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Signal amplification strategies for DNA and protein detection based on polymeric nanocomposites and polymerization: A review

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Graphical abstract

Highlights

1. We review the innovative advances in polymer-based signal amplification.
2. Conceptual connectivity between different amplified methodologies is illustrated.
3.Examples explain the mechanisms of polymers/polymerizations-based amplification.
4. Several elegant applications are summarized that illustrate underlying concept.

ABSTRACT

Demand is increasing for ultrasensitive bioassays for disease diagnosis, environmental monitoring and other research areas. This requires novel signal amplification strategies to maximize the signal output. In this review, we focus on a series of significant signal amplification strategies based on polymeric nanocomposites and polymerization. Some common polymers are used as carriers to increase the local concentration of signal probes and/or biomolecules on their surfaces or in their interiors. Some polymers with special fluorescence and optical properties can efficiently transfer the excitation energy from a single site to the whole polymer backbone. This results in superior fluorescence signal amplification due to the resulting collective effort (integration of signal). Recent polymerization-based signal amplification strategies that employ atom transfer radical polymerization (ATRP) and photo-initiated polymerization are also summarized. Several distinctive applications of polymers in ultrasensitive bioanalysis are highlighted.

Key words:

Signal amplification; Biosensor; Polymerization; Polymeric DNA dendrimers; Conjugated polymers; Atom transfer radical polymerization

1. Introduction

In recent years, highly sensitive detection of DNA, proteins and other disease-related biomolecules has attracted considerable interest in a wide range of research areas, including disease diagnosis [1,2], clinical therapy [3,4], environmental monitoring [5,6], and food analysis [7,8]. However, disease-related biomarkers in body fluid or tissues usually show low expression levels in the early stages of disease. These are hard to detect with conventional detection methods. As a result, the development of novel ultrasensitive detection strategies and biosensors is of great interest. In past decades, great efforts have been made to maximize the signal output and to realize ultrasensitive or even single-molecule detection by using signal amplification strategies and by correlation results from multiple analytical techniques [9-12]. The sensitivity of any analytical strategy is determined by the correlation between the analyte concentration and the intensity of the output signal. Therefore, a number of

nanostructured materials and long chain polymeric materials have been incorporated into these strategies. This is to increase the loading of signal tags or target biomolecules for further amplification of the signal readout associated with specific recognition or/and signal-triggered elements.

Signal amplification based on polymers or polymerization is a process that makes good use of the dynamic growth of macromolecules. Similar to rolling circle amplification (RCA), polymerization is conducted (instead of enzyme-based replication) to obtain polymers with abundant repeating moieties for subsequent coupling of signal tags. As a result, significant signal amplification is achieved by employing polymer-based detection strategies.

In addition to acting as signal molecule carriers, some polymeric materials have excellent optical or electrochemical responsiveness. These have been extensively employed for developing detection strategies with lower and lower detection limits for clinical diagnosis via recognition of target molecules. For example, 4-acetoxystyrene was chosen (as a monomer) and used to simplify the assay process and to improve detection output due to its electrochemical activity [13]. Through a simple hydrazine hydration process, the acetoxy groups involved in the monomer (or subsequent polymer) were converted into phenolic hydroxyl groups. These were further oxidized to form the corresponding catechol and o-quinone derivatives with the help of tyrosinase-catalyzed reactions, which generated electrochemical signals in the presence of O_2. In addition, some types of polymers also possess the ability to produce a synergic or transformed effort among conductivity, fluorescence, optical properties and biocompatibility to accelerate the signal transduction for significant signal amplification.

A critical factor that affects the sensitivity of biosensors is the signal to noise ratio (SNR). In some polymer-based amplification strategies, although the local accumulation of targets or probes maximizes the detection signal, this may also cause increased noise due to nonspecific adsorption and side reactions. However, the optimizations can be combined to reduce the background signal and raise the SNR [14-16]. Usually the SNR is defined as 3 (based on the detection limit), and amplification is accomplished when compared to other un-amplified methods based on the same SNR level.

We note that all of the above-mentioned strategies are essentially highly efficient signal amplification processes with controllable enhancements. These strategies

provide promising insights for the development of electronic, optical, and photoelectrochemical biosensors. It is believed that the design and synthesis of functionalized macromolecules for molecular recognition, tag loading and/or triggering a polymerization reaction will be conducive to polymer-based signal amplification. In this review, we intend to highlight significant innovative advances in polymer-based signal amplification strategies. Some of these methods focus on novel functionalization approaches for efficient immobilization of biomolecules or active tags on polymeric materials as recognition or/and signal generating elements. Other approaches emphasize hybrid methods that rely on the integration of other signal amplification strategies such as nanoparticles, enzyme catalysis and sensitive analytical techniques. Rather than providing a detailed description of related approaches, the aim of this review is to illustrate the conceptual connectivity between various amplification methodologies for ultrasensitive bioanalysis and biosensing. Polymer-based approaches have been extensively studied in recent years. However, only representative results are summarized here to illustrate the underlying concepts and to indicate the advantages and critical issues. In this review, we first discuss the applications of as-synthesized polymers as carriers for increased loading of signal probes or biomolecules. And then, the direct use of polymers as signal molecules or energy donors is summarized. Finally, in situ polymerization-based amplification (PBA) in which the polymerization is triggered after target-ligand recognition by an initiator labeled molecular probe is described. As far as polymerization-based signal amplification is concerned, we have already provided a summary of these strategies, including target-triggered ATRP and activators generated by electron transfer (for atom transfer radical polymerization (AGET ATRP)). These are mainly from the research of He’s group at North Carolina State University and our laboratory [17]. Therefore, in this overview, other PBA strategies such as photopolymerization and enzyme-mediated polymerization, and the latest follow-up research in our group are summarized.

2. Polymers Used as Carriers

Based on the traditional signal amplification strategies, it is known that nanoparticles (NPs), such as carbon and metal-based NPs, can act as catalysts to trigger a detectable signal or as carriers for high loading of signal probes. Similarly, all of the polymeric materials have abundant sites or groups for functionalization with

biomolecules and/or probe molecules in their interiors or on their surfaces. These can help to amplify the signal and therefore enhance the detection sensitivity (Fig. 1). Most of the polymer-based signal amplification strategies are performed on the basis of this mechanism.

Fig. 1 here

2.1. Polymeric Microspheres

If the polymers form into spherical or granular nanoparticles at a nanometer scale, they can be excellent candidates for carriers to enhance the probe loading (due to their good conductivity, biocompatibility and functionality). The polymeric microspheres, either at the bottom or on the surface, can be employed as supports for depositing and concentrating the capture molecules and/or significantly loading signal molecules. Kathryn and co-workers made good use of polystyrene microspheres which were internally loaded with organic media-soluble redox tags for amplifying DNA measurements [18]. The electroactive beads caused significant amplification of single hybridization events. This was the result of their ability to carry a large number of ferrocenecarboxaldehyde probes. The detection limit of the target DNA (based on chronopotentiometry) was remarkable (down to the 5.1×10^{-21} mol level after 20 min of hybridization and "release" of the ferrocene probes into the organic medium).

In addition, polymer nanocomposites have many interesting optical, electronic, magnetic and mechanical characteristics. As a result, polymer materials have been developed into powerful tools for modifying and tailoring the surface properties of various nanostructured functional materials (including metal and semiconductor nanospheres, oxide nanospheres, and so on [19,20]). Grafted fibrous polymer chains also increased the adsorption capacity of the support via multilayer binding of biomolecules [21]. Surareungchai and co-workers [22] prepared 338 nm poly(styrene-*co*-acrylic acid) microspheres. They modified the latex spheres by layer-by-layer assembly of avidin-labeled gold nanoparticles (AuNPs). The as-synthesized nanocomposite showed an increase of 2 orders of magnitude in the number of Au atoms delivered per complex sphere. Then, the nanocomposite was coupled to DNA probes specific to *E. coli* and immobilized on an electrode surface via DNA hybridization. This was followed by dissolution of the Au latex particles and

the detection of Au^{3+} ions using anodic stripping voltammetry (ASV). A single DNA hybridization signal was successfully translated into hundreds of AuNP signals by using this strategy. This enabled ultrasensitive detection of DNA with a detection limit of 0.5 fM. Ju and co-workers [23] also demonstrated an amplification method for electrochemical detection of DNA hybridization by employing functionalized poly(styrene-*co*-acrylic acid) (PSA) microbeads with CdTe quantum dots (QDs). The CdTe-labeled signal tags were prepared by a layer-by-layer deposition of three-layer polyelectrolyte films and subsequent self-assembly of the CdTe QDs onto the PSA cores (Fig. 2A). The huge coverage of 9540 QDs per polybead enabled the material to be an efficient candidate for signal amplification. After the CdTe tags were dissolved with HNO_3, DNA hybridization was measured by means of sensitive square-wave voltammetry (SWV) of Cd^{2+}. This resulted in a low detection limit of 0.52 fM as well as a wide linear range of five orders of magnitude (Fig. 2B). Yang's group [24] has developed a robust immune-label based on encapsulation of Fe_3O_4 nanoparticles by poly(ethylene glycol)-poly(lactic acid) (PEG-PLA) polymeric vesicles. The advanced mechanical properties of such polymeric vesicles facilitated a high Fe_3O_4 loading of 37 wt.%. This displayed a high catalytic activity of the PEG-PLA-Fe_3O_4 toward H_2O_2. With the conjugation of a great amount of antibodies onto the hybrid vesicle's surface, the immune-labels were successfully used for the fabrication of ultrasensitive sandwich immunosensing of prostate specific antigen (PSA). Liu and co-workers [25] also grafted poly(glycidyl methacrylate) (PGMA) onto the surfaces of silica nanospheres for a further increase of CdTe QD loading per immunoassay event. This resulted in highly efficient signal amplification as well.

Fig. 2 here

2.2. Polyelectrolyte

As we known, some electroactive polymers can be used as biocompatible films for entrapping and immobilizing biomolecules and/or signal tags. They can also generate a large number of electrons during electrochemical redox processes to amplify the output signal and improve the detection sensitivity [26]. Of the various types of electroactive polymers, polyelectrolytes are a group of water-soluble high-molecular polymers. They are stable in aqueous solution in the form of polyions with multilayer

structures (despite the presence of abundant equal charges [27]). Due to the strong electrostatic attraction between the surface and an oppositely charged molecule, the adsorption of polyanions and polycations on a charged surface can form multilayers with approximately equal charge-to-charge distances. Bulk polyion complexes of flexible polyelectrolytes possess high charge density and similar molecular weights (useful for supporting films). The linear increase of film thickness with the number of deposited layers is often the same even if different substrates are used. This makes the supporting film properties rather independent of the substrate. By nature, polyelectrolytes are also nontoxic and harmless, which endows them with excellent biodegradability and biocompatibility. As a result of the electrostatic layer-by-layer technique, the film architectures are completely determined by the deposition sequence. Therefore, proteins can be incorporated in a single layer or in individual multilayer films. In addition, a wealth of different biomolecules or signal tags can also be functionally immobilized on the polyelectrolyte material surface under mild conditions [28-30]. In the case of non-covalent electrostatic adsorption, the unsteady coupling provides new possibilities for reversible immobilization of targeted materials and for reusing the biosensors.

Renneberg and co-workers [31] encapsulated crystalline fluorescein diacetate (with an average size of 500 nm) in ultrathin polyelectrolyte layers of poly(allylamine hydrochloride) and poly(sodium 4-styrene sulphonate) *via* layer-by-layer assembly (Fig. 3). Subsequently, the fabricated multilayer was used to attach antibodies by adsorption. The number of luminophores for per binding site ranged from 5×10^4 to 2×10^5, generating remarkable amplification rates for this polyelectrolyte-nanocrystal label-based assay. The amplification rates were 70- to 2000-fold those of conventional immunoassays that employed directly fluorophore-labeled antibodies. After the immunological reaction, the nanocrystals were dissolved by using a release reagent for detection. This polyelectrolyte/nanocrystal based amplification method was introduced into the immunoassay of mouse IgG. This lowered the detection limit by a factor of 5 ~ 28, generating a remarkable increase in sensitivity (by 400- to 2700-fold compared with conventional fluorescein isothiocyanate conjugates [32]). In addition, when ferrocene microcrystals were encapsulated in the polyelectrolyte multilayers, a high signal molecule to antibody ratio (of $10^4 \sim 10^5$) was achieved for electrochemical immunoassay. Abundant signal-generating molecules in the microcrystal system diffuse across the capsule film into the outer environment for a significant

enhancement of the final detection sensitivity [33].

In addition to entrapping the probe molecules internally, the polyelectrolytes were employed as carriers for increased loading of signal tags on their surfaces (in Chen's group [34]). In this highly effective amplifying strategy, magnetic Fe_3O_4 nano-clusters were coated with a bilayer composed of two types of polyelectrolyte: polystyrene sulfonate sodium salt (PSS) and poly(diallyldimethylammonium chloride) (PDDA). PSS and PDDA support high loadings of Au NPs on their surfaces by means of electrostatic layer-by-layer self-assembly. The Fe_3O_4/PSS/PDDA/Au composites were used as signal labels for DNA hybridization. A signal was detected by electrochemical ASV measurement of multiple Ag NP tracers after catalytic deposition of silver on the gold tags. A dramatic improvement of the sensitivity was achieved by increasing the loading of signal probes by use of the polyelectrolytes. The DNA detection limit was reduced to 100 aM. This was 800 times lower than that obtained by using only Au NPs as labels.

Fig. 3 here

2.3. Dendritic High-Branched Polymers

In recent years, interesting dendritic polymers (including dendrimers and hyperbranched polymers (HBPs), with their fractal structure and multitude of branches) have been widely exploited for various applications [35,36]. Dendrimers are composed of dendritic units and terminal units with a three-dimensional (3-D) highly branched globular and monodisperse structure. The multiple surface functional groups render the dendrimers amenable to a wide range of chemical modifications with various cellular components. In addition, the numerous modifiable terminal groups (located on the exterior of the branching units) are able to increase the loading of biomolecules and signal tags. This helps to enhance the signal output [37-39]. Therefore, dendritic polymers show excellent performance as nanocarriers for biological applications in the fields of drug and protein delivery, antimicrobial and antifouling materials, gene transfection, cytomimetic chemistry, bioimaging and tissue engineering [40].

With the use of fluorescent dendrimers, the minimum detectable amount of target DNA was lowered from hundreds to tens of attomoles by using flow cytometry

techniques [41]. Dendrimers labeled with different organic dyes can also serve as strongly fluorescent probes for the detection of biomacromolecules [42]. Zhu and co-workers developed poly(amidoamine) dendrimer (generation 4.0，G4 PAMAM) monolayers for the measurement of DNA hybridization with high selectivity, sensitivity and stability [43]. Qing's group recently reported a chemiluminescent (CL) biosensor for highly sensitive sequence-specific detection of DNA [44]. Capture probe DNA was firstly immobilized on magnetic beads covered with AuNPs, and then the target DNA and signal DNA were introduced onto the surface via hybridization. A CL signal was generated by adding H_2O_2 and Co(II) ions as the catalyst. The immobilization of G4 PAMAM dendrimers onto the gold nanoparticles significantly enhanced the sensitivity. This resulted in a detection limit of 6 fM of target DNA.

A nanocomposite of PAMAM dendrimers and CdS QDs (prepared by an electrodeposition method) showed 55-fold enhanced electrochemiluminescence (ECL) compared with that of QD films without dendrimers [45]. For example, abundant CdSe-ZnS QD solutions were directly added into the dendrimers. After modification with DNA, the as-synthesized nanocomposites could be used as signal probes to produce extremely strong and stable ECL. Based on the high specificity of aptamers to the target cells and a biobarcode technique, significant signal amplification was achieved for the ECL detection of cancer cells. The resulting detection limit was 162 cells mL^{-1} (Fig. 4) [46]. Similarly, Ju's group developed an ultrasensitive ECL biosensor for detection of near single DNA molecules [47]. The resultant QD-dendrimer nanocomposite (in their assay) gave rise to an amplified ECL signal with a detection limit of 2.5 aM, corresponding to 15 DNA molecules in a volume of 10 mL.

Fig. 4 here

An electrochemical immunosensor based on a dual signal amplification strategy of dendrimers and Au nanoparticles was proposed for sensitive detection of α-synuclein (α-SYN) [48]. The electrode surface was first modified by a layer of poly-o-aminobenzoic acid (poly-o-ABA, PAB), providing numerous carboxyl groups for subsequent covalent binding of G4 PAMAM-encapsulated AuNPs (PAMAM-Au). The abundant amino groups endowed the PAMAM-Au nanocomposites with highly dense immobilization of target antigen (due to a favorable electron transfer process).

Then, horseradish peroxidase-labeled antibodies (HRP-Ab2) were immobilized on the surface of the AuNPs. After the immunoassay process, the HRP-Ab2-AuNPs were captured onto the electrode surface. These generated an electrocatalytic response toward the reduction of H_2O_2, with a detection limit of 14.6 pg mL^{-1} for α-SYN (Fig. 5).

Fig. 5 here

2.4. DNA Dendrimers

Recently, DNA dendrimer technology has been established as a robust system to provide signal amplification [49-52]. In this system, two DNA strands that share part of a sequence form a stable double strand under the right conditions, and the remaining portion will be free for further hybridization or modification. Such an "X"-like structure (called a DNA monomer) is the basic unit of the DNA dendrimers. Basically, identical or different DNA monomers are integrated through their single-stranded regions to form a dendritic structure. DNA dendrimers with numerous terminal groups or multiple binding sites (approximately 5~1000 on each dendrimer unit), are expected to significantly increase the density of immobilized molecules. Due to being heavily negatively charged, the DNA dendrimers can be further incorporated into conjugated polymers for further outstanding functionality. Robert's group has designed a type of DNA dendrimer, which is composed of both oligonucleotide-conjugated antibodies (for selectivity) and enzymes or dyes (for signal). These can be used in protein microarrays and in enzyme-linked immunosorbent assays (ELISA) for improving the detection limit [53]. The use of conjugated DNA dendrimers for cytokine detection yielded up to 10-fold improvement of the detection limit with no additional steps or equipment.

In addition, the DNA dendrimers can be easily incorporated into abiotic conducting polymer matrices and are biocompatible with various biological species. Due to these advantages, DNA dendrimers have been integrated with conjugated polymer interfacial films (at the nanoscale) to increase the assay sensitivity by orders of magnitude [54]. In addition, DNA dendrimers are already commercially available, and these include various functionalized derivatives. Briefly, DNA dendrimers are pre-modified with streptavidin and then mixed with pyrrole monomers. Then, DNA

dendrimer embedded polypyrrole (DDPpy) is formed on the electrode surface by a common electrochemical polymerization, followed by coupling with biotinylated antibodies (Fig. 6a). The target antigen and the secondary HRP-labeled antibodies are immobilized on the surface (in turn) by using traditional ELISA, followed by simple amperometric detection (Fig. 6b). A detection limit of 100 ~ 200 fg mL^{-1} was achieved for salivary protein, which was three orders of magnitude higher than that of a Ppy-only treated surface.

Fig. 6 here

3. Polymer Itself Used as Signal Tags and Energy Donors

Some polymeric materials have abundant repeat units, and they also possess excellent optical and electrochemical responsiveness, or the ability to serve as energy donors. The most representative example of this is a class of polymers called conjugated polymers (CPs). CPs are constructed with a backbone with a highly delocalized electronic structure, exhibiting unique electronic and optical properties [55]. The polymer backbone can keep the short conjugated units in close proximity. This special structure yields effective electronic coupling and thus produces quick intra- and inter-chain energy migration along the polymeric backbone [56]. The energy that is transferred from the conjugated chain to an adjacent acceptor causes significant amplification of the fluorescence signal.

3.1. Signal Amplification of CPs

It is known that CPs always have better light harvesting abilities than their common small molecule counterparts. CPs can trap energy and/or electron migration along with the backbone due to their delocalized electron backbone. Once a quencher (or analyte) is bound locally to a receptor of a CP, the electronic properties of the backbone and the entire CP are both affected. Upon proper excitation, the single quencher molecule (that binds to a single receptor) is sufficient to trap and transfer almost all of the exciton energy along the entire backbone. Thus by using a molecular wire approach, it is possible to quench many repeated units in the chain. This amplifies the quenching effect [57,58]. In other words, the recognition event on a

small molecule only causes a single chromophore to change its fluorescence signal. However, a CP binding event can affect the fluorescence of an entire chain of macromolecular chromophores. This signal amplification makes the CPs sensitive to minor perturbations from analytes, even in extremely diluted concentrations (at nanomole or subpicomole levels). This has promoted the rapid development of CP-based sensor applications [57].

3.2. Classification of CP-based biosensors by signal amplification

Based on the different interactions between analytes and CPs, the CP-based amplification strategies can be divided into three main categories: polymer aggregation [59,60], conformational and geometry change of the CP backbone [61,62], and electron energy transfer [63,64]. Fluorescence resonance energy transfer (FRET) is used in all of these fluorescent modes to amplify the fluorescent signal. In these, the CPs serve as the energy donors and the signal chromophores serve as the energy acceptors [65]. As excellent energy donors with high extinction coefficients, the CPs can amplify the acceptor signal after energy is transferred from a CP to a target fluorophore. This results in high sensitivity and selectivity with minimized background signals. In addition, if the resultant change (due to a binding event) occurs within the conjugation length of a CP backbone, this will change the wavelengths of light that is absorbed by the CPs. As a result, the generated signal can also be conveniently and clearly seen by the naked eye. This results in a visible and useful colorimetric sensor. In addition, CP-based sensors can also be subdivided into three modes based on their output signal patterns: fluorescence turn-on (enhancing), turn-off (quenching), fluorescence color change and visible color change [66].

3.3. Application of CP-based signal amplification

The fluorescence properties of CPs can be affected and changed by different mechanisms, including changes in the chemical nature, effective conjugation length, intramolecular conformation and intermolecular packing. These can be implemented in various sensing applications [67-69].

CP-based biosensing platforms have been developed based on the incorporation of specific functional moieties that can couple with target molecules of interest, showing a change in either fluorescence or color. For example, Bazan’s group demonstrated

that cationic poly(fluorene)s (CP1) can be used as a highly sensitive optical sensor for signal amplification of DNA assays [70]. In their work, peptide nucleic acid (PNA) labeled fluorescein (PNA-C*) was designed as a probe to favor FRET from CP1 (donor) to C* (acceptor). No desirable FRET was observed in the initial solution between positively charged CP1 and neutral PNA-C*. However, when a targeted ssDNA (which was complementary to the PNA probe) was brought into the system, the subsequent hybridization endowed the PNA-C* with multiple negative charges. Then, a PNA-C*/ssDNA duplex could form due to electrostatic interactions. The resulting decrease in the average distance between CP1 and C* would trigger effective energy transfer from CPs to fluorescein (Fig. 7, situation A). In contrast, PNA-C* could not form a duplex with negatively charged DNA in the presence of a noncomplementary ssDNA. The CP1-C* distance remained so large that it caused ineffective FRET and no emission from the signaling fluorescein (Fig. 7, situation B). As a result of CP excitation, the fluorescent intensity was increased more than 25-fold compared to direct excitation of the dye using its adsorption maximum. An extremely low concentration (of 10 pM) was established as the detection limit (for ultrasensitive detection of target DNA).

Fig. 7 here

Some CPs, such as cationic poly(thiophene)s (PTs), have been reported to undergo conformational change upon association with oligonucleotides. This leads to a solution color change or fluorescence alterations in the presence of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). In addition, there has not been any requirement for sophisticated chemical modification of polymers and analytes. In view of these advantages, Leclerc’s group has developed an approach to detect a human α-thrombin by a cationic PT and an aptamer of thrombin [71]. As shown in Fig. 8, the specific aptamer binds to the thrombin and forms a compact unimolecular quadruplex structure. Then, the PTs wrap around the quadruplex to form a complex. This exists in a less aggregated conformation and exhibits blue-shifted absorption and enhanced fluorescence (Fig. 8, path A). In the absence of thrombin, the PT complex and free aptamer adopt a planar formation, yielding red-shifted absorption and weak fluorescence (Fig. 8, path B). This simple sensing system lowered the detection limit for human α-thrombin to the femtomole level (~10 pM). Recently, Liu and co-workers

demonstrated a nanoparticle-based protein assay for goat IgG and thrombin detection with signal amplification by CPs. The proteins were pre-immobilized on the surfaces of NPs, followed by the attachment of cationic poly(fluorene-cophenylene)s (PFPs) through sandwiched immunoreactions [72]. Then, FRET occurred upon excitation of the PFPs, producing high sensitivity and selectivity of the assay with a detection limit of 1 ng mL^{-1}. If the antibodies that were immobilized on the NPs' surfaces were replaced by relevant aptamers, this assay method could be employed for thrombin and lysozyme measurement in serum [73].

Fig. 8 here

On the basis of the polymer aggregation mechanism, a new type of polymer called a cationic conjugated polyelectrolyte (CPE) (combining the signal amplification advantages of polyelectrolytes and CPs) was employed to enhance the sensing response of a fluorogenic substrate (fluorescein diacetate, FDA). This was used for a better study of esterase activity [74]. Although the FDA was nonfluorescent, it could be transformed into a negatively charged fluorophore via hydrolyzation by the pig liver esterase (PLE). After that, the electrostatic interaction would allow this newly formed negatively charged fluorophore to form a complex with positively charged CPE. The presence of CPE not only shortened the readout time but also allowed effective FRET to amplify the fluorescence signal (by up to approximately 19-fold). In addition, using CPE as a signal amplifier could also enable real-time monitoring of enzyme activity with extremely low interference.

4. In-Situ Polymerization for Signal Amplification

Polymerization-based amplification has opened the door to the investigation of a series of concepts for basic research. It has also showed promise as a brilliant and robust technique for the detection of trace amounts of a wide variety of analytes in molecular diagnostics. A growing body of work is in progress, devoted to bridging the gap between disease diagnosis criteria and currently available detection methods in the lab. PBA integrates various fundamental benefits of the specific, rapid and repetitive nature of radical polymerization with macromolecule-linked dynamic growth to amplify the presence of targeted biomolecules. The method is developed

and aimed to realize improved biological detection [75].

In this approach, a small initiator molecule is first labeled with the detection species prior to ligand-target binding. This links a signal amplification reaction to molecular recognition events, as described conceptually in Fig. 9. By means of this specific recognition, the initiating molecules (i.e., initiators) (as polymerization reaction centers) are immobilized on the measuring substrate surface. This is followed by triggering in situ polymerization after the addition of the monomer solution and other reaction necessities. The polymer growth leads to a local accumulation of monomers that alter the optical and/or electrochemical properties of the substrate or the supporter. The active groups embedded in the polymer provide numerous sites for the binding of optical or electroactive tags for a wider measurement technique. Consequently, a single identification of a biological molecule by a small monomer is amplified hundreds to millions of times through the propagation chain. This polymerization and amplification (after specific molecular recognition) will also generate a better signal-to-noise ratio by minimizing nonspecific interferences [76-79].

Fig. 9 here

4.1. PBA using atom transfer radical polymerization

In recent years, a strategy for highly sensitive detection of target proteins based on a combination of tyramide signal amplification (TSA) and PBA was proposed by our group [80]. In this work, initiator-labeled protein was immobilized on the surface of an electrode via traditional sandwich immunoreactions. Subsequently, surface-initiated atom transfer radical polymerization (SI-ATRP) of GMA was triggered. The resulting long chain polymers provided numerous epoxy groups for coupling of HRP. The increased amount of HRP significantly increased the accumulation of QD signal probes in the presence of hydrogen peroxide due to the TSA mechanism. This combination demonstrated dramatically increased signal amplification (by one-order of magnitude) (Fig. 10, Route 3). ECL and square-wave voltammetric (SWV) measurements that employed this dual signal amplification strategy showed 9.4- and 10.5-fold increases in the signal outputs in comparison with the unamplified method, respectively. The detection limits for human IgG antigen

were 0.73 pg mL^{-1} and 0.09 pg mL^{-1} for ECL and SWV, respectively. Prior to this, we also developed a label-free immunosensing method for sensitive detection of tumor necrosis factor-alpha antigen (TNF-α) via consecutive SI-ATRP of ferrocenylmethyl methacrylate (FMMA) and GMA on the surface of an Au electrode [81]. The redox-active ferrocene moieties in the PFMMA segment were localized on the surface to play an important role of an electron transfer mediator, while the epoxide groups in the PGMA segment was utilized for the coupling of TNF-α. The final antigen-coupled electrode displayed good electrochemical properties, as measured by cyclic voltammetry. This sensing platform enabled the detection of TNF-α at a low detection limit of 3.9 pg mL^{-1}. The use of SI-ATRP and block polymerization in the manufacturing process enabled flexible tuning of the amount of surface functionalized FMMA and GMA units in the immunosensor. Controlled adjustment of the spatial distribution of redox mediators was also possible. All of these merits enabled a versatile pathway for the design of electrochemical biosensors with extraordinary sensitivity. The modifiable groups in the polymeric materials were capable of coupling a variety of detection probes. These could potentially expand the range of applications for clinical diagnostic research and biological assays.

Fig. 10 here

4.2. PBA using photo-initiated polymerization

In Bowman's research, another signal enhancement strategy was developed, based on PBA. The method utilized the fundamental advantages of radical chain photopolymerization reactions and was demonstrated to be a rapid, non-enzymatic method of signal amplification. It showed the capability of offering visual detection of the presence of labeled polynucleotide targets on a biochip surface [82,83]. In this method, amplified signal output was achieved by specifically allocating photoinitiator molecules only to those locations (on the biosensor surface) where biotinylated oligonucleotide targets experienced a biorecognition event. A dual-functional molecule was used to simultaneously bind relevant biological sites and to initiate a polymerization reaction.

In one PBA detection system, for example, ultraviolet (UV) light sensitive photoinitiators were conjugated with Neutravidin protein to test oligonucleotides tethered to a silicon surface [82]. Another PBA process used an eosin visible-light

sensitive photoinitiator to couple with streptavidin (Fig. 11) [83]. Due to the gentle and low-cost excitation source (of visible light), this eosin-based photoinitiation system is a dominant candidate for PBA. When the photoinitiator immobilized biochip was exposed to the proper irradiation and some monomer was added, surface initiated polymerization was triggered only at the binding sites. The resulting polymer films had high molecular weights and could be easily detected by the naked eye. This strategy showed outstanding amplification and was demonstrated to be a sensitive visual assay platform for the detection of biotinylated DNA under favorable implementation conditions. Based on dilution-study data, even in a case where only a mere 1000 biotinylated DNA oligos were present, a polymer film was formed and could be visually observed [82]. The detection limit was three orders of magnitude lower than that of comparative enzymatic assays. More specifically, visible light irradiation is likely to promote the eosin dye to an excited state. After this, further energy transfer (with an amine co-initiator) produces initiating radical species [84]. Recently, the eosin system was employed for the detection of hybridization events and single-base pair mismatches [85-87].

Fig. 11 here

4.3. PBA using enzyme-mediated radical polymerization

One of the classical issues that faces radical polymerization is that molecular oxygen inhibits the polymerization by reacting with initiators and by propagating radicals to form peroxy radicals. These are unreactive towards further propagation and thus terminate chain growth. In this respect, Iwata and co-workers reported a glucose oxidase (GOD)-mediated initiation of a radical polymerization reaction. This was found to be an ideal biological approach for benefiting interfacial polymerization [88]. This initiation used multiple components to realize fast and significant polymerization. In particular, the reaction between β-D-glucose and oxygen was catalyzed by GOD in the presence of ferrous ions, producing H_2O_2 and generating hydroxyl radicals. These are highly efficient for initiating common radical polymerization of (meth)acrylate [89]. The light-independent mechanism and oxygen-free inhibition in this unique polymerization system made it excellent for use in fabricating hydrogels (several millimeters thick) for cellular encapsulation. Because of the continuous regenerating feature of GOD (for radical generation), this method was implemented to design

highly sensitive polymerization-based amplification devices [90]. GOD-mediated PBA (for biological detection) was always performed on a biotinylated microwell surface with a GOD-avidin conjugate. This was used to immobilize the enzyme and initiate subsequent polymerization (Fig. 12). During the polymerization process, external fluorescent monomers were also copolymerized directly into the polymer matrix, and these enabled the visual measurement of the biometric recognition events. Enzyme-mediated polymerization could proceed in an ambient atmosphere without any external energy supply. This was an improvement compared with previous PBA platforms, and the method also resulted in higher signal output. This strategy led to a signal to noise ratio of 14 with a detection limit of 156 ng mL^{-1} for the detection of transforming growth factor-beta (TGF-β). It also exhibited signal amplification that was more than 3 times greater than that of the traditional enzymatic method. In a word, this dual amplification system (combining both enzymatic and polymerization amplification) was demonstrated to be a more robust approach with greater specific and enhanced assay sensitivity. More recently, a novel capillary flow device was fabricated to block a microfluidic channel in response to target biomolecules, on the basis of this enzyme-mediated PBA strategy [91]. In this assay system, a polymer undergoing gelation within the microfluidic channel played the role of a barrier to be tested by multiple modes of detection. These included visual observation, pressure recording and redirection of fluid flow by virtue of capillary action without an external power source. A dramatic difference (> 15-fold) in the measured pressure between positive and negative results made this a simple and sensitive method. The readout could be interpreted with little or no supplementary instrumentation. This was promising for good performance for different tasks due to its potential for use in more complex microfluidic architectures.

Fig. 12 here

5. Conclusions and perspective

Sensitive detection is needed to detect prognostic and predictive biomarker levels in earlier disease stages and to guide further treatment. A wide variety of polymers or polymeric materials (with different densities, shapes and compositions) have been used to achieve signal amplification for bioanalysis. Polymers possess collective properties that are absent in monomers. This fact is used to enable efficient

amplification of the detection signal by the functionalization of anchored groups in side polymer chains (and/or by using inherent unique characteristics of the polymers). This review explores the potential and advantageous of such polymer-based amplification. Different polymeric materials have been used as carriers or tracers, catalysts, optical emitters and electronic conductors, with low-toxicity. Such materials have been successfully used to construct excellent and sensitive biosensors. These are powerful and universal tools that yield synergic effects among catalytic activity, conductivity and biocompatibility. Such materials are enabling the design of a new generation of biosensing devices and applications for the detection of important biomolecules such as DNA and proteins, as well as cells.

Highly robust and flexible polymerization techniques (ATRP, etc.) are particularly suited for the preparation of functional bioactive surfaces, including antifouling, antibacterial, stimuli-responsive, biomolecule-coupled and micro-patterned surfaces. In addition to bioactive surfaces, the preparation of functional bulk biomaterials in the forms of stimuli-responsive micelle delivery systems, hydrogels, cationic gene carriers, and polymer–protein conjugates has also been conveniently accomplished by ATRP. All of the above also warrant further studies with the aims of improving detection sensitivity and biosensing mobility.

Considering polymer-based signal amplification strategies, combining various amplification approaches (based on more than one analyte-recognition principle) for the design of biosensors may be a good approach to improve sensor performance. For instance, cascading amplification events by utilizing two or more types of functional materials (e.g., polymers, nanoparticles and enzymes) can offset the insufficiencies of each individual material. This can produce superior results. In additionally, the new eATRP technique has enormous potential for the development of novel polymerization-based electrochemical sensors.

Biosensing requires much interdisciplinary effort due to its complex requirements. Good collaboration between polymer scientists and other scientists will promote new discoveries and advanced technologies for ultra-sensitivity and even single-molecule detection. Scientists, engineers and clinicians must operate in unison to determine the clinical needs for a biosensor and then design a nanosensor with robust signal amplification.

Acknowledgments

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BIOGRAPHICAL INFORMATION

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Legends

Fig. 1 Schematic illustration of signal amplification strategies using polymeric materials as carriers.

Fig. 2 (A) Schematic diagram of the assembly process for the preparation of streptavidin/CdTe-tagged polybeads. The polybead templates were prepared by emulsifier-free copolymerization of styrene with acrylic acid to form PSA cores and layer-by-layer growth of a three-layer polyelectrolyte film. The film consisted of a poly(sodium 4-styrenesulfonate) (PSS) layer sandwiched between two poly(allylamine hydrochloride) (PAH) layers. The positively charged template facilitated the chemical adsorption of negatively charged mercaptoacetic acid-capped CdTe QDs and covalent binding of streptavidin. (B) Procedure for detection of DNA hybridization using streptavidin/CdTe-tagged polybeads (Formed from Ref. 23).

Fig. 3 (A) Schematic illustration of the preparation of polyelectrolyte-encapsulated fluorescent microcrystals. FDA was ball milled into micrometer-sized crystals in an aqueous surfactant (sodium dodecyl sulfate, SDS) medium (to introduce charges onto the originally uncharged microcrystal surface), followed by (a) consecutive encapsulation with polyelectrolyte multi-layers

with nanometer thicknesses, and (b) coupling of the specific antibody. (B) Schematic illustration of the procedure for a sandwich immunoassay using the polyelectrolyte-encapsulated FDA particulate labels. The analyte was first coupled by the capture antibody pre-immobilized on (a) the surface of a solid substrate, and then (b) exposed to antibody-coated microparticle labels. Each microparticle contained approximate 10^8 FDA molecules. (c) Significant signal amplification was achieved after dissolution, release and conversion of the precursor FDA into fluorescein molecules by the addition of DMSO and NaOH (Formed from Ref. 31).

Fig. 4 Schematic representation of the ECL sensing platform for ultrasensitive signal-off detection of cancer cells by using dendrimers/QDs-DNA as labels (Formed from Ref. 46).

Fig. 5 Schematic representation of the functioning principle of an electrochemical immunosensor for detection of α-synuclein. Highly branched polyamidoamine (PAMAM) dendrimer-encapsulated Au nanoparticles (PAMAM-Au) were immobilized onto a carboxyl group-terminated glassy carbon electrode (GCE). After the traditional immunoassay protocol, gold nanoparticle (GNP) linked horseradish peroxidase-secondary antibodies (HRP-Ab2-GNPs) were introduced on the electrode surface. Subsequently, these amplified the signals via an enzymatic catalytic response of HRP related to the H_2O_2-thionine (TH) system. (Formed from Ref. 48).

Fig. 6 Schematic process of a) DDPpy formation and b) its application in the DDPpy directed multiplexing immobilization of biomolecules (Formed from Ref. 54).

Fig. 7 Schematic representation for the use of a water-soluble CP1 with a specific PNA-C* optical reporter probe to detect a complementary ssDNA sequence (Formed from Ref. 70).

Fig. 8 Schematic illustration of the detection of human α-thrombin by the use of an ssDNA thrombin aptamer and a cationic PT (Formed from Ref. 71).

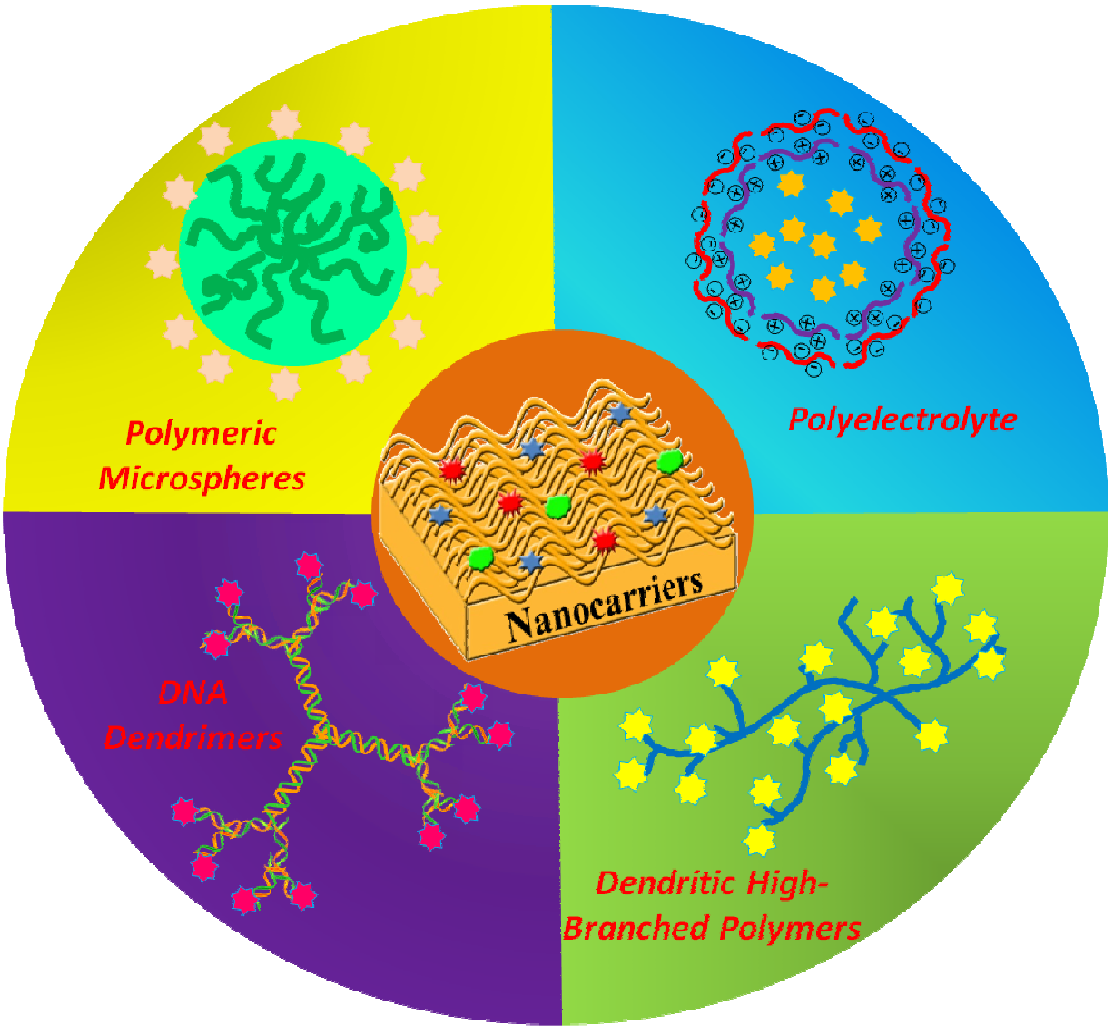
Fig. 9 Schematic representation of the process of PBA (involving biomolecular recognition events) used to introduce initiators on a biochip surface. After the polymerization, abundant functional groups in side chains increase the local concentration of signal tags (either inherently or through covalent binding), yielding an enhanced signal readout. High sensitivity and exceptional selectivity are achieved by integrating polymerization with ligand-target binding events.

Fig. 10 Schematic representation of tyramide signal amplification based on common enzyme-linked immunosorbent assays (ELISA) using quantum dot (QD)-tyramide conjugates as labels (Route 1); polymerization-based amplification in s sandwich immunoassay via surface-initiated ATRP of glycidyl methacrylate (GMA) and subsequent direct binding of CdTe QDs (Route 2); and, a sandwich immunoassay using QD−tyramide conjugates as labels via surface-initiated ATRP and tyramide signal amplification (Route 3). The o-aminobenzoic acid (*o*-ABA) was used to produce a poly(*o*-ABA) (PAB) film modified surface with abundant

carboxyl groups via electropolymerization for immobilization of antibodies. Electrochemiluminescence (ECL) and square-wave voltammetric (SWV) measurements were employed for the demonstration of signal amplification, respectively. (Formed from Ref. 80).

Fig. 11 Schematic representation of photopolymerization for signal amplification from a biochip surface. Varied amounts of biotin-labeled oligonucleotide are present on each row of spots on the chip surface. The photoinitiator is stabilized exclusively at positive sites through biotin-streptavidin binding. In cases where the photoinitiator molecule is fluorescent, the allocation of initiator can be verified and quantified through fluorescence scanning. Polymerization is then started by adding monomer solution and applying irradiation. A cross-linked hydrogel then grows and becomes apparent to the unaided eye. In the absence of the target polymer, growth is not observed at negative spots (Formed from Ref. 83).

Fig. 12 Schematic representation of a signal amplification approach using enzyme-mediated polymerization for biodetection. A biotinylated secondary antibody (B) is coupled to the prior antigen (A) which is recognized by the primary antibody. The biotin functionalized surface is incubated with a GOD-avidin conjugate (C) endowing the surface with specifically bound GOD that triggers the polymerization after addition of a monomer solution containing poly(ethylene glycol) diacrylate, hydroxyethyl acrylate, rhodamine B-methacrylate (indicated by stars), glucose, $FeSO_4$, and MES. Finally, the resulting fluorescent polymer can be qualitatively observed by the unaided eye or can be quantified by using a plate reader (Formed from Ref. 90).



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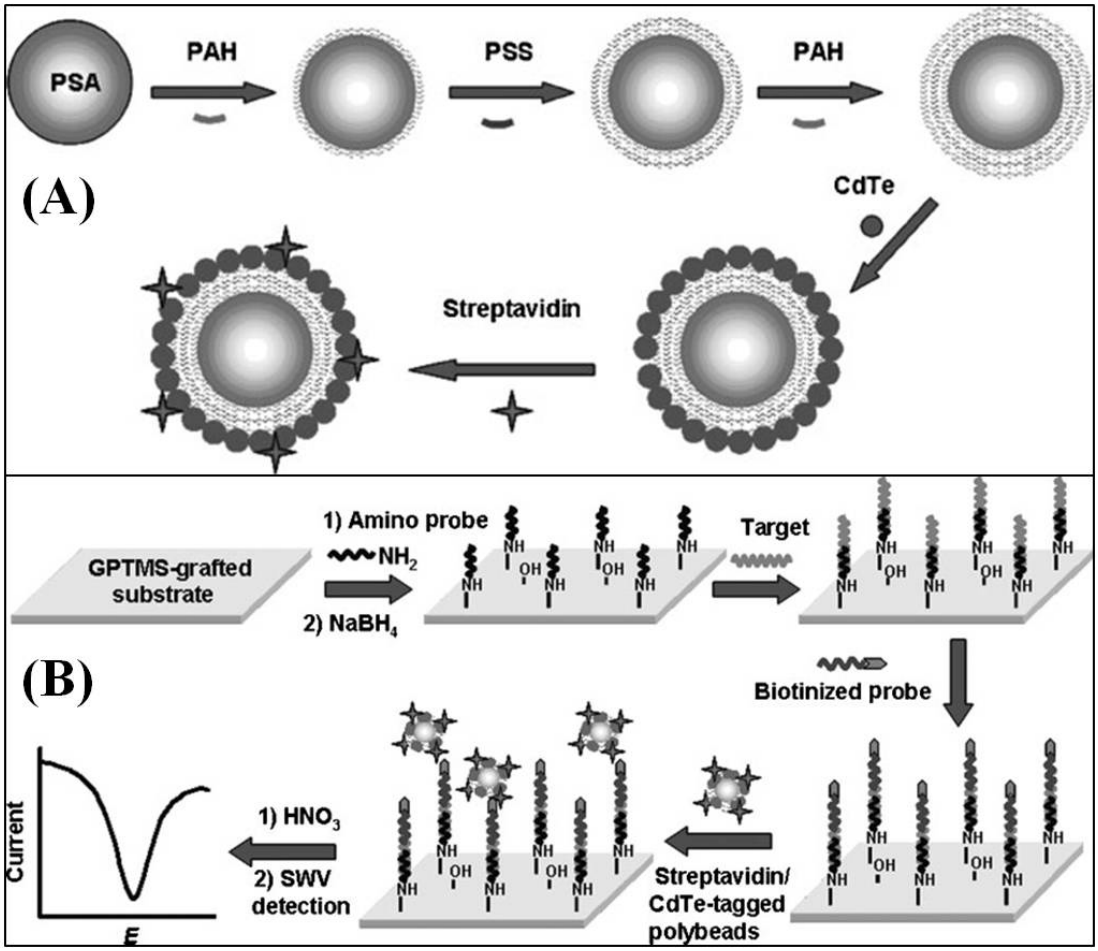

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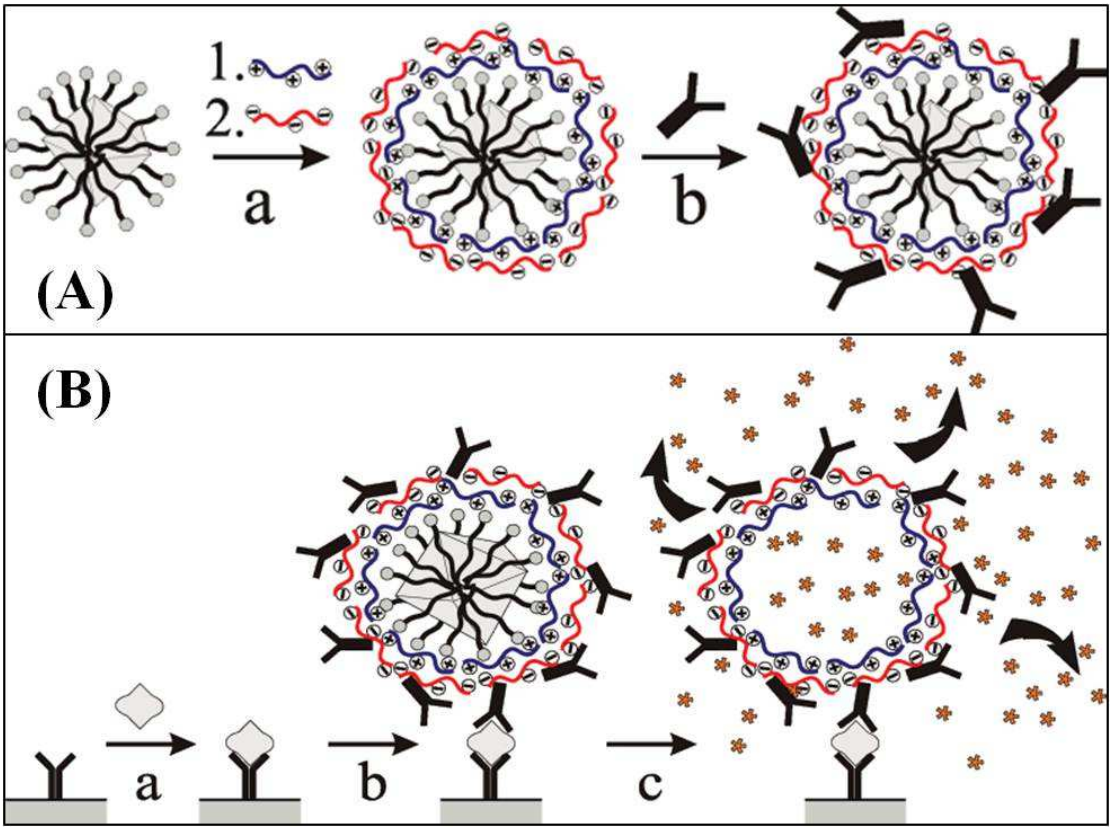

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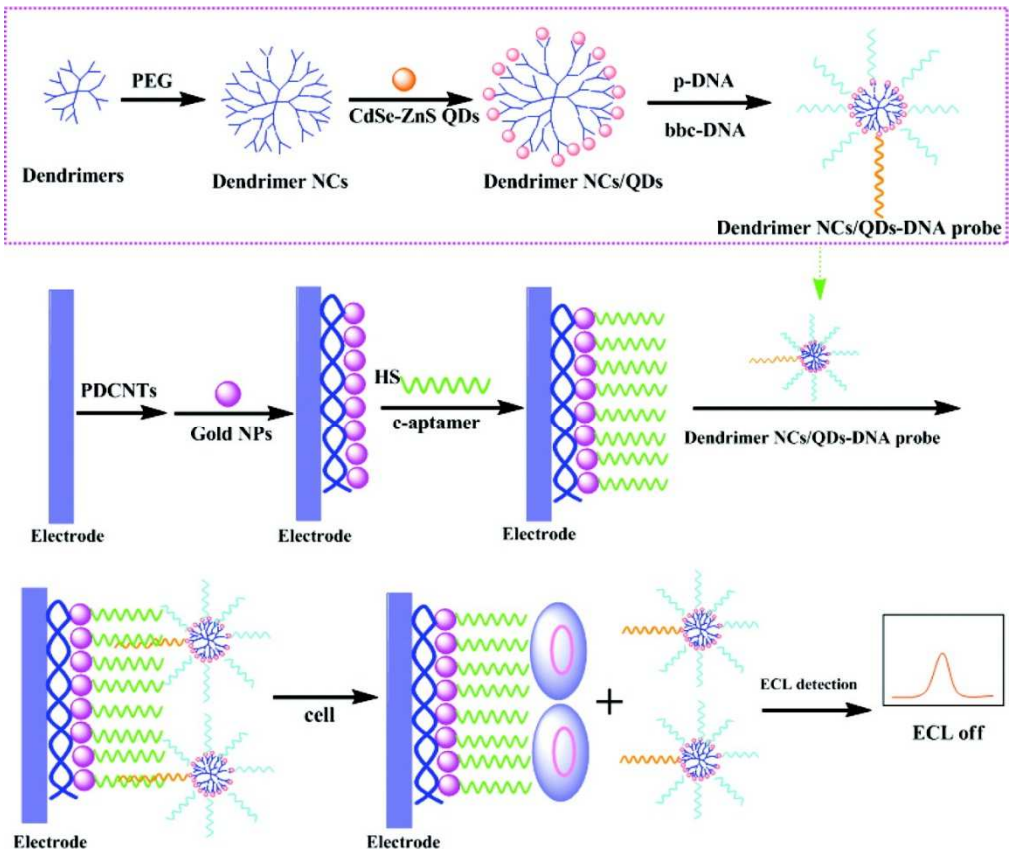

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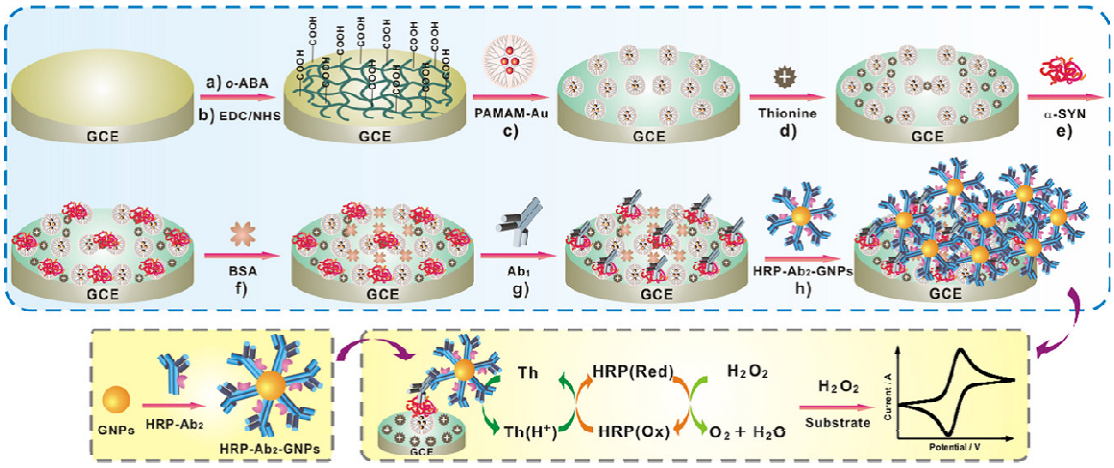

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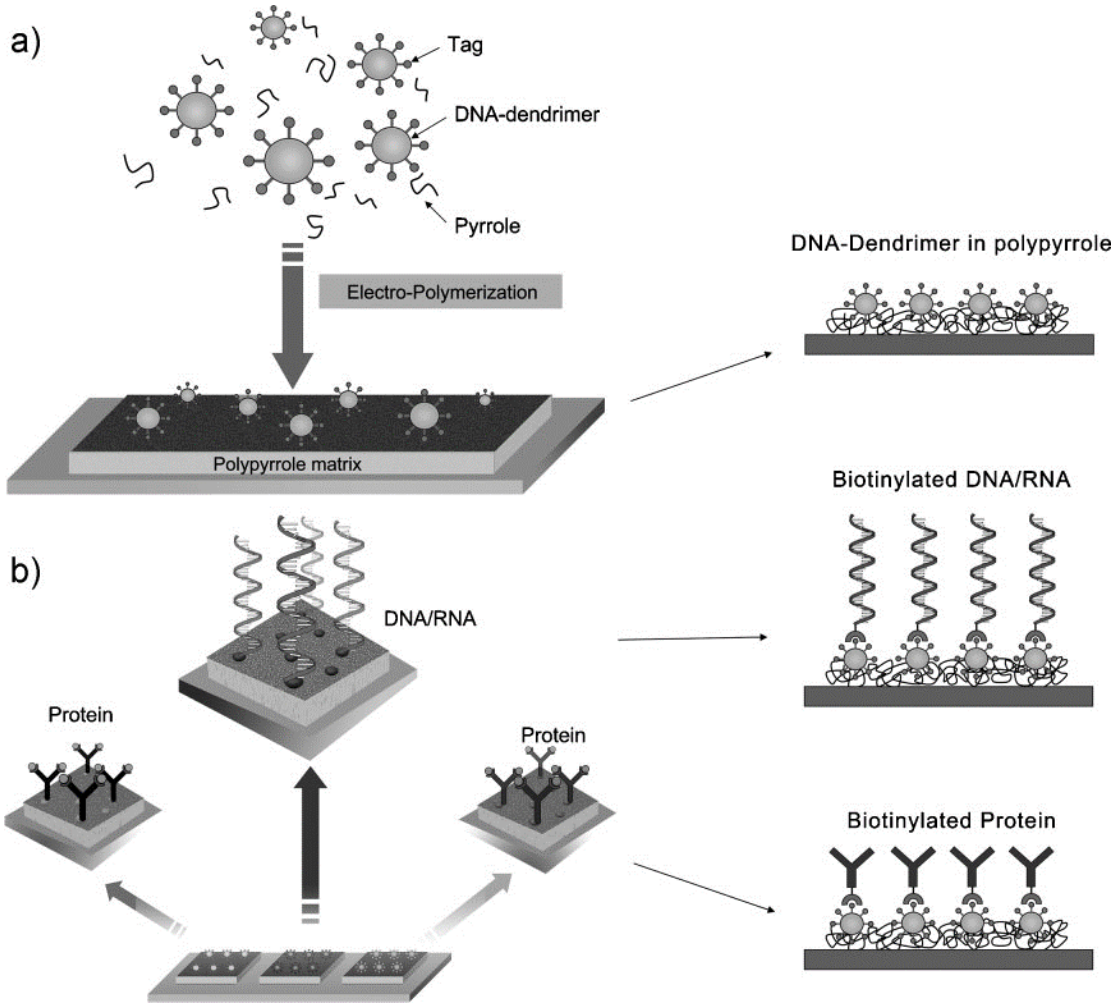

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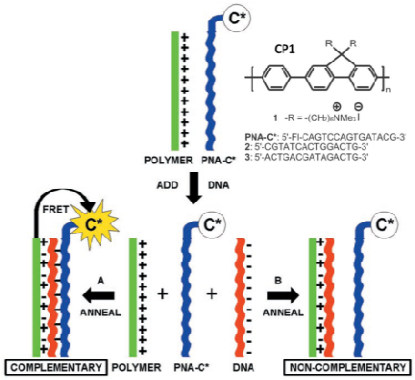

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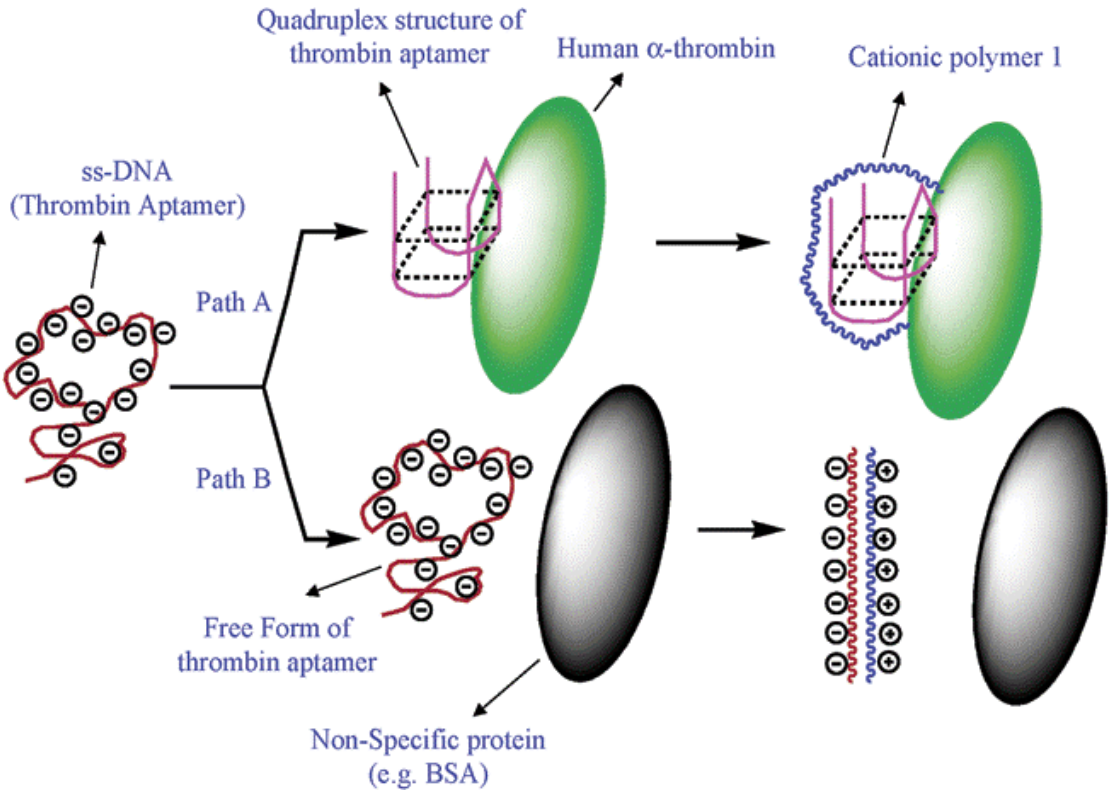
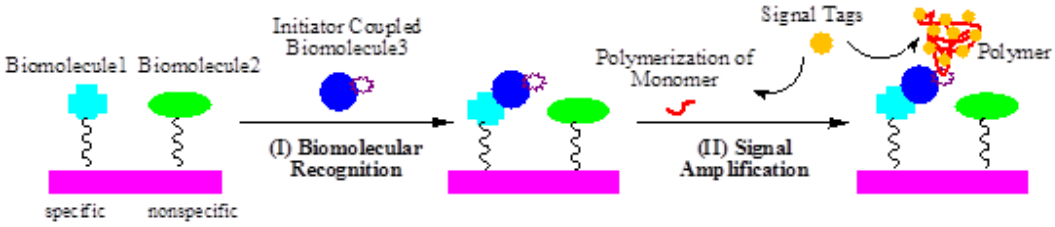


Fig 8 .



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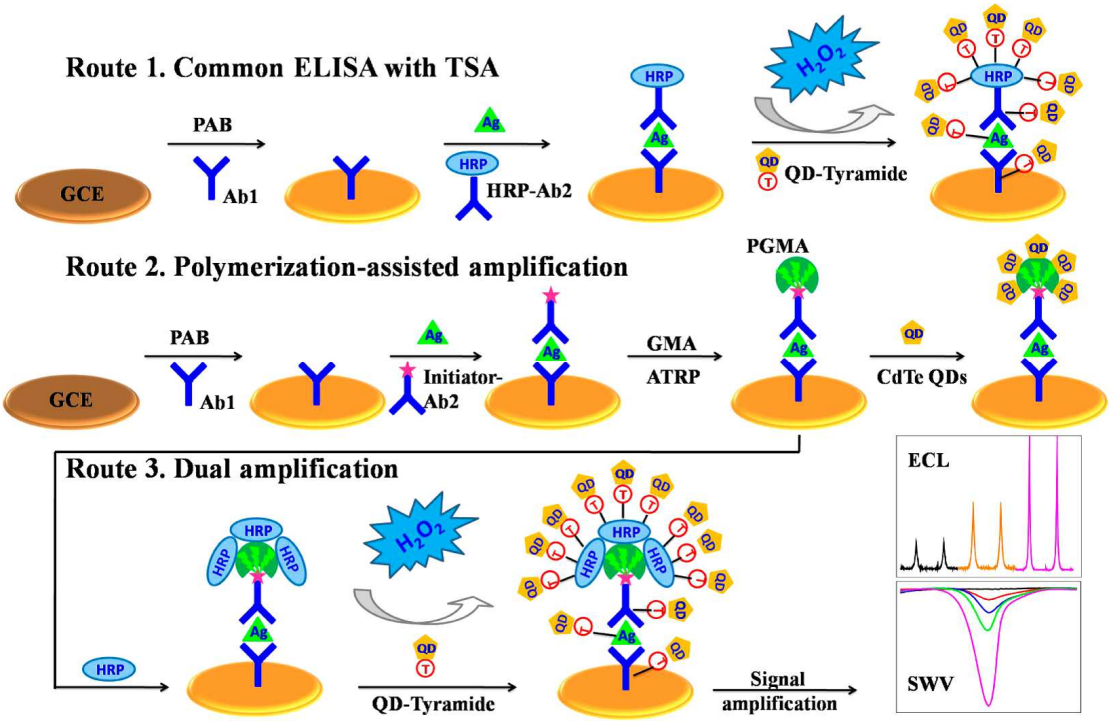

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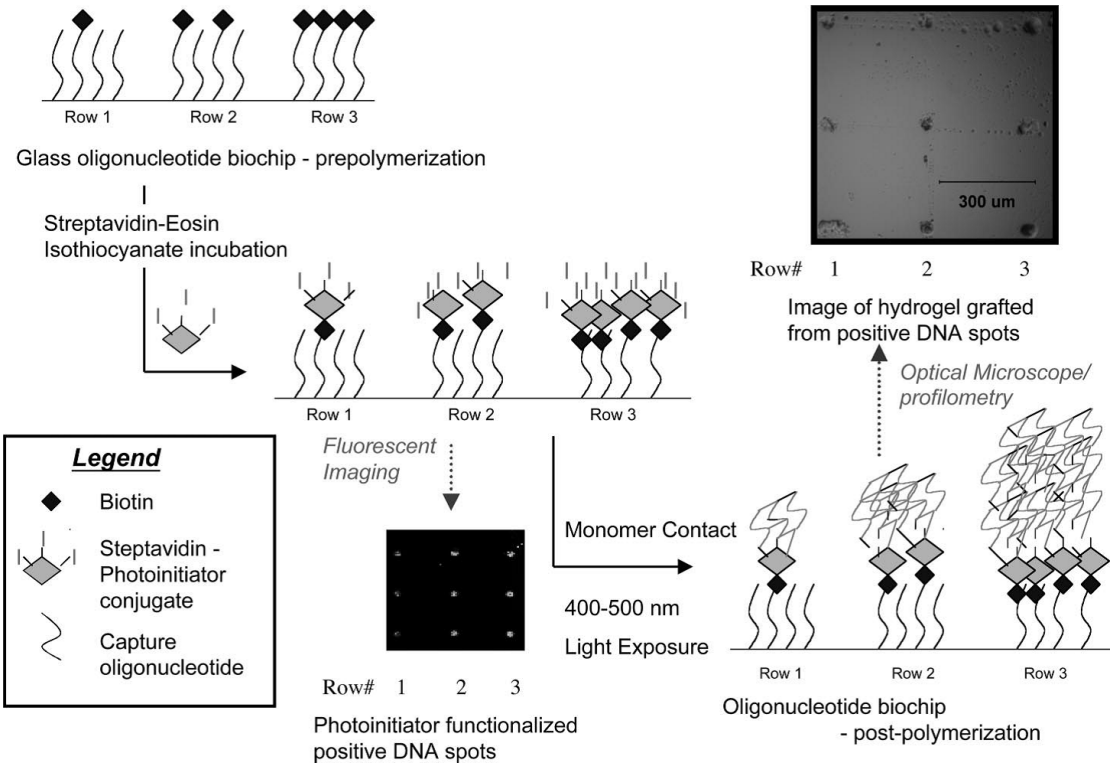

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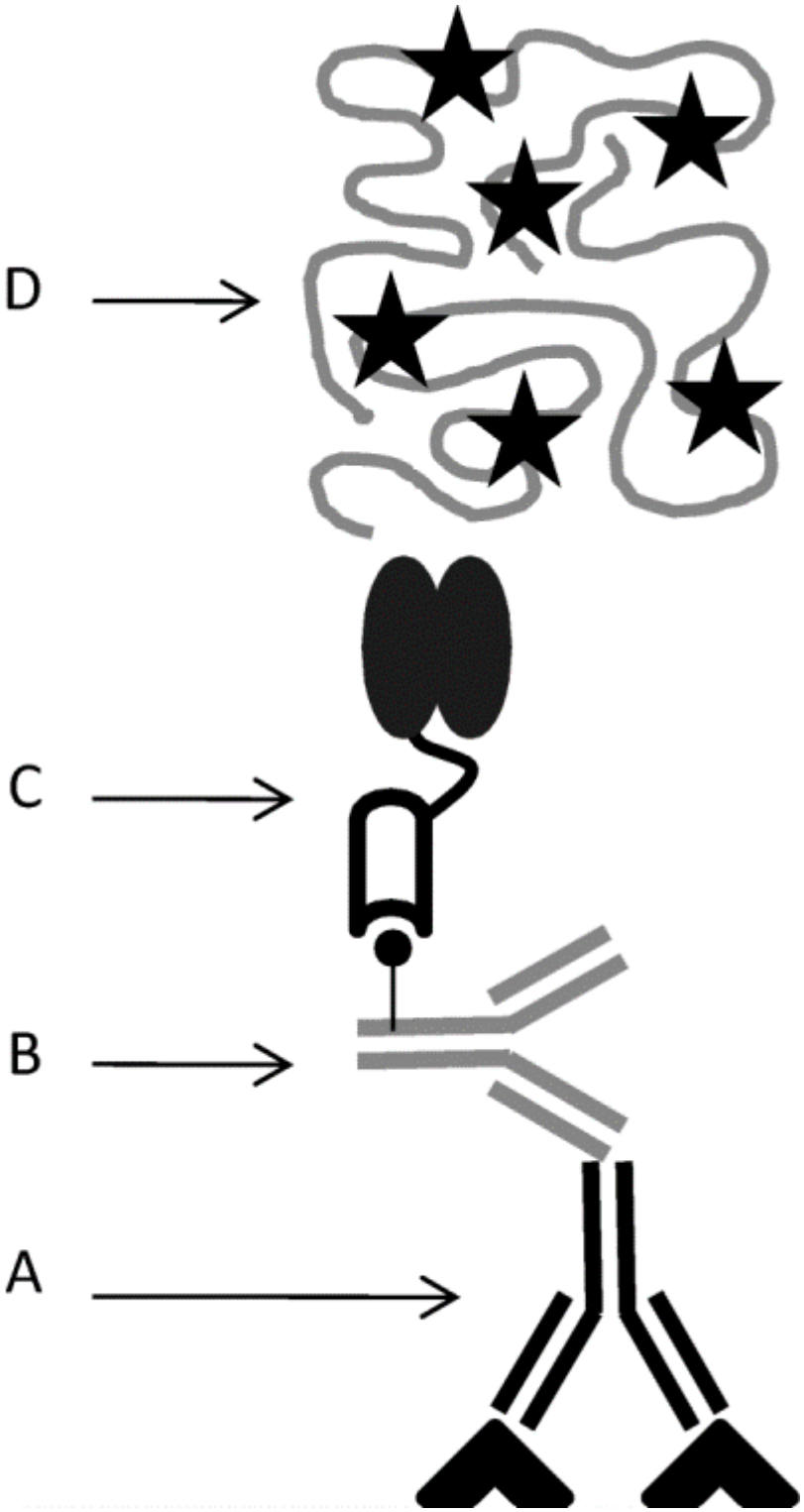

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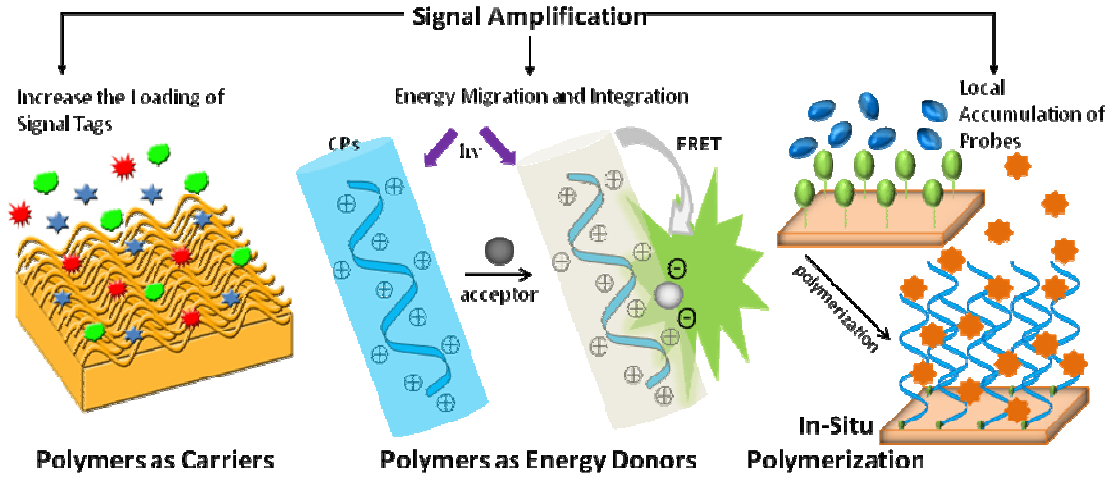

Graphical abstract