterson, Science 232 (1986) 1001–1004.
[32] F. Manca, Ann. Ist. Super. Sanita 27 (1991) 15–19.
[33] E. Etienne, C. Creminon, J. Grassi, D. Grouselle, J. Roland, P. Pradelles, J. Immunol. Methods 198 (1996) 79–85.
[34] W.B. van der, V.M. Nikodem, H.J. Cahnmann, Proc. Natl. Acad. Sci. U.S.A. 79 (1982) 3508–3512.
[35] E. Etienne, C. Creminon, P. Lamourette, J. Grassi, P. Pradelles, Anal. Biochem. 225 (1995) 34–38.
[36] L. Buscarlet, J. Grassi, C. Creminon, P. Pradelles, J. Dupret-Carruel, M. Jolivet, S. Mons, Anal. Chem. 71 (1999) 1002–1008.
[37] Braun, B. Merrick, Photochem. Photobiol. 21 (1975) 243–247.
[38] G. Czapski, Methods Enzymol. 105 (1984) 209–215.
[39] H.J.H. Fenton, J. Chem. Soc. 65 (1894) 899–910.
[40] F. Haber, J. Weiss, Proc. R. Soc. Lond. A 147 (1934) 332–351.
[41] S.I. Liochev, Met. Ions Biol. Syst. 36 (1999) 1–39.
[42] M. Marsawa, H. Cohen, D. Meyerstein, D.L. Hickman, A. Bakac, J.H. Aspenson, J. Am. Chem. Soc. 110 (1988) 4293–4297.
[43] B. Halliwell, J.M. Gutteridge, Methods Enzymol. 186 (1990) 1–85.

[44] L. Buscarlet, H. Volland, J. Dupret-Carruel, M. Jolivet, J. Grassi, C. Creminon, F. Taran, P. Pradelles, Clin. Chem. 47 (2001) 102–109.
[45] L. Buscarlet, Ph.D. Thesis, University of Paris VI, 2000.
[46] S. Goldstein, G. Czapski, Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 46 (1984) 725–729.
[47] R.C. Scaduto Jr., Free Radic. Biol. Med. 18 (1995) 271–277.
[48] J.E. Brown, H. Khodr, R.C. Hider, C.A. Rice-Evans, Biochem. J. 330 (1998) 1173–1178.
[49] B. Frei, R. Stocker, B.N. Ames, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 9748–9752.
[50] V. Fogliano, V. Verde, G. Randazzo, A. Ritieni, J. Agric. Food Chem. 47 (1999) 1035–1040.
[51] D.M. Goldberg, B. Hoffman, J. Yang, G.J. Soleas, J. Agric. Food Chem. 47 (1999) 3978–3985.