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Enzymatic Growth of Metal and Semiconductor Nanoparticles in Bioanalysis

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In the recent past, the application of metal and semiconductor nanoparticles (NPs) has gained attention in analytical chemistry for various advantages over other analytical tools. Most frequently nanoparticles are extensively employed as labels in optical and electrochemical assays. They also are utilized as scaffolds for assembling of small molecules and macromolecules. The conventional approach consists of synthesis and modification of nanoparticles with recognition elements followed by their application to an analytical assay. Another approach relies on the synthesis of nanoparticles in situ triggered by a recognition event. The enzymatic synthesis of NPs in situ allows to address shortcomings related with the nonspecific adsorption of pre-synthesized NPs on surfaces and improve significantly the signal to noise ratio improving significantly sensitivity of analytical assays. This review deals with conventional and unconventional applications of metal and semiconductor to analytical chemistry.

1. Introduction

Bioanalytical assays serve to detect analytes of interest using biological components, such as bacteria or biological products, enzymes, antibodies, proteins, DNA and RNA oligonucleotides, oligosaccharides, and so on. These biological components specifically recognize their target through affinity interactions and enzymatic reactions. The event of recognition is followed by transduction to yield a measurable signal. In most cases, the initial signal is so weak that it needs appropriate amplification. The amplified signal can be measured employing UV–vis and fluorescence spectroscopy, surface plasmon resonance, Raman spectroscopy, quartz crystal microbalance, electrochemical methods, etc.

Nanoparticles (NPs) of different nature, for instance, semiconductor and metal NPs are very broadly utilized as labels to read out biorecognition events by optical, electrochemical, and other physical methods.[1] Amplified surface plasmon-resonance imaging of complexes with biological molecules is based on the coupling of a localized plasmon with the surface plasmon wave on gold surfaces.[2] In most cases, pre-synthesized metal NPs were linked to recognition elements proteins, polysaccharides, antibodies, RNA and DNA aptamers, DNA oligonucleotides, which have affinity for respective target analyte molecules such as proteins, DNA fragments, small organic and inorganic molecules, cations and anions and so on.[3,4]

The key advantage of semiconductor NPs is their intrinsic capacity to become photoexcited to produce electron/hole couples, which can recombine to yield fluorescent emission of light. The size, nature, and environment of semiconductor NPs define the wavelength and intensity of emitted light.[3] The emission of light by these NPs is explained by quantum effects, therefore they are referred to in the literature as quantum dots (QDs).

2. Conventional Applications of Nanoparticles to Bioanalysis

Professor A. P. Alivisatos (University of California, Berkeley, USA) pioneered study of semiconductor NPs. He managed to synthesize and derivatize semiconductor NPs.[5,6] His group reported self-assembled monolayers of semiconductor NPs on metal surfaces.[7] His laboratory developed protocols to synthesize highly stable and luminescent CdSe/CdS core/shell nanocrystals.[8] They found experimental conditions for the synthesis of rod, arrow, tetrapod-shaped CdSe nanocrystals,[9] and semiconductor nanorod liquid crystals.[10] They described conjugation of DNA to semiconductor QDs[11] and sorting flourescent NPs with immobilized oligonucleotides.[12] The most important advantages of QDs over organic fluorophores are higher brightness, reduced photobleaching, and longer lifetimes.[13] QDs have been generally used as fluorescent labels in biosensing, especially in assays based on detection of analytes by affinity binding.

The research group of professor Hedi Mattoussi (Optical Sciences Division and Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, Washington) used QDs as labels very extensively. They described conjugating protein molecules to luminescent CdSe–ZnS QDs for use as bioactive fluorescent probes.[14] They also reported conjugation of QDs with antibodies through an engineered protein,[15] through the use of an avidin bridge adsorbed to the surface of CdSe–ZnS core–shell QDs,[16] and QD–biotin conjugates.[17] This group found out that luminescent QDs act as acceptors in fluorescence resonance energy transfer (FRET) assays with organic dye donors,[18] and utilized this phenomenon to develop

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solution-phase sensing assemblies for the specific detection of the explosive 2,4,6-trinitrotoluene.[19] Assemblies for detection of protease activities were constructed by linking CdSe-ZnS QDs and metal complexes[20] or red fluorescent protein.[21] They demonstrated the simultaneous detection of cholera toxin, ricin, shiga-like toxin, and staphylococcal enterotoxin B from a single sample using conjugates made with highly luminescent semiconductor nanocrystals (CdSe–ZnS core–shell QDs) and antibodies.[22]

The laboratory of professor Wolfgang J. Parak (Fachbereich Physik and WZMW, Philipps Universität Marburg, Germany) also contributed to development of methods for the synthesis and modification of QDs. His group described strategy allowing for transferring hydrophobically capped CdSe/ZnS nanocrystals from organic to aqueous solution by wrapping an amphiphilic polymer around the particles.[23] They studied cytotoxicity of CdSe and CdSe/ZnS NPs with different surface modifications such as pre-treatment with mercaptopropionic acid, silanization, and polymer coating.[24] They reported procedure to grow PbSe nanocrystals on the tips of colloidal CdS and CdSe nanorods.[25] They synthesized and characterized CdTe nanocrystals.[26,27] They showed that the photocurrent generated at CdSe/ZnS quantum dot-modified electrodes can be modulated by NADH and glucose biosensors employing glucose dehydrogenase can be built on this principle.[28]

The laboratory of professor Uri Banin (Institute of Chemistry and the Center for Nanoscience and Nanotechnology, The Hebrew University, Jerusalem, Israel) is another important player in the field of the synthesis and characterization of bright and stable QDs. This group studied the advantageous optical gain characteristics of CdSe/ZnS core/shell colloidal quantum rods and compared the gain properties with spherical QDs.[29] They applied near-field scanning optical microscopy, along with time-resolving capabilities, to produce optical imaging and spectroscopy measurements of single-semiconductor nanocrystals.[30] They probed with scanning tunneling spectroscopy the electronic level structure of InAs QDs in 2D arrays.[31] They embedded InAs and CdSe QDs in a thin polymer and performed electrical transport measurements through single simeconductor nanocrystals.[32] They studied growth of gold onto CdSe-seeded CdS nanorods and identified preferential growth of gold onto the position of CdSe seeds.[33] Then, they discovered synergetic properties arising from the coupling between the two materials in terms of the second harmonic generation in CdSe-Au hybrid NPs.[34]

Bakalova et al.[35] (National Institute for Advanced Industrial Science and Technology, Hayashi-cho, Takamatsu, Japan) introduced QDs as photosensitizers in photodynamic therapy of cancer. They treated leukaemia cells with soluble CdSe nanocrystals conjugated with anti-CD antibody to demonstrate that quantum dot anti-CD conjugates sensitized leukemia cells to UV irradiation.[35] They reported quantum-dot-based Western blotting protocols for ultrasensitive detection of "tracer" proteins in cell lysates using biotinylated QDs crosslinked with primary biotinylated antibody through avidin.[36] They described fabrication of QD hybridization probes for the selection of target-specific siRNA sequences.[37] They produced highly bright and stable fluorescent QDs (CdSe, CdSe/ZnS, or CdSe/ZnSe/ZnS) by hydrophobic silica precursors and an extension of the silica layer to form a silica shell around them.[38] They synthesized uncoated, broad fluorescent, size-homogeneous water-soluble CdSe QDS in the absence of the most popular coordinating ligands. The obtained CdSe core QDs were further conjugated with antibodies and applied in biochemical analyses.[39]

Valeri Pavlov obtained his Ph.D. degree in chemical engineering in January 2005 from the University Rovira I Virgili (Spain). He worked in the Hebrew University of Jerusalem (Israel) in the group of professor Itamar Willner as a postdoctoral researcher. In October 2005, he began conducting postdoctoral study at the Chemistry Department of the University of Heidelberg, Germany. Then, in February 2007, he joined the new research institute CIC BiomaGUNE in San Sebastian as a group leader. His research interests include enzymatic generation of metal and semiconductor nanoparticles, production of new recombinant mutated enzymes, and optical bioanalytical assays.

The group headed by professor Arben Merkoçi (Catalan Institute of Nanotechnology, Barcelona, Spain) also took part in development of biosensors based on NPs and QDs. They described novel protocol for detection of DNA hybridization using a magnetically trigged direct electrochemical detection of gold quantum dot tracers.[40] They reported electrochemical detection of cadmium sulfide QDs (CdS QDs)–DNA complex connected to paramagnetic microbeads without the need for chemical dissolving,[41] and DNA hybridization assays based on the use of different quantum dot tracers for the simultaneous electrochemical measurements of multiple DNA targets.[42]

Professor Joseph Wang (Department of Nanoengineering, University of California San Diego, La Jolla, USA) employed QDs as labels in electrochemical biosensors. He designed a bioelectronic method for coding single nucleotide polymorphisms based on the use of different encoding nanocrystals such as ZnS, CdS, PbS, and CuS. The recognition events were amplified by the stripping voltammetric technique.[43] He developed the multi-analyte electrochemical assay in which a self-assembled monolayer of several thiolated aptamers conjugated to proteins carrying CdS and ZnS NPs was displaced by analytes competing for these immobilized aptamers. Electrochemical stripping was utilized to detect nondisplaced nanocrystal tracers.[44] His group presented the biosensor for glycan markers of diseases. This novel methodology relied on immobilization of lectin, followed by the competition between a CdS-tagged sugar and the target sugar for the binding sites on the modified surface. Electrochemical detection of the captured CdS NPs was perfomed.[45] Wang's group described electrochemical detection of DNA hybridization. Analyte DNA was captured on the

gold surface pre-modified with a monolayer of probe DNA. CdS NPs bound on a secondary oligonucleotide were dissolved and detected with cadmium-selective microelectrodes.[46]

The group of Dr. Yuehe Lin (Pacific Norwest National Laboratory, Richland, Washington, USA) developed electrochemical and fluorescent bioanalytical assays based on conjugates of QDs with antibodies. He described QD-based electrochemical immunoassay of Interleukin-1a,[47] magnetic electrochemical immunoassays with QD labels for detection of phosphorylated acetylcholinesterase in plasma,[48] electrochemical immunoassay for screening of the prostate-specific antigen with QDs labels,[49] immunochromatographic fluorescent biosensor for detection of trichloropyridinol with QD nanocrystals,[50] electrochemical immunoassay for the detection of phosphorylated serum albumin employing CdSe/ZnS QDs.[51]

3. Growth of Metal NPs Modulated by Enzymes

The above-mentioned analytical NPs-based assays relied on pre-synthesized NPs, which could be split into two subsets of methods: assays based on NPs decorated with recognition elements, for detection of affinity interactions, and assays for enzymatic activities, employing donor/quencher fluorescence resonance energy transfer (FRET) pairs in which one of the elements is an NP. However, such assay systems frequently are limited by high background signals caused by nonspecific adsorption of decorated NPs on surfaces or poor quenching of donor couples. Generation of NPs in situ can address these drawbacks of relevant analytical systems by decreasing the background signal. The seminal article published by the group of Willner (The Hebrew University of Jerusalem)[52] demonstrated that the reduced form of the well-known enzymatic cofactor nicotinamide adenine dinucleotide (NADH) is able to reduce $AuCl_4^-$ ions to yield Au^I cations, which are finally converted to Au^0 according to the following reactions:

$$AuCl_4^- + NADH \rightarrow Au^I + 4Cl^- + NAD^+ + H^+$$

$$2Au^I + NADH \rightarrow 2Au^0 + NAD^+ + H^+$$

Resulting Au^0 ions enlarge seeding Au NPs in solutions and on the surface of glass slides in the presence of cetyl trimethylammonium bromide (CTAB) surfactant (**Figure 1**). The changes in the concentration and shape of Au NPs can be followed by UV–vis spectroscopy. The enlargement of Au NP seeds by NADH/$AuCl_4^-$ system detected by optical methods can be applied to follow biocatalytic processes catalyzed by NAD^+-dependent dehydrogenases, for example, lactate dehydrogenaase according to **Scheme 1**.

NADH can reduce Cu^{2+} to yield metallic copper NP:[53]

$$NADH + Cu^{II} \rightarrow NAD^+ + H^+ + Cu^0$$

Cu^0 atoms readily enlarge Au seeds immobilized in indium tin oxide (ITO) electrodes as one can see in **Figure 2**. Growth of copper

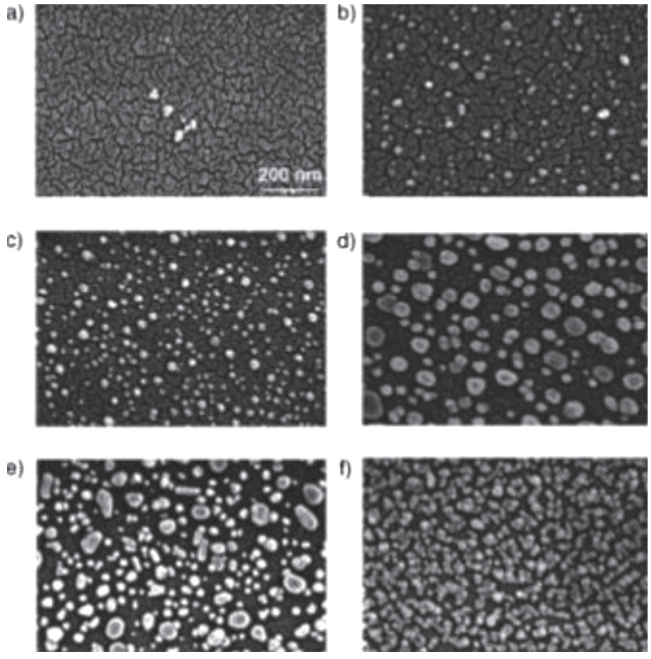
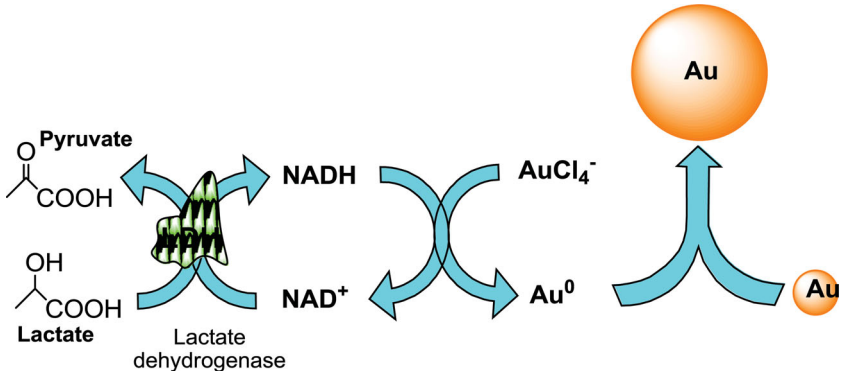


Figure 1. SEM images of enlarged Au particles generated on a Au nanoparticle/3-amino-propylsiloxane interface on a glass support using $AuCl_4^-$, CTAB, and variable concentrations of NADH: a) 14×10^{-5} M; b) 27×10^{-5} M; c) 41×10^{-5} M; d) 54×10^{-5} M; e) 61×10^{-5} M; f) 1.36×10^{-3} M. Reproduced with permission.[52]

NPs on Au seeds caused by NADH was followed by UV–vis spectroscopy and chronocoulometry. The charge associated with the stripped-off Cu^0 is higher as the concentration of NADH in the analyzed sample increases, since the rate of the formation of the metallic copper on the electrode is proportional to the NADH concentration. The successful analysis of NADH with chronocoulometry allowed detection of biocatalitic processes, which involve NAD^+-dependent dehydrogenases. Hence, alcohol dehydrogenase (AlcDH) was interacted with a constant concentration of NAD^+ and variable concentrations of ethanol, which yielded the reduced state of the cofactor, NADH (**Scheme 2**).

In addition, it was found that NAD^+/NADH redox couple is able to affect the shape of gold NPs produced by the reduction



Scheme 1. Enzymatic enlargement of Au NPs for the detection of lactate dehydrogenase activity. (This Scheme was prepared by the author).

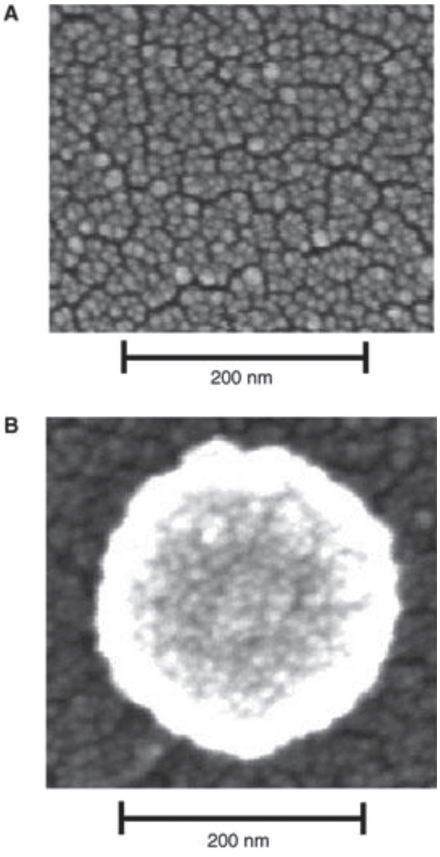


Figure 2. SEM images of the Au-NP-functionalized glass slides: A) prior to the copper deposition; B) after the copper deposition in the presence of NADH (0.84 mm) for 2 h. Reproduced with permission.[53]

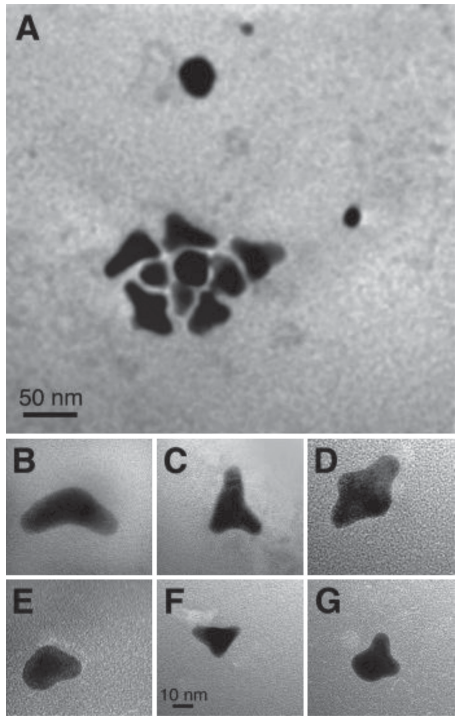
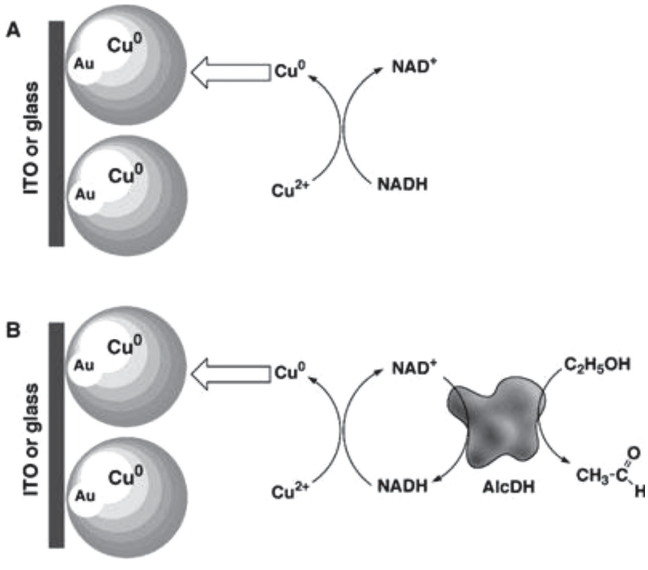


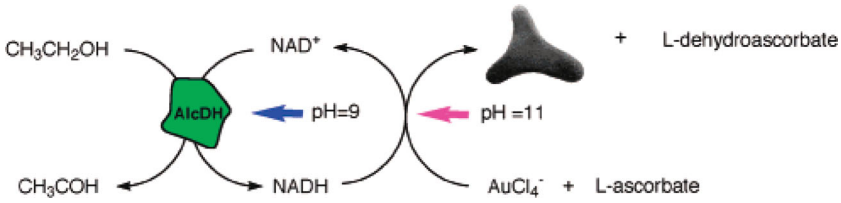
Figure 3. A) Typical TEMimage of shaped Au NPs generated in the presence of NADH, 4.0×10^{-6} M. B–D) Typical images of dipod-, tripod-, and tetrapod-shaped Au NPs formed in the presence of NADH, 4.0×10^{-6} M, respectively. E–G) "Embryonic-type" images of dipod-, tripod-, and tetrapod-shaped Au-NPs formed in the presence of NADH, 5.4×10^{-7} M, respectively. Reproduced with permission.[54] Copyright 2013 American Chemical Society.

of $AuCl_4^-$ with ascorbic acid resulting information of dipods, tripods, and tetrapods.[54] (**Figure 3**). NADH and NAD^+ bind with different affinities to the faces of Au nanocrystals. NADH binds with a predominantly favored affinity to the (011) face. Thus, the affinity association of NADH to the particles increases the local concentration of the reduced cofactor at the Au NP, facilitating the catalyzed reduction of $AuCl_4^-$ to Au(0) and the enlargement of the Au NP. The resulting NAD^+, however, binds effectively to the (011) face, thus, blocking the further NADH-mediated growth of the gold crystals on that face. Adsorption of CTAB to the Au NP controls the direction of growth of the particles in the <211> directions. Modulation of the ascorbate reduction of $AuCl_4^-$ ions with NADH allows improvement of detection limit by two orders of magnitude. The enzymatic system employing NADH modulation for sensitive detection of alcohol using alcohol dehydrogenase is depicted in **Scheme 3**.

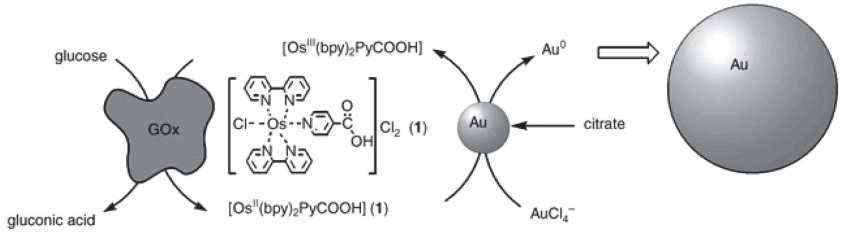


Scheme 2. Enlargement of Au-NP seeds by the biocatalyzed deposition of Cu^0 shells. Reproduced with permission.[53]

The scope of these simple enzymatic assays was limited to dehydrogenases, nevertheless, another class of redox enzymes, oxidases, can also produce compounds capable to reduce $AuCl_4^-$. Glucose oxidase is a robust and available enzyme catalyzing oxidation of β–D-glucose with oxygen to yield D-gluconolactone and hydrogen peroxide. The later reduces $AuCl_4^-$ to Au(0) enlarging Au seeding NPs.[55] The generated Au NPs have size between 15 and 20 nm. Unfortunately, this system suffered from low signal/noise ratio and consequently bad detection limit of glucose. The significant improvement in signal to noise ratio and detection limit was achieved by using the redox couple $[Os^{II}(bpy)_2PyCO_2H]/[Os^{III}(bpy)_2PyCO_2H]$ as mediator competing with oxygen for the redox center of glucose oxidase



Scheme 3. Colorimetric detection of ethanol based on the shape-controlled growth of the Au NPs. Reproduced with permission.[54] Copyright 2013 American Chemical Society.



Scheme 4. Schematic $[Os(bpy)_2PyCO_2H]^{2+}$-mediated growth of the Au NPs in the glucose/GOx/citrate/Au NP seeds system. Reproduced with permission.[56]

(**Scheme 4**).[56] The resulting Au NPs have maximum diameter of 200 nm as shown in **Figure 4**.

Another enzyme used broadly as an enzymatic label in ELISAs, immunosensors, DNA hybridization assays is alkaline phosphatase (ALP). So, the development of methods for enzymatic reduction of metal ions catalyzed by this enzyme is very attractive for scientific community. Costa-García and co-workers[57] (University of Oviedo, Spain) reported that the product of enzymatic hydrolysis of 3-indoxyl phosphate is able to reduce silver ions in solution into a metallic deposit, localized where the enzymatic label ALP is attached. The deposition of silver was followed by anodic stripping voltammetry. Compared with the direct voltammetric detection of indigo carmine, the anodic stripping voltammetry of silver ions was 14-fold more sensitive.

Potentially, all enzymes that are able to catalyze the decomposition of artificial enzymatic substrates to reducing agents can promote generation of metal NPs. Acetyl choline esterase (AChE) in nature hydrolyzes the decomposition of acetylcholine, a central neurotransmitter. The inhibition of AChE, e.g., by nerve gases, leads to the perturbation of the nerve conduction system and to the rapid paralysis of vital functions of the living systems. AChE also enhances hydrolysis of acetylthiocholine (ATCh) to yield acetate and thiocholine. The latter reduces $AuCl_4^-$ to give Au NPs as shown in **Scheme 5**.[58] As the concentrations of AChE or ATCh increase, the rate of conversion of ATCh to thiocholine is enhanced. The latter product acts as the reducing agent for the reduction of $AuCl_4^-$ and the deposition of gold on the Au NP seeds. As the amount of reductant increases, larger Au particles with increased nanoclustering on the particle surface are formed, giving rise to intensified absorbance bands. Inhibition of AChE by analogues of nerve gases such as 1,5-bis(4-allyldimethylammoniumphenyl) pentane-3-one dibromide and paraoxon decreases the rate of thiocholine formation and consequently the amount of Au NPs formed in the assay mixture. Thus, the drop in the absorbance indicates the presence of AChE inhibitors.

The main drawback of using enzymatic generation of metal nanoparticles for bioanalysis is the consumption of reducing agents produced in the course of enzymatic reactions. Therefore, relatively great amounts of reducing agents should be produced enzymatically in order to initiate formation of metal NPs detectable by UV–vis spectroscopy, hence enzymatic assays based on NPs were not sensitive enough. An alternative approach to detection of AChE inhibitors was based on the enzymatic modulation of the growth of metal NPs generated by the interaction of metal ions with the strong reducing agent, ascorbic acid[59] as described in **Scheme 6**. The hydrolysis of ATCh by AChE produces thiocholine, which binds to the surface of the Au seeds to hinder the deposition of silver (Ag^0) reduced by ascorbic acid from $AgNO_3$ (Ag^+). As a result, the formation of silver-coated gold nanoparticles is blocked (route A). In the presence of an AChE inhibitor, hydrolysis of ATCh by the enzyme is blocked, seeding Au NPs are left intact and the formation of Au–Ag NPs is promoted (route B). This was the first enzymatic assay for detection of nerve gases in which the increase in inhibitor concentration was correlated with the increase in the readout signal allowing sensitive optical detection of inhibitors in the range between 10^{-9} and 10^{-11} M.

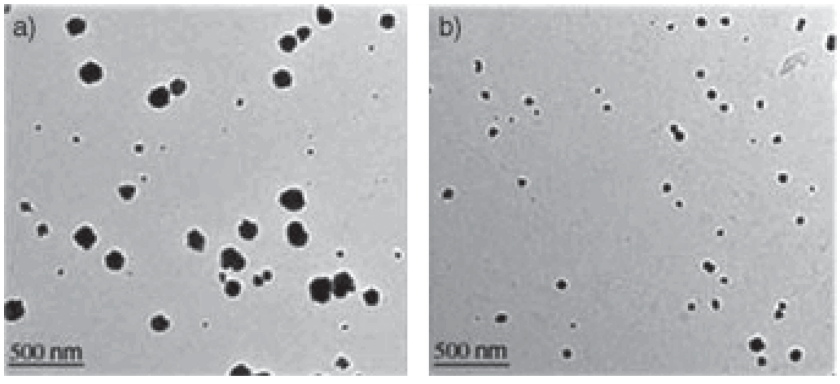
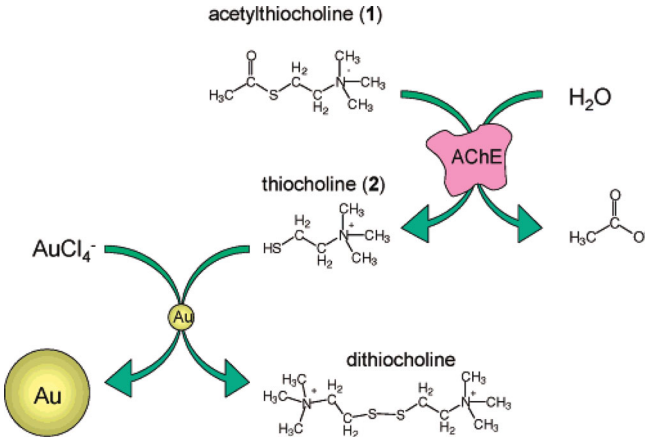


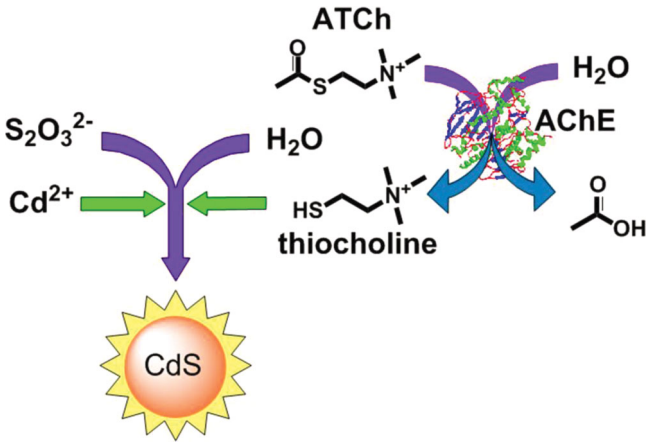
Figure 4. TEM images of: a) the Au NPs formed in the glucose/$[Os^{II}(bpy)_2PyCO_2H]$/$[Os^{III}(bpy)_2PyCO_2H]$ GOx system by using 5.9×10^{-3} M glucose; b) 7.4×10^{-5} M glucose. Reproduced with permission.[56]

4. Growth of Semiconductor NPs Modulated by Enzymes

The drawback of the systems based on the enzymatic generation of metallic NPs is that only UV–vis spectroscopy and



Scheme 5. Detection of acetylcholine esterase activity by growing Au nanoparticles. Reproduced with permission.[58] Copyright 2013 American Chemical Society.



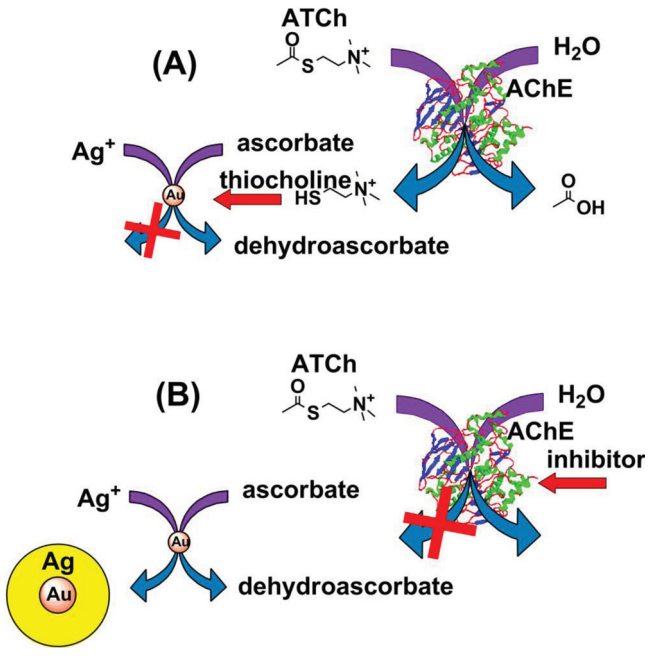
Scheme 7. Enzymatic generation of CdS QDs for the detection of AChE activity. Reproduced with permission.[60]

electrochemistry can be utilized for reading of a signal. Bioanalytical enzymatic systems producing fluorescent NPs can employ much more sensitive techniques such as fluorescence spectroscopy or photoelectrochemistry to register the read out signal as demonstrated in the seminal work.[60] Two analytical assays for detection of enzymatic activities in which generation of fluorescent CdS NPs was induced by products of bio-catalytic reactions were reported. The first assay for enzymatic activity of AChE from *Escherichia coli* is shown in **Scheme 7**. This enzyme breaks the artificial substrate ATCh to acetate and thiocholine. The latter catalyzes decomposition of $S_2O_3^{2-}$ according to equation: $S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + H_2S$. The resulting hydrogen sulfide interacts with Cd^{2+} to yield CdS NPs: $Cd^{2+} + H_2S \rightarrow CdS + 2H^+$.

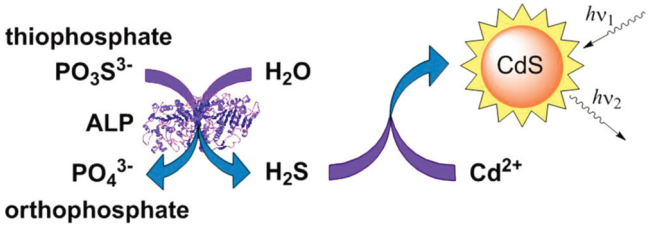
It was found out that the formation of CdS NPs in this system follows auto-catalytic mechanism. Another assay was designed to detect enzymatic activity of ALP, see **Scheme 8**. This enzyme finds wide application in bioanalysis as a label in enzyme-linked immunosorbent assays. ALP hydrolyzes thiophosphate to orthophosphate and H_2S. The latter reacts immediately with cadmium cations to give CdS QDs. When these CdS QDs were excited at 360 nm a strong fluorescence signal was observed as shown in **Figure 5**A.

The detection limit for ALP was 8 ng mL^{-1} signifying that this method was four times more sensitive than the commercial assay using chromogenic substrate p-nitrophenyl phosphate. The diameter of the QDs was calculated to be between 1 and 3 nm as found by TEM (Figure 5B).

In those reported assays, hydrogen sulfide was the product of enzymatic reactions catalyzed by hydrolyzing enzymes AChE and ALP. Redox enzyme also can generate H_2S and consequently CdS QDs as reported by the same research group.[61] It was found that glucose oxidase (GOx) is able to catalyze oxidation of 1-thio-β-D-glucose by oxygen to yield finally hydrogen sulfide. Thus, the assay method consists of the generation of CdS QDs in the presence of GOx, 1-thio-β-D-glucose and cadmium nitrate (**Scheme 9**). GOx oxidizes the substrate 1-thio-β-D-glucose to 1-thio-β-D-gluconic acid, which is hydrolyzed to D-gluconic acid and H_2S. The latter reacts with Cd^{2+} to produce fluorescent CdS QDs. Another redox enzyme horseradish



Scheme 6. Detection of acetylcholine esterase inhibitors by growing silver-coated gold nanoparticles. Reproduced with permission.[59] Copyright 2013 American Chemical Society.



Scheme 8. Enzymatic generation of CdS QDs for the detection of ALP activity. Reproduced with permission.[60]

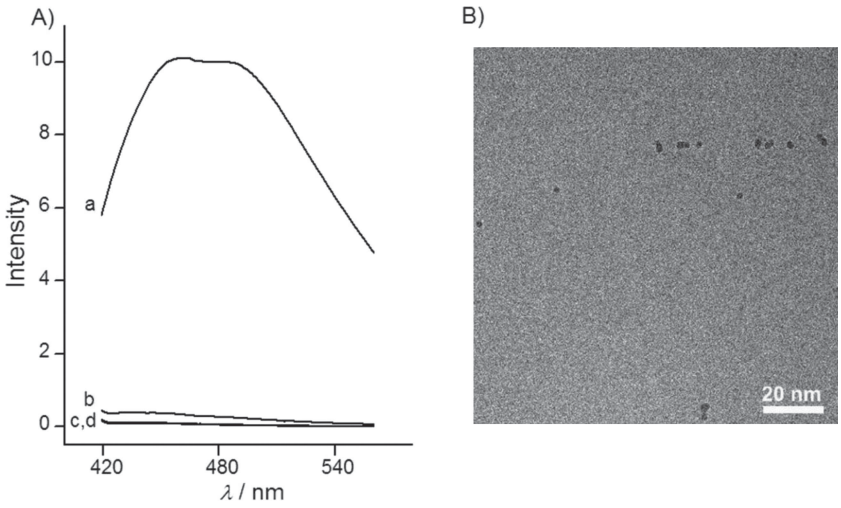
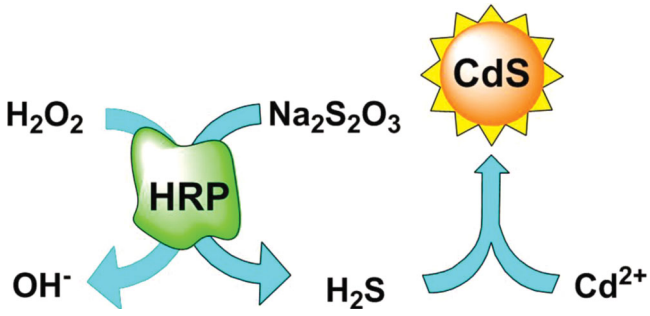


Figure 5. A) Emission spectra of the CdS QDs formed in the presence of: a) ALP (228 ng mL^{-1}), sodium thiophosphate (75 × 10^{-6} M) and $Cd(NO_3)_2$ (2.5 × 10^{-3} M); b) sodium thiophosphate (75 × 10^{-6} M) and $Cd(NO_3)_2$ (2.5 × 10^{-3} M); c) ALP (228 ng mL^{-1}) and $Cd(NO_3)_2$ (2.5 × 10^{-3} M); d) ALP (228 ng mL^{-1}) and sodium thiophosphate (75 × 10^{-6} M). B) TEM image of the CdS QDs formed in the presence of ALP (228 ng mL^{-1}), sodium thiophosphate (75 × 10^{-6} M) and $Cd(NO_3)_2$ (2.5 × 10^{-3} M). Reproduced with permission.[60]

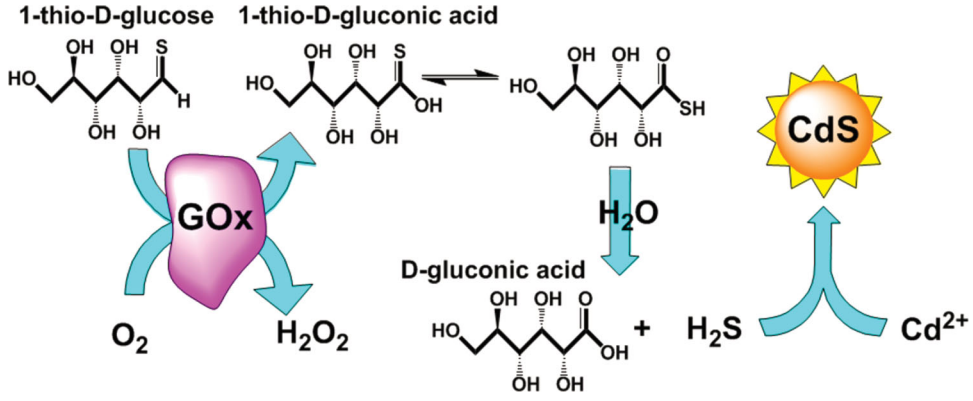
peroxidase (HRP), a robust and stable enzyme extensively employed in bioanalytical chemistry as a label in enzyme-linked immunosorbent assays, can generate QDs too. It was reported[61] that the rate of hydrogen peroxide reduction with sodium thiosulfate can be significantly enhanced by the active center of HRP yielding H_2S as one of the reaction products. Interaction of enzymatically produced H_2S with Cd^{2+} ions results in the formation fluorescent CdS nanocrystals according to **Scheme 10**. TEM images of the resulting CdS nanoparticles disclose they have spherical shape and a diameter between 1 and 2 nm. The assay demonstrated the detection limit of HRP equal to 1.88 ± 0.2 μg mL^{-1}.

The above-mentioned enzymatic assays rely on artificial sulfur containing substrates in order to generate H_2S. The turnover rate of their enzymatic decomposition is usually lower than the turnover rate of natural substrates. Application of natural substrates to QDs generating enzymatic assays was published too.[62] The cascade of two enzymes S-adenosyl-L-homocysteine hydrolase (AHCY) and Methionine gamma-lyase (MGL) process in nature decomposition of S-adenosyl-L-homocysteine (AdoHcy) to hydrogen sulphide, 2-oxobutanoate and NH_3 by the following reaction route. AHCY hydrolyzes AdoHcy to L-homocysteine (Hcy). MGL catalyzes the decomposition of Hcy to hydrogen sulfide. Generation of CdS QDs by this natural enzymatic cascade is straightforward and does not require any artificial substrates therefore the turnover rates are hot hindered by them (**Scheme 11**). This fluorogenic assay for MGL activity demonstrated 200-fold better sensitivity than the conventional chromogenic assay based on interaction of 2-oxobutanoate, generated by MGL, with 3-methyl-2-benzothiazoline hydrazone leading to azine derivative.

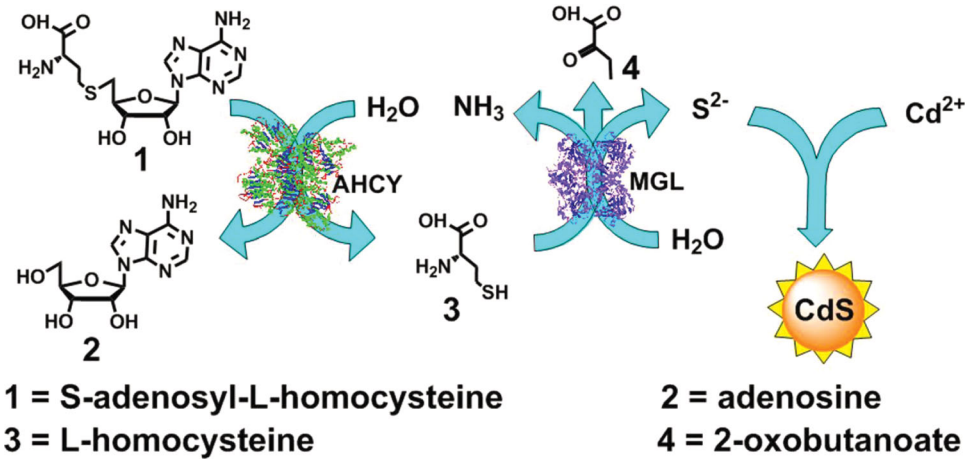
Presence of citrate or orto-phosphate ions is crucial for the formation of CdS NPs in the enzymatic systems due to its stabilizing effect. It turned out that other compounds like thiols, organic and inorganic acids produced by enzymatic reactions can influence the stabilization of CdS NPs. This finding extends significantly the range of enzymatic pathways suitable for generation of



Scheme 10. Enzymatic generation of CdS QDs for the detection of HRP activity. Reproduced with permission.[61]

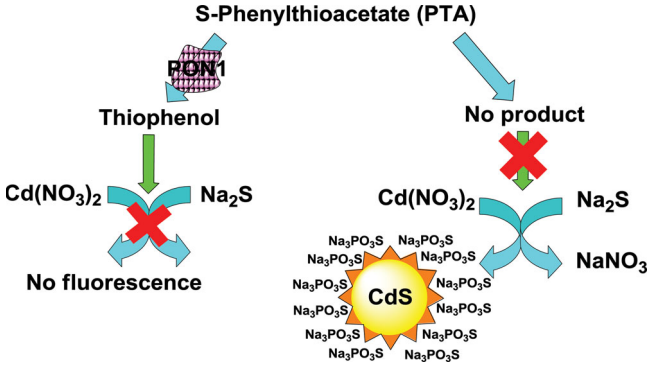


Scheme 9. Enzymatic generation of CdS QDs for the detection of GOx activity. Reproduced with permission.[61]



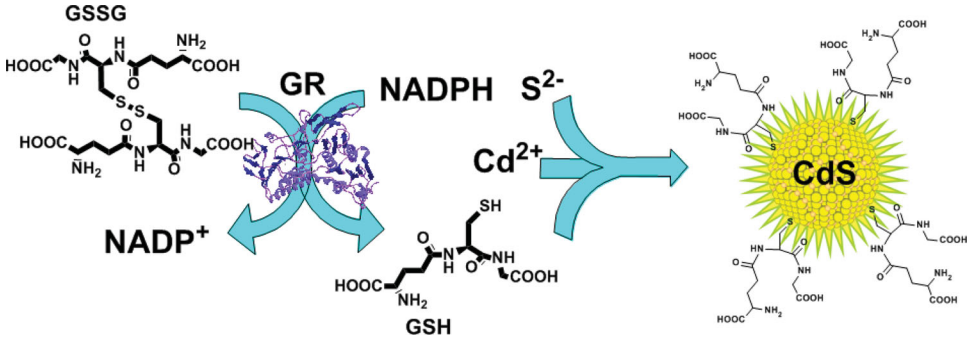
Scheme 11. The reaction process for quantification of enzymatic activities of MGL and AHCY. Reproduced with permission.[62] Copyright 2013 American Chemical Society.

QDs, allowing enzymes to be used that do not generate S^{2-} ions. An ultrasensitive assay for detection of serum paraoxonase by modulating the growth of fluorescent semiconductor nanoparticles was reported.[63] Serum paraoxonase (PON1) is an enzyme associated exclusively with high-density lipoproteins and seems to be an antiatherogenic agent that prevents initiation and progression of atherosclerosis. PON1 hydrolyzes S-phenylthioacetate to acetate and thio-phenol, which blocks the surface CdS NPs preventing their growth driven by the interaction of Cd^{2+} with S^{2-}. In the absence of thio-phenol, the growth of fluorescent CdS NPs is stabilized by orto-phosphate ions according to **Scheme 12**. The lowest PON1 activity that could be detected by this system was 0.625 mU mL^{-1}, with a dynamic range up to 5 mU mL^{-1}. This system showed improvement of the limit of detection by 15 times in comparison with the conventional assays for determination of PON1 arylesterase activity.

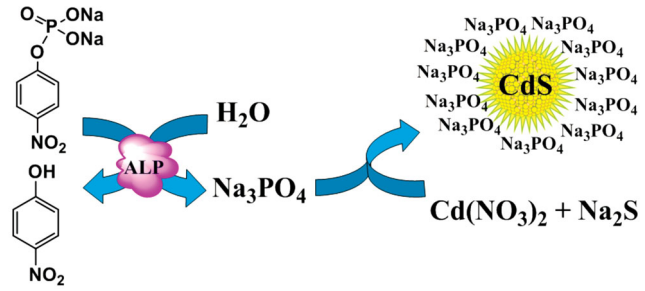


Scheme 12. Detection of PON1 activity by the enzymatic modulation of growth of fluorescent CdS QDs. Reproduced with permission.[63] Copyright 2013 American Chemical Society.

Assays for enzymatic activity depending on the inhibition of NPs growth by the enzymatic product intrinsically are not so sensitive. An example of the highly sensitive and inexpensive fluorogenic assay for glutathione reductase (GR) in which the product of enzymatic reaction enhances the growth of QDs was published too.[64] GR is the NADPH-dependent dehydrogenase reducing glutathione (GSSG) to its reduced form (GSH), which stabilized growth of CdS NPs produced by the interaction of Cd^{2+} with S^{2-}. This concept is represented in **Scheme 13**. The rate of CdS growth directly depends on the concentrations of GS, NADPH, and GR, therefore this versatile and extremely sensitive assay can be applied for the detection of those three components. The sensitivity of this new assay to GR is 5 pM, which is three orders of magnitude better than that of other



Scheme 13. Detection of GR activity through enhancing the growth of fluorescent CdS nanoparticles with the enzymatic product, reduced glutathione. Reproduced with permission.[64] Copyright 2013 American Chemical Society.



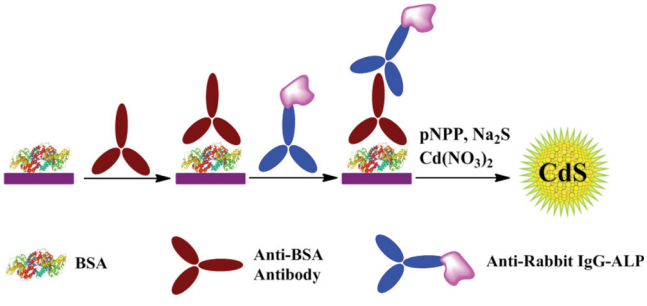
Scheme 14. Detection of ALP activity by enzymatic growth of CdS QDs. Reproduced with permission.[64] Copyright 2013 American Chemical Society.

fluorogenic methods previously reported in the literature. The resulting flourescent CdS NPs have spheroidal shape and a medium diameter of 1.82 ± 0.2 nm.

The concept of QDs stabilization with the products of enzymatic reaction was feather extended and re-applied to the detection of ALP activity. It was demonstrated in the literature that ALP from *E. coli* can hydrolyze artificial substrate thiophosphate to H_2S, which interacts with Cd^{2+} to yield CdS QDs.[60] Regrettably, thiophosphate is a potent inhibitor of bovine ALP widely used as a label in ELISA. Nevertheless, the standard chromogenic substrate of bovine ALP p-nitrophenyl phosphate (pNPP) undergoes enzymatic hydrolysis to give p-nitrophenol (pNP) and orthophosphate ions. They stabilize fluorescent CdS NPs formed by after addition of Cd^{2+} and S^{2-} to the assay mixture according to **Scheme 14**. This technique allowed monitoring the specific interaction of analyte antibody with ALP-labeled antibody in a sandwich ELISA (**Scheme 15**) with fluorescence spectroscopy.[65] The sensitivity of that heterogeneous assay was at least one order of magnitude better than that of the standard procedure using chromogenic p-nitrophenyl phosphate assay.

5. Conclusion

Enzymatic generation of metal and semiconductor nanoparticles in situ opens new vistas in the area of bianalysis. This concept allows development of relatively inexpensive, simple, and available assays. This technique offers alternatives to conventional chromogenic and fluorogenic organic enzymatic substrates, which usually are not stable and require storage in a freezer.



Scheme 15. Detection of anti BSA antibody based on enzymatic growth of Cds QDs. Reproduced with permission.[64] Copyright 2013 American Chemical Society.

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