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Detection methods of microsphere based single-step bioaffinity and in vitro diagnostics assays

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Abstract

Microspheres provide a solid phase substrate for bioaffinity binding similar to the walls of traditional test tubes and the wells of microtiter plates. The coated microsphere concentrates analyte molecules in the reaction volume on its surface. When the bioaffinity binding reaction has reached an equilibrium, the local concentration of the analyte in close proximity of the microsphere is orders of magnitude higher than the concentration of the analyte in the total reaction volume. The preparation and quality control of microspheres coated with bioactive material is less costly and labour intensive when compared to test tube or microwell plate coating procedures. In addition, the cost for logistics and transportation of microsphere reagents is lower than that of coated tubes or plates. Moreover, microspheres can be easily used in miniaturised assay formats and several different detection schemes can be employed in the measurement of microsphere-based assays. Several different types of microspheres are commercially available. The microspheres can be manufactured in different sizes from many materials, such as polystyrene, acrylate, and glass. The surface of the microspheres can be activated to enable covalent binding of biomolecules. Further, the microspheres may contain internal fluorochrome or magnetic material, for identification or separation purposes. In this paper we review different assay formats for single-step measurement of bioaffinity assays emplying microspheres. The term single-step is used to describe assays where all reagents and the sample are mixed, incubated and measured without separate washing steps.

Keywords: Diagnostic assays; Microsphere based bioaffinity; Biomolecules

1. Flow cytometry-based bioaffinity assay concepts

Optical flow cytometers are designed for characterization of different cell types, based on specific fluorochrome labelling of cell antigens. Suggestion to use flow cytometer and microspheres of specific biological binding activity for detection of analytes in serum or other fluids was made already in the mid-1970s [1–3]. Since then, a multitude of flow fluorometric single analyte assays have been developed for the analysis of serum antibodies. The flow cytometer has the capability to discriminate particles on the basis of both the size and the colour of the particle, thus giving the possibility for multiplexed measurement of several analytes simultaneously.

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The use of microspheres of several size categories in the simultaneous analysis of several analytes was proposed by Horan and coworkers [2,4,5]. This concept was later demonstrated in the detection of two antibodies, antibodies to cytomegalovirus and herpes simplex virus, and later in the detection of antibodies to four different HIV-virus antibodies [6–10].

The Copalis™ system (Sienna Biotech, Inc., Columbia, MD, USA) differentiates among monomeric latex microspheres in a flow cytometer on the basis of the light-scattering signal. The concentration of analyte is also measured by scattering and is proportional to the broadening of the microsphere scattering signal distribution. This broadening occurs due to binding of colloidal gold tracer particles on the microsphere surface [11–14]. Benecky et al. have detected up to four viral antibodies using microspheres of different sizes ranging from 1.1 to 1.9 μm.

Multiplexed assays have also been commercialised, and the multiplexing capacity of the instrumentation have been expanded to cover even more analytes. An example of such a system is that made by the Luminex Corporation (Austin, TX, USA.) [15–17]. This system originally named FlowMetrix™ and later LabMAP™, is based on the use of microsphere subclasses, each having a unique combination of two internal identification fluorophore concentrations. The system discriminates among microsphere subclasses on the basis of two longer wavelength fluorescence identification signals (orange and red) leaving the third shorter wavelength fluorescence signal (green) for the determination of the bioaffinity reaction.

This method involves a risk that the identification signals of two or more microspheres will overlap. The microspheres used in the LabMAP™ system have been carefully selected to have low variance in the size [16]. While there exists small heterogeneities even in the sets of selected microspheres [18], the side-scattering signal is carefully analysed for the determination of the correction factor for the fluorescence identification signals.

According to publications of Luminex Corp., the LabMAP™ technology allows for fast, separation free measurement of tens of multiplexed analytes. The sample volume can be as small as 10–50 μl. However, as is typical for flow cytometer based analysis concepts, the sample is lost during the measurement, thus measurement of real time reaction kinetics is not easily done. The LabMAP™ system and reagents are now available through different companies, including Qiagen GmbH (Hilden, Germany), and Bio-Rad Laboratories (Hercules, CA, USA).

Approaches similar to the LabMAP™ system have later been introduced by other manufacturers, for example, by Becton–Dickinson Biosciences under the trademark CBA, Cytometric Bead Array (BD PharMingen, San Diego, CA, USA). The Cytometric Bead Array system is based on the use of the FACSCalibur flow cytometer. BD PharMingen has developed a multiplexed assay for IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ with, according to the commercial sales material, assay sensitivities of 6.6, 6.5, 2.8, 4.7, 4.3 and 15.6 pg/ml, respectively. However, these results were not obtained using single-step assay procedure, but a protocol that included intermediate washing of excess unbound tracer prior to measurement.

The measurement of electrolytes in patient serum is of great importance in routine clinical chemistry. Recently, fluorophores that display ion selectivity has been incorporated into microspheres and measured in flow cytometry [19]. This allow assay miniaturisation, while ion specific electrodes are not required. Bakker's group has developed ion selective microspheres for assaying of Na^+, K^+ and Pb^{2+} ions using flow cytometry [19,20].

2. Scintillation proximity assay

The Scintillation Proximity Assay, SPA, uses a radioisotope as an energy donor to microspheres having a scintillant-coated surface [21,22]. In the typical SP-assay, tritiated ligand molecules bind to the surface of the scintillating microspheres (Fig. 1). The scintillant microspheres emit detectable photons under bombardment of beta-radiation that is originating from the close proximity of the microspheres. Since beta-radiation is quickly absorbed by the aqueous assay buffer, radiation originating from unbound ligand molecules has a very low probability of reaching the microspheres.

The advantage of SPA is, as with all radioimmunoassays using tritium labeling, that the binding properties of the labelled reagent (ligand, etc.) remain unaltered by the labeling. The SPA concept is in wide use within high-throughput screening in drug

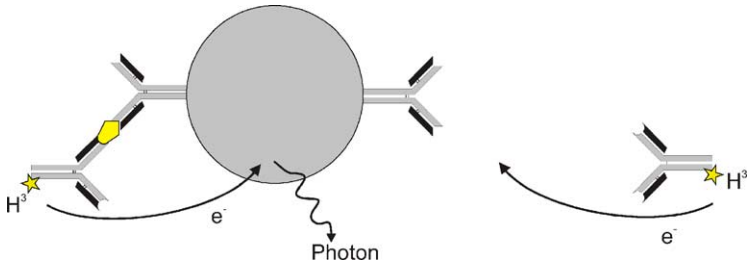

Fig. 1. Tritiated tracer antibodies emit beta-radiation that is absorbed either by the assay buffer for unbound tracer molecules (right) or by a scintillant nanoparticle for bound tracer molecules (left). Upon absorption of beta-radiation, the scintillant nanoparticle emits a photon in the visible or UV-range.

discovery, where studies of ligand–receptor interactions are common [23–27].

3. Electrochemiluminescence

Electrochemiluminescence (ECL) is one of the most sensitive means for detection of bioanalytes. Electrochemiluminescence is generated electrochemically on the surface of an electrode. The system is stable and has a reported sensitivity down to 200 fmol/l and a dynamical range up to six-orders of magnitude [28,29]. Of several existing electrochemiluminescent chemical compounds, the organometallic complexes of Ruthenium and Osmium are most commonly used [28,30].

Blackburn et al. have devised an immunometric and competitive ECL assays, where magnetic microspheres are used as the solid phase for the bioaffinity reaction. Ruthenium(II) tris(bipyridyl), $Ru(bpy)_3^{2+}$, (Fig. 2), was used as the labelling reagent to develope immunoassays for the detection of TSH, AFP, CEA, and digoxin, as well as hybridisation assays for post-PCR detection of viral genes and oncogenes [29,31–33].

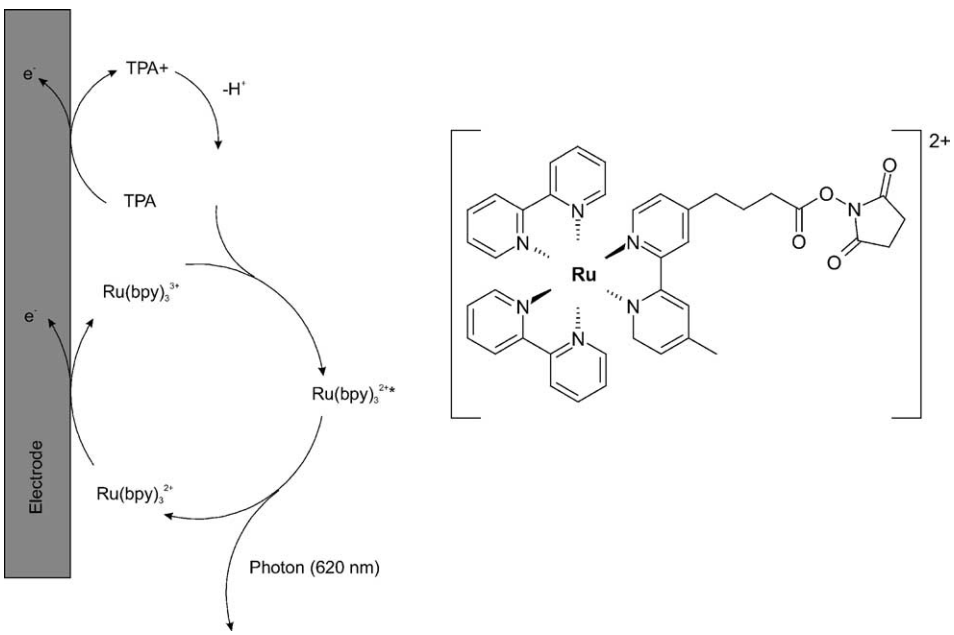

Fig. 2. States of the Origen and Elecsys ECL-system (left) and the structure of the ruthenium(II) tris(bipyridyl) labelling reagent.

Electrochemiluminescence has been commercialised by Igen, Inc. (Origen™, Igen, Inc., Gathersburg, MD, USA) for research applications and Roche Diagnostics GmbH (Elecsys™, Roche Diagnostics GmbH, Mannheim, Germany) for clinical diagnostics. The Origen™ ECL-technology is based on the use of magnetic microspheres. This enables magnetic concentration of microspheres on the surface of an electrode and a quick dilution of unbound tracer molecules by simultaneously washing the surrounding measurement volume by continuous flow of assay buffer. Thus, the method is limited to a single-point measurement of a given sample of microspheres. Origen™ has the benefit of low instrumental and assay background noise, while no optical excitation of luminescence is needed. Thus, Origen™ is one of the most sensitive detection methods available for bioaffinity measurements. Assay sensitivity can be enhanced by increasing the sample reaction volume. The ECL-electrode suffers from wear and must be calibrated and replaced regularly.

4. Chemiluminescence

The luminescent oxygen channelling immunoassay (LOCI(tm), Dade Behring, Inc., Deerfield, Illinois, USA), was introduced by Ullman and co-workers [34–37] and later registered also as the “Amplified Luminescent Proximity Homogeneous Assay”, the AlphaScreen(tm) technology (Packard Instrument Company, Meriden, CT, USA). The LOCI technology is based on the use of nanometer scale latex particles similar to those that are used in existing particle aggregation or agglutination assays. The method is targeted to single-step bioaffinity assays in order to overcome the sensitivity problems of the existing assays, where measurement of light scattering by turbidometry or nephelometry is used for detection.

The luminescent oxygen channelling immunoassay monitors the formation of latex particle pairs by chemiluminescence [34]. One of the particles has a photosensitiser dye and an antenna dye. Upon illumination with laser light at 680 nm, a photosensitiser in the donor bead converts ambient oxygen to the singlet-state oxygen radical, $^1\Delta_gO_2$. The excited singlet-state oxygen molecules are ejected out of the donor particle and diffuse maximally about 250 nm (one bead diameter) before rapidly decaying. If the acceptor bead is in close proximity of the donor bead, by virtue of a biological interaction, the singlet-state oxygen molecules react with chemiluminescent olefin groups dissolved in the acceptor beads. This chemiluminescence reaction transfers energy to fluorescent acceptors in the same bead. These fluorescent acceptors shift the emission wavelength from 520 to 620 nm. The whole reaction has a 0.3 s half-life of decay and can be measured in a time-resolved mode, by switching of the illuminating laser before measurement of fluorescence. The assay principle is presented in Fig. 3.

Due to time-resolved measurement and the upconversion of the emission light, the luminescent oxygen channelling immunoassay clearly has a great

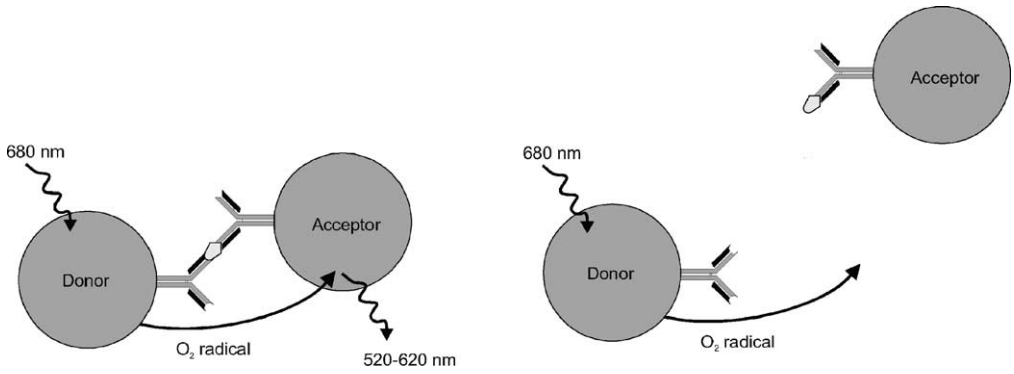

Fig. 3. The principle of the LOCI assay. A donor nanoparticle converts the 680 nm illumination light to a single oxygen radical, which is absorbed by a bound acceptor nanoparticle and a photon is emitted (left). Whereas, the ejected oxygen radical oxidises solvent molecules if no bound acceptor nanoparticle is in the vicinity (right), and thus, decays rapidly.

potential in terms of sensitivity and miniaturisation and can, in principle, be used in real time monitoring of bioaffinity binding kinetics. The time resolution of kinetic measurement is, however, limited by the 0.3 s lifetime of oxygen radicals.

Ullman's approach has further been developed by Roelant [38,39]. Similar to Ullman's approach, Roelant's proposal is also based on the transfer of energy via diffusion of a singlet oxygen radical. However, in the latter case the donor of the singlet oxygen is a single proto-porphyrin molecule. Thus, the concept enables small molecule labelling of the bioactive compounds. The concept is marketed as the Constant-Quanta^{TM} technology by the Packard Instrument Company (Meriden, CT, USA).

5. Confocal microscopy techniques

Confocal microscopy can be used to enhance the signal to background ratio of the fluorescence detection. One major sources of background in homogenous microsphere based immunoassays is the fluorescence emission from free unbound tracer molecules. Confocal microscope set-up allows collection of emitted fluorescence photons only from reduced focal volume element of the microscope. Thus, the background caused by free unbound tracer molecules outside the focal volume is suppressed and the measurement of homogenous microsphere and cell based immunoassays is possible with high sensitivity.

The volumetric capillary cytometer is a scanning fluorescence detection system developed by Biometric Imaging, Inc. (Mountain View, CA, USA) [40,41]. The instrument utilises a He:Ne-laser for fluorescence excitation. The optical set-up is macro-confocal, where the half-width of the laser focus is about 8 μm laterally and 100 μm axially (Fig. 4). The laser is used to excite the CY5 and CY5.5 fluorochromes at a wavelength of 633 nm, and the fluorescence emission in the wavelength ranges 654–680 and 680–730 nm is detected using two photomultiplier tubes. The total area that is imaged is around 1 mm^2. There is a capability to automatically focus on the inner bottom surface of the reaction chamber. Besides the measurements in capillaries, the instrument has been adapted for imaging

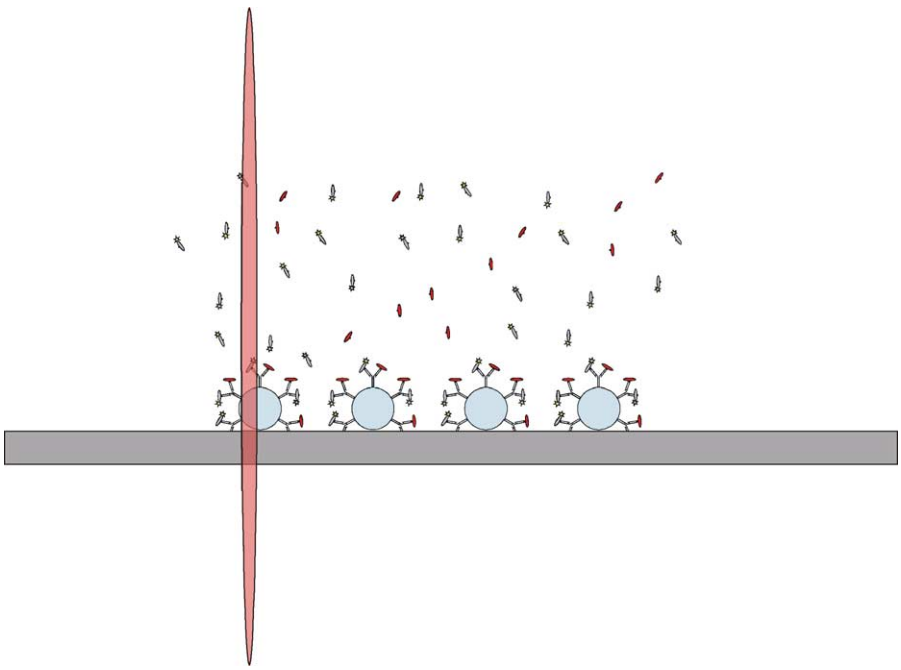

Fig. 4. Detection principle of volumetric capillary cytometry. The bottom surface of the reaction cuvette is imaged using laser scanning confocal microscope. The system has an auto-focus set-up to focus on the inner bottom surface. The half-width of the laser focus is about 8 μm laterally and 100 μm axially.

signals from standard 96, 384 and 864-well microtiter plates [42]. The instrument is commercialised under the trademark IMAGN® 2000 by Biometric Imaging and as FMAT system (Applied Biosystems, Foster City, CA, USA). Several high throughput screening assays for the volumetric capillary cytometer have been developed.

Swartzman et al. demonstrated multiplexed immunometric IL-6 and IL-8 assays [43]. The assay reached a sensitivity of 100 and 10 pg/ml for IL-8 and IL-6, respectively, both assays having a dynamic range of ∼2 log units. The assay was based on the use of 6 μm diameter polystyrene microspheres.

The same group has demonstrated a multiplexed competitive cell-based assays used to determine receptor-ligand interactions of ligands for two different seven-transmembrane receptors [44]: the ligands Substance P and Neurokinin A for the neuronal tachykinin receptors and neuropeptide Galanin for receptor subtypes GalR1 and GalR2. The sensitivities of the cell-based assays reached 0.01 nM having a dynamical range of ∼2 log units. This technology is marketed as the FMAT-technology by the Applera Corp. (Norwalk, CT, USA).

Martens et al. from the Affymax Research Institute (Palo Alto, CA, USA) demonstrated a multiplexed competitive assay for the receptors IL-1 and IL-5. The detection sensitivities of these assays where around 0.01 nM and the dynamical range >2 log units [45].

The third group utilising the same approach is the group of Zuck [46], from Pharmacopeia, Inc. (Princeton, NJ, USA). Zuck et al. show a dual analyte assay for leptin and IL-6 and a single analyte phosphatase enzyme activity assay.

All of these above mentioned assays were either single or dual analyte assays. The assays have been shown to function in microplate formats down to 16 μl reaction volume. The macro-confocal approach allows sensitive and relatively quick measurement of both bead- and cell-based assays and has high potential value. The benefits of an elongated focus is the capability to position the focal measurement volume directly on and above the optically clear cuvette bottom and, thus, sensitive measurement of particles or cells in close vicinity to bottom surface is possible, when the free unbound label concentration is low. The use of fluorochromes that are excitable with a red laser and emitting in deep red wavelengths further reduces scattering and autofluorescence background noise. The multiplexing capability of the set-up can be extended to imaging particles of several sizes, but the measurement of microspheres within the solution phase of the assay would probably not reveal as good a signal to noise ratio and sensitivity.

Recently, the nanoparticle counting concept, the nanoparticle immunoassay (NPIA), has been used to enhance the signal to background noise ratio of the bioaffinity assay [47]. In this assay concept, the nanoparticles are used to concentrate the analyte on the particle surface. Measurement is performed directly in the reaction volume using confocal microscopy. Additionally, a fluorescence intensity distribution analysis (FIDA) technique is applied to improve the assay performance [48].

6. The two-photon excitation fluorescence—TPX-technology

In the TPX-technology [49,50], uniform microspheres of around 3 μm diameter are functioning as the solid phase for bioaffinity reaction. The TPX-measurement device is a microfluorometer that is capable of measuring the fluorescence signal from individual microspheres [51]. The optical set-up can precisely discriminate the fluorescence signal of tracer molecules that are bound by bioaffinity reaction to microsphere surface from the unbound tracer molecules in solution. This is achieved by limiting the fluorescence excitation to a focal volume of ∼1 fl, i.e. about the volume of a single microsphere, and by activating the fluorescence measurement only when a microsphere is within the focal volume (Fig. 5). The limited volume of the fluorescence excitation is due to the two-photon excitation of fluorescence, where the half-width of the focal volume is around 0.7 μm laterally and 4 μm axially. The microspheres are traced by optical scanning from a total measurement volume of ∼10 nl. Thus, the optical set-up of the TPX-fluorometer enables measurement of bioaffinity assays without physically separating or washing the unbound tracer molecules from the bound molecules on the microsphere surface. Furthermore, the measurement can be made in microvolumes and microcavities. The method has been applied, for example,

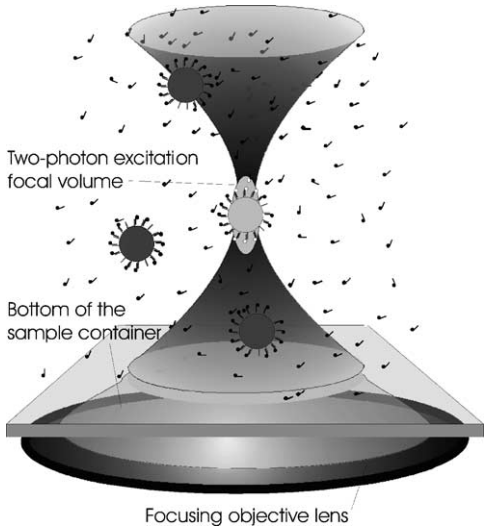

Fig. 5. Fluorescence excitation occur only within the limited two-photon excitation focal volume. Fluorophores residing out of the focal volume do not contribute to the measured signal.

to measurement of C-reactive protein immunoassay [52].

7. Time-resolved microfluorometry

An approach for the detection of bioanalytes by utilising time-resolved fluorometry together with lanthanide chelate labels and polystyrene microspheres of 50–100 μm diameter has been demonstrated by Hakala et al. [53] and Lövgren et al. [54]. Lövgren et al. devised a special time-resolved fluorescence microfluorometer for use in microsphere based bioaffinity assays [54]. The instrument has been used for rapid detection of prostate specific antigen (PSA), C-reactive protein (CRP), and thyrotropin (TSH) immunoassays [54,55]. The assays use microspheres of 50–60 μm diameter as the solid phase for bioaffinity binding reaction. The results show excellent sensitivity that is around 10 pg/ml for PSA from an ∼1 μl reaction volume.

Hakala et al. has used the same instrumentation and porous of 50 μm [56]. The first experiments were made with single analyte hybridisation assays, but later expanded to multiplexed hybridisation assays and to immunoassays [56,57]. The categorisation of microspheres for multiparameter assays was done by applying several concentrations of dansyl and fluorescein dyes [56]. The multiparameter measurements show a dynamical range of three orders of magnitude and crosstalk of ∼1% between the different parameters.

The total signal yield in measurements of long decay time fluorochrome labels typically suffer from the limited excitation frequency. This limitation is due to the availability and cost of suitable pulsed light sources for the excitation wavelengths and due to the practical upper limit of the excitation pulse frequency that is in the range of the fluorochrome decay time half life. The lanthanide chelates and metalloporphyrin compounds that have been used have typical decay times in the range 100–1000 μs.

Time-resolved microfluorometry is a potential method for miniaturised multiplexed bioaffinity assays, however, all the assays mentioned above were performed using wash steps prior to measurement.

8. Conclusions

New microsphere based assay applications are continuously developed, for both, in vitro diagnostics and bioanalytical research applications. Due to possibility for assay manufacturing in batch processes, the microspheres bring an economical benefit in production, when compared to traditional coated tube technology. Further, the microspheres allow miniaturization of assay reaction volumes and measurement of multiplexed simultaneous analytes.

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