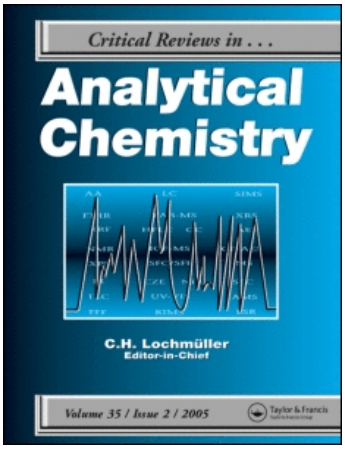

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Chemiluminescence Flow-Injection Immunoassays

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ABSTRACT: The state of art in chemiluminescence flow-injection immunoassays is reviewed. Different approaches and manifolds are described. Advantages of chemiluminescence flow-injection immunoassays, which are discussed in detail, are speed, selectivity, and sensitivity. The major benefit of such systems is the simple and economical set up, which is easily adapted to automation.

KEY WORDS: flow-injection immunoassays, sequential injection immunoassays, chemiluminescence immunoassays.

I. INTRODUCTION

Immunoassays have found widespread application in pharmaceutical analysis, toxicological analysis, bioanalysis, clinical chemistry, and environmental analysis. Conventional immunoassay techniques carried out manually are difficult in handling and rather time consuming. Therefore, automation of immunoassays became of increasing interest.

There are several systems for automating microtiter plate technology; however, these systems require complicated robotics and are rather expensive. Flow-injection systems consist of a very simple instrumental set up and very easy to automate. Because in immunoassays the components are usually added sequentially, FIA is predestinated for carrying out immunoassays. Whereas in conventional immunoassays the measurements are equilibrium based, in FIA non-equilibrium based measurements can be performed. The development of flow-injection immunoassays (FIIAs) therefore has attracted great attention in recent years.[1-4] A new variation of FIA, sequential injection analysis (SIA), introduced by Ruzicka and Marshall,[5] also found application in immunoassay.[6] While in FIA all components are injected into a flowing stream produced by a peristaltic pump, in SIA the different solutions are sequentially aspirated into the single reaction channel using a cam-driven sinusoidal flow piston pump in connection with a multiport-multiposition valve. SIA systems can be automated even more easily.

Both homogeneous and heterogeneous FIIAs have been developed. In homogeneous assays the signal has to be modulated during the formation of the antigen-antibody complex. For example, one of the earliest FIIA described uses a fluorescence energy transfer reaction.[7] Heterogeneous assays are more suitable for FIA, as the separation step can be carried out on-line in the FIA-system. Typically, a heterogeneous FIIA system consists of a peristaltic multichannel pump, an injection valve, the immunoreactor column, and the detector. The immunoreactor can be a small column or cell containing immobilized antibodies (or antigens, respectively, depending on the mode). As carriers polymers, glass beads, or more recently membranes or magnetic particles are used. The advantages of heterogeneous FIIAs is the higher sensitivity. Furthermore, no sample pretreatment is required, as the selective binding in the immunoreactor represents a preconcentration and clean-up step. Wash-

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ing of unbound components and regeneration is continuous. Heterogeneous FIIAs show extremely high binding kinetics. There is a very high surface-area-to-volume ratio in the immunoreactor. In contrast to static systems, in FIIAs the binding reactions are not based on passive diffusion; the flowing stream actively transports the sample to the immobilized antibodies (or antigens, respectively). Therefore, despite the sample's short residence time in the immunoreactor, binding is nearly quantitative.

Different modes are possible for heterogeneous FIIAs. Briefly, in the competitive assay sample antigens and labeled antigens compete for the limited binding sites on the immobilized antibodies. In the two-site sandwich assay, which is only applicable to large molecules such as proteins, the sample antigen binds to the immobilized antibodies in the first step. In the next step labeled antibodies are injected that bind to second binding sites of the sample antigen. Another mode is a "noncompetitive" assay for haptens, where an antigen is immobilized and a mixture of sample antigen with excess labeled antibodies is injected after preincubation. Excess antibody is captured on the immobilized antigen. Finally, there is the double antibody assay, which can also be carried out in the sandwich mode. In this case second antibodies, which are directed against the first antibodies, are labeled. In a modified form this approach can also be used for the determination of antibodies. Several variations of these basic formats are possible. Detection takes place either directly on the immunoreactor ("on column") or after the reactor in the flow-cell. Fluorescent dyes, chemiluminescence reagents, or enzymes are usually used as labels. When an enzymatic reaction is involved, the signal is drastically amplified. An amplification of sensitivity can also be obtained by involving the biotin-streptavidin binding system. Streptavidin has a high binding affinity to biotin. Because one molecule streptavidin can bind up to 12 acridinium ester molecules,[8] sensitivity can be significantly increased. Another advantage is the circumvention of direct labeling of antibodies with dyes, which may cause denaturation when antibodies are labeled to a high degree. Labeled streptavidin can be used as a universal reagent for different types of assays. Several biotinylated antibodies are commercially available or can be prepared easily with commercially available activated biotin labeling reagents.

There is a large choice of detection modes for FIA systems. Basically, all detection principles used in HPLC or in other dynamic systems can be used. For FIIAs, electrochemical, photometric, fluorimetric, and, more recently, chemiluminometric methods are usually employed.

Chemiluminescence detection shows several advantages. It requires only a very simple and not expensive set up. No lamp is needed, and therefore there is no interference from light scatter, resulting in a very low blank, and sensitivity is extremely high. Interferences from the matrix are minimized due to the high selectivity of CL reagents. Chemiluminescence detection has found broad application in HPLC and FIA due to its sensitivity and selectivity. The most commonly used chemiluminescence systems are the luminol, peroxyoxalate, acridinium, and firefly-luciferase systems.

The introduction of chemiluminescence labels in conventional immunoassay techniques significantly enhanced detection sensitivity. Among others, luminol,[9,10] isoluminol,[11] or acridinium derivatives[12] were used as labels.

II. STATE OF THE ART IN CHEMILUMINESCENCE FLOW-INJECTION AND SEQUENTIAL-INJECTION IMMUNOASSAYS

The first application of CL detection in a flow-injection system in connection with an immunoassay was described by Maeda and Tsuji.[13] The authors developed an enzyme immunoassay for α-fetoprotein, insulin, and 17-α-hydroxyprogesteron (17-OHP) using a flow-injection system with CL detection. Glucose oxidase is used as the labeling enzyme for anti-α-fetoprotein, insulin, and 17-OHP. However, because the immunoreactions are carried out off-line by overnight incubation of the components and only the CL reaction and detection is done by FIA, this assay cannot be regarded as a real flow-injection immunoassay. In the assay for α-fetoprotein the serum sample or standard is transferred to a bead coated with anti-α-fetoprotein and glucose oxidase conjugated anti-α-fetoprotein is added. Af-

ter overnight incubation, glucose is added as a substrate, and after a further incubation the reaction mixture is injected into the flow-injection system. The hydrogen peroxide formed is detected by CL after adding luminol and hexacyanoferrate (III). For the determination of insulin, the serum sample or standard, glucose oxidase conjugated insulin and insulin antibodies are added to a second antibody-coated bead. The CL detection is carried out in the same way as described for α-fetoprotein. Similary, an assay for 17-OHP is carried out.

Recently, authors from the same laboratory[14] reported the development of enzyme immunoassays for human choriongonadotropin (HCG) in urine using CL-FIA to detect the products formed. The method makes use of the CL reaction of cortisol with lucigenin. Alkaline phosphatase (ALP) is used as a label which cleaves cortisol-21-phosphate (C-21-P) to cortisol, which is detected by the lucigenin CL reaction. The immunoreactions and enzymatic reactions were again done off-line and only the final reaction mixture was injected into the FIA system. For the determination of HCG, the sample and anti-HCG-ALP conjugate are added to a microtiter plate coated with anti-HCG antibodies and incubated for 2 h. C-21-P is added and after 30 min the reaction mixture is injected into the flow system where the CL reaction is initiated by adding lucigenin-NaOH-cetyltrimethylammonium bromide solution. 17-OHP is determined by adding the sample, ALP labeled 17-OHP, and anti-17-OHP antibodies to a anti-rabbit-IgG coated microtiter plate. After overnight incubation, ALP activity is determined analogously to the HCG assay.

Osipov et al.[15] developed a two-site sandwich CL-FIIA for human-IgG using antibodies immobilized on a polystyrene bead and horseradish peroxidase (HRP)-labeled antibodies. The instrumental set up consists of a two-pump system with a double-valve injector. The assay is carried out by injecting the sample and peroxidase conjugated rabbit anti-human IgG through the double-valve injector. After mixing, the solution is transported to a reaction cell containing the polystyrene bead with the immobilized antibodies. Flow is stopped for 2 min for incubation. After washing, the bead is transported automatically to the detection cell where the CL produced by the addition of luminol, hydrogen peroxide, and iodophenol is measured. The total assay time is 10 to 15 min, and the LOD 10^{-9} *M*. In another paper[16] the same group described studies on the rate constant for this enhanced CL reaction of peroxidase with hydrogen peroxide and luminol using different enhancers. In addition to IgG this basic principle is applied to the development of a CL-FIIA for thyroxine T4. In the assay for T4, BSA-T4 conjugate is immobilized on a cyanobromide activated Sepharose column and peroxidase-labeled antibodies are used. Sample T4 and peroxidase-labeled antibodies are injected together through the two-channel injector. When the reaction zone of the sample and the conjugate reaches the centre of the loop, the pump is stopped for two minutes. Then the reaction zone is transferred to the column containing the immobilized T4, and the pump is again stopped for 2 min. The linear range of the calibration curve is 10^{-11} to 5×10^{-10} *M* with a LOD of 10^{-11} *M*. Furthermore, the authors propose a homogeneous assay for insulin, antibodies to insulin, and antibodies to trinitrophenyl group based on the enhanced CL reaction.

Shellum and Gübitz[17] developed a CL flow-injection immunoassay for mouse IgG with on-column detection using acridinium ester CL. All reactions, both the immunoreaction and the CL reaction, take place in a flow-cell consisting of a transparent 20 µl piece of Teflon® tubing packed with immobilized antibodies. This immunoreactor cell is placed in front of the PMT window of a Kratos fluorescence detector and the chemiluminescence signal is measured directly on the reactor cell. Sheep anti-mouse IgG is immobilized to a Pierce carbonyldiimidazole activated Trisacryl GF 2000. Anti-mouse antibodies are labeled with 4-(2-succinnimidyloxycarbonylethyl) phenyl-10-methylacridinium-9-carboxylate fluorosulfonate. A two-site sandwich assay for IgG is carried out by injecting sample IgG, which binds to the immobilized antibodies, in the first step. In the second step, acridinium-labeled antibodies are injected to form an immunosandwich. In the last step alkaline hydrogen peroxide is injected to initiate the CL reaction. Regeneration is carried out by washing with a pH 1.8 buffer. The sensitivity is shown to be dependent on the residence

time regulated by the flow rate. With a residence time of 15s a LOD of 500 amol (10^{-11} *M*) is obtained; with a 60 s residence time the LOD is 50 amol (2.5×10^{-12} *M*). The linear range is about two orders of magnitude. The within day RSD with manual injections is 4.9% and the interassay RSD is 5.9%. The total assay time is between 10 and 18 min, depending on the residence time.

A further increase in sensitivity is obtained when the biotin-streptavidin binding system is involved. In this case the antibodies are biotinylated and the streptavidin is labeled with acridinium ester. The detection limit was improved to 20 amol IgG/ injection by applying a residence time of 60 s.[18]

The same group also tested a competitive format.[19] For a competitive assay for human IgG, a reactor is used that contains a limited amount of immobilized antibodies and acridinium-labeled IgG. Sample and labeled antigens (human IgG) are injected together and compete for the limited binding sites on the immobilized antibodies. This mode is simpler and faster than the sandwich assay but not as sensitive. The detection limit for this mode was found to be 7 fmol. One assay cycle in a fully automated system takes 7 min, including regeneration. The intraassay reproducibility is 1 to 3% RSD, and the interassay reproducibility within 3 days below 4%. The authors have shown that this approach can be applied to serum samples without interferences. The specific binding of the sample antigens to the immunoreactor represents a clean up and preconcentration step and thus obviates sample pretreatment. The recovery from spiked serum samples was shown to be 103%. One immunoreactor could be used for about 50 injections over a period of 1 week. The reactor's activity was observed to decrease slightly after 3 days, which is not dramatic because with bioassays calibration is usually done every day.

Recently, two new applications of the competitive mode of this basic approach to small haptens such as digoxin and triiodothyronine (T3) were reported.[20] Two different instrumental configurations are compared in these studies: a normal FIA system and a modified sequential injection analysis (SIA) set up. The SIA system consisted of a Gilson syringe pump in combination with a Gilson liquid handler acting as an autosampler. On-column CL detection is carried out with a Jasco FP920 luminescence detector containing the flow cell packed with immobilized antibodies. While in the FIIA mode all components are injected into a flowing stream and are transported by the stream to the immunoreactor cell, in the SIIA mode the components are applied by sequentially injecting plugs directly into the reactor cell. The assay for digoxin is based on competitive binding of sample digoxin and biotinylated digoxin to the immobilized antidigoxin antibodies. Acridinium-labeled streptavidin binds to the biotin moiety and the chemiluminescence reaction is initiated by hydrogen peroxide. In the SIIA mode sample or digoxin standards and biotinylated digoxin are premixed by the autosampler and a plug is injected followed by plugs of acridinium-labeled streptavidin and alkaline hydrogen peroxide. Between the plugs of the components, plugs of buffer are injected. Regeneration is done by injecting a pH 2.2 glycine buffer. The total assay time in the SIIA mode is 8 min. A substantial advantage of the SI over the FI technique is the possibility of individually regulating injection volume and injection rate of every single component. Furthermore, the instrumental set up of an SIA system is simpler and is predestinated for automation. While the detection limits are comparable for the FIIA and SIIA modes (10 and 5 fmol/ injection, respectively, corresponding to 0.4 and 0.2 ng /ml), the precision is superior with the SIIA mode. The intraassay RSD for 2 ng/ml is 5.6% in the FIIA mode and 2.2 in the SIIA mode, the interassay RSDs within 3 days are 11.% and 5.5%, respectively. The recovery from serum samples spiked with 2 ng/ml is 90% with precision of 12%.

The T3 assay is similar, but does not involve the biotin-streptavidin binding system.[21] T3- and acridinium labeled T3 are injected together and compete for the binding sites on the immobilized antibodies. The CL reaction is again initiated by injecting hydrogen peroxide. The assay is carried out in both the FIIA and SIIA mode. One assay cycle in a fully automated SIA system takes 7 min. The detection limit for both systems is about 0.45 ng/ml. An attempt to enhance sensitivity by increasing the incubation time is described by implementing a flow-reversal step in the SIIA mode. Thus, after passing the immunreactor once,

the sample plug is moved back and forward again by changing the flow direction of the pump to enhance the contacts of the haptens with the immobilized antibodies. This slightly increases sensitivity but at the expense of precision. The authors have shown that the procedure is applicable to serum samples without any sample pretreatment.

Drawbacks associated with packed tubings of small diameter could be the formation of channels through the packing and light scattering might cause some loss in sensitivity.

These problems might be overcome by changing the geometrics of the flow cell. Work along this line was done by Liu et al.[22] They developed an interesting reactor system consisting of a thin-layer flow-cell in combination with a membrane. This flow cell is made by cutting a channel of 5 ul volume in a 0.1-cm-thick piece of Teflon®. The channel is covered by a Millipore Immobilison® membrane on which bovine IgG is immobilized. These parts are sandwiched between two transparent Plexiglas plates. The assembly is directly mounted in front of the PMT window to allow direct CL detection. The authors demonstrated the performance of this new approach by carrying out a sandwich assay for mouse anti-bovine IgG antibodies. The assay is done by injecting sequentially sample or standard mouse anti-bovine IgG, excess HRP labeled goat anti-mouse IgG followed by a mixture of luminol, hydrogen peroxide, and *p*-iodophenol. The CL signal is proportional to the amount of HRP-labeled antibodies and consequently to the amount of sample bound. The immunoreactor is regenerated by washing with a pH 2.2 buffer. After three regenerations the signal decreases by 20% and the membrane should be replaced. The total time for one assay cycle is 12 min. The LOD is 1 fmol with a linear range of 1 fmol to 3 pmol. The precision for spiked serum samples is 8.7% RSD.

Hage and Kao[23] used acridinium ester CL for the development of a sandwich FIIA for parathyroid hormone (PTH). The authors call this approach "high performance immunoaffinity chromatography", which is basically identical to an FIIA. The set up consists of three pumps with a valve-switching system. Antibodies to PTH are immobilized to diol-bonded Nucleosil and packed into 2.0 or 4.0 (i.d.) × 2.0 cm columns. To avoid interferences from different PTH-fragments, antibodies that bind one end of PTH at its 1-34 N-terminal region are used for immobilization and acridinium-labeled antibodies that bind a second portion, such as PTH's 44-68 midmolecular region. Sequential and simultaneous injection modes are compared. In the sequential injection mode PTH sample or standards are first injected and bind to the immobilized antibodies, followed by labeled antibodies that bind to second binding sites of PTH. The immuno complex is then disrupted by a pH 3 elution buffer and hydrogen peroxide/sodium hydroxide/Triton X-100 is added as a postcolumn reagent to initiate the CL reaction. In the simultaneous injection mode PTH sample or standards are mixed with labeled antibodies in the autosampler and incubated for 1 h. The immunocomplex formed is then injected automatically onto the immunoreactor column, where the immobilized antibodies extract it from other sample components and excess labeled antibodies. The sequential injection mode is simpler and faster; however, with the simultaneous mode a higher sensitivity can be obtained due to the longer incubation time. One assay cycle requires 6 min following 1 hour of incubation. A fully automated assay has been developed on this basis. The LOD obtained with the latter approach is 16 amol. The within –day precision for 10 repeated injections of spiked plasma samples containing 4.1, 13.3, 30.2, and 106.0 pmol/L was found to be ± 9. 3%, 3. 4%, 6.0%, and 10.2%, respectively. About 200 injections of clinical samples could be made with one and the same immunoreactor column.

Recently, Marquette et al.[24] described the development of a semiautomated flow immunosensor with CL detection for the determination of okadaic acid (OA) in mussels. The sample is injected together with horseradish peroxidase-labeled antibodies and are transported to a membrane containing immobilized OA. Sample OA competes with the immobilized OA for the binding sites of the labeled antibodies. Detection is carried out with the p-iodophenol enhanced luminol CL system. The detection limit was shown to be 0.2 μg/100 g mussel homogenate. The membrane was found to be stable for more than 30 measurements.

This basic principle was adapted also to the determination of 2,4-dichlorophenoxyacetic acid (2,4-D).[25]

In the same lab an assay for 2,4-D was developed using electrochemiluminescence.[26] In this case 2,4-D is immobilized on a glassy carbon electrode surface via a C6 spacer arm and the antibodies are labeled with luminol. The detection limit for 2,4-D obtained in this system is 0.2 µg/L.

An electrochemiluminescence FIIA for atrazine was published by Wilson et al.[27] Atrazine caproic acid is bound to a transparent amino silanized indium tin oxide-coated glass electrode via an amino dextran spacer. A mixture of sample and glucose oxidase-labeled antibodies is injected followed by a solution consisting of glucose as a substrate and luminol to produce chemiluminescence with the hydrogen peroxide formed. One assay cycle takes about 20 min. With this technique it is possible to detect less than 0.1 ppb atrazine in drinking water. The authors propose the development of multianalyte immunoassays by using an electrode array with different individual electrodes.

DeLavalle and Grayeski[28] observed that the chemiluminescence signal from an aqueous peroxyoxalate CL reaction can be enhanced in the presence of hydrophobic proteins. Based on this phenomenon, they developed a method for the determination of albumin and IgG in human serum using 4,4'-oxalylbis (trifluoromethylsulfonylimino)ethylene *bis* (4-methyl morpholinium trifluoromethane sulfonate), 8-anilino-1-naphthalene sulfonic acid, and hydrogen peroxide as components for the CL reaction. The authors suggested that this basic principle could be a potential tool for carrying out FIIAs.

III. DISCUSSION

The power of FIIA is certainly its flexibility and speed for single assays. The system can be adapted very quickly to different analytes and types of assays. This is advantageous if rapid results are required for different analytes and varying problems. Of course, the sample throughput of the existing prototypes of FIIA and SIIA systems is not comparable with that of automated random access carrousels. A solution to increase sample throughput in FIA systems certainly would be the development of multichannel systems to process several parallel runs. Compared with robotic systems, automation of such FIA systems is much easier and less cost intensive. SIA in particular would readily lend itself to automation.

A particular advantage of heterogeneous FIIAs and SIIAs is that biological samples can be applied without any sample pretreatment due to the specific binding of the sample in the immunoreactor. Unwanted components are washed off. The binding in the immunoreactor results at the same time in a concentration of the sample. Contrary to static systems, unspecific binding is very low in dynamic systems.

The major drawback of heterogeneous FIIAs is the limited lifetime of the immunoreactor containing immobilized antibodies. To overcome this drawback, Palmer et al.[29,30] used a protein A reactor and loaded it with fresh antibodies before each assay. Stöcklein and Schmid[31] used a membrane as immunoreactor that was exchanged automatically after each assay. Another alternative described by several groups is the use of magnetic particles to immobilize antibodies that are also exchanged automatically with an electromagnet.[31-33] Pollema and Ruzicka introduced a flow-injection renewable surface immunoassay (FIRSI).[34] In this approach beads containing immobilized antibodies are retained in a "jet-ring cell", where detection takes place and are released after the assay.

Recent trends are miniaturization of FIA systems with the goal of reducing reagent consumption, decrease of waste production and increase sensitivity and sample throughput.

A new challenging field is certainly the development of microfabricated FIA manifolds.[35] An interesting alternative to the use of pumps in such µ-FIA manifolds is electroosmotic flow generation.[35,36] The development of an electrophoretic micro-chip immunoassay has been described recently by Koutny et al.[37]

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