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Synthetic materials capable of reporting biomolecular recognition events by chromic transition

Patrick Englebienne

Department of Nuclear Medicine, Free University of Brussels, Brugmann Hospital, Place Van Gehuchten 4, B-1020 Brussels, Belgium. E-mail: patrick.englebienne@skynet.be

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This article presents a survey of synthetic materials capable of reporting in real time by chromic transition the recognition event occurring between a specific ligand and a biomolecule such as a receptor or an antibody. The review focuses on conductive polymers and colloidal gold. Besides their mode of synthesis and coupling to biomolecules, it explores their physicochemical reactivity, emphasizing the mechanisms underlying the chromic transition occurring upon the biomolecular recognition event. The future prospects offered by composite materials based on these polymers and colloids are further discussed.

Introduction

Biomolecular recognition occurs when a biomolecule such as a receptor or an antibody meets its specific ligand. The encounter leads to the binding of one species by the other. The biomolecular recognition event is usually monitored by the introduction of a label into one of the complementary binding molecules, which transduces the recognition event into a signal.

As a result of important advances made these last few years in the field of synthetic polymers and colloids usually qualified as 'intelligent' in the literature,[1] the means for monitoring biomolecular recognition events have evolved quite tremendously. These advances have allowed the design of efficient biosensors capable of a high level of user-friendliness and automation.[2]

A biosensor is a device comprising either a ligand or a receptor (antibody) species coupled to a signal transducer, which detects the binding of the complementary species. As illustrated by the sketch presented in Fig. 1, an indirect biosensor uses a separate labelled complementary species which is detected after the binding event has occurred, by measuring the activity of the label (*i.e.* radioactivity, fluorescence, chemiluminescence) associated with the complex. In the field of immuno- and receptor-assay technology, an indirect biosensing system is termed heterogeneous because the ligand–receptor complex must be sequestered from the free fractions before recording the signal of the label. On the other hand, a direct biosensor (*i.e.* homogeneous immuno- or receptor-assay) comprises a ligand or a receptor respectively coupled to a signal transducer detecting in real time the binding of the complementary species by a change in potential difference, current, resistance, mass, heat, or optical properties.[3]

Chemical materials able to serve as transducers in direct biosensors are currently hot topics in biomedical research. The so-called 'intelligent' materials are particularly interesting candidates for direct biosensing systems. These are polymers or colloids sensitive to external stimuli, which experience respective changes in their structure or in either their chemical or physical properties in response to changes in their environmental conditions such as temperature, mass, pH, light, electric field, or oxido-reduction. As a result of their physical responsiveness to temperature, many of these new materials have been primarily explored as new controlled drug-delivery systems.[1] However, the modifications experienced by these materials from ground to new environmental conditions can be as varied as a change in volume,[4] in color,[5] from solid to liquid, from water-soluble to water-insoluble,[6–9] from extinct to light emitting.[10–13] Consequently, many of the modifications in their properties as induced by changes in their environmental conditions can be detected by common means, which make them suitable candidates for transducer elements for biosensors. The concept is illustrated in Fig. 2. Some of these materials have been used successfully in various direct biosensing systems in which the binding event induced respectively either a change in solubility,[14] a change in volume,[15] or the fusion,[16] of the polymeric materials. Among these new materials, those reporting a change in their environmental conditions by a chromic transition are of major interest for application in direct biosensors because the change in their optical properties occurring upon a biomolecular recognition event can be detected by a simple photometer and sometimes even by the naked eye. Among such materials, conductive polymers and colloidal solutions of gold particles are very promising and will be considered in this article.

Conductive polymers

Nature and properties

Conductive polymers are extensively conjugated organic macromolecules made of repetitive sequences of respectively alkyne, phenyl or heterocyclic monomers. These polymers can gain near-metallic conductivity by oxidation (p-doping) or reduction (n-doping) from their insulating state.[17] The structures of the most extensively studied conductive polymers, along with the conductivities they can attain, are displayed in Fig. 3. For comparison, the conductivity of metallic copper is 5.8×10^7 S cm^{-1}.[18] Another important feature common to the conductive polymers is their capacity to undergo chromic transitions upon changes in their oxidation state.[19] The conductive polymers are intrinsically semi-conductors in which the highest occupied (HOMO) and lowest unoccupied molecular orbitals (LUMO) can give rise to valence (π) and conduction (π^*) bands. The energy gap (E_g) between the HOMO and LUMO electron bands determines the intrinsic electrical and optical properties of a given polymer.[20] The doping process modifies the electronic band structure of the polymer by producing new electronic states that are localized in the band gap (polarons and bipolarons) and cause its passage from insulating to conducting as well as its chromic transition.[21,22] In cyclic conductive polymers, these transitions are indicative of the passage from aromatic to quinonoid molecular forms. The process of interband electronic transition is schematically illustrated in Fig. 4. In chemical terminology, a polaron is a radical ion (spin 1/2) and a bipolaron is a dication (pair of like charges, spinless). In their insulating conformation, the

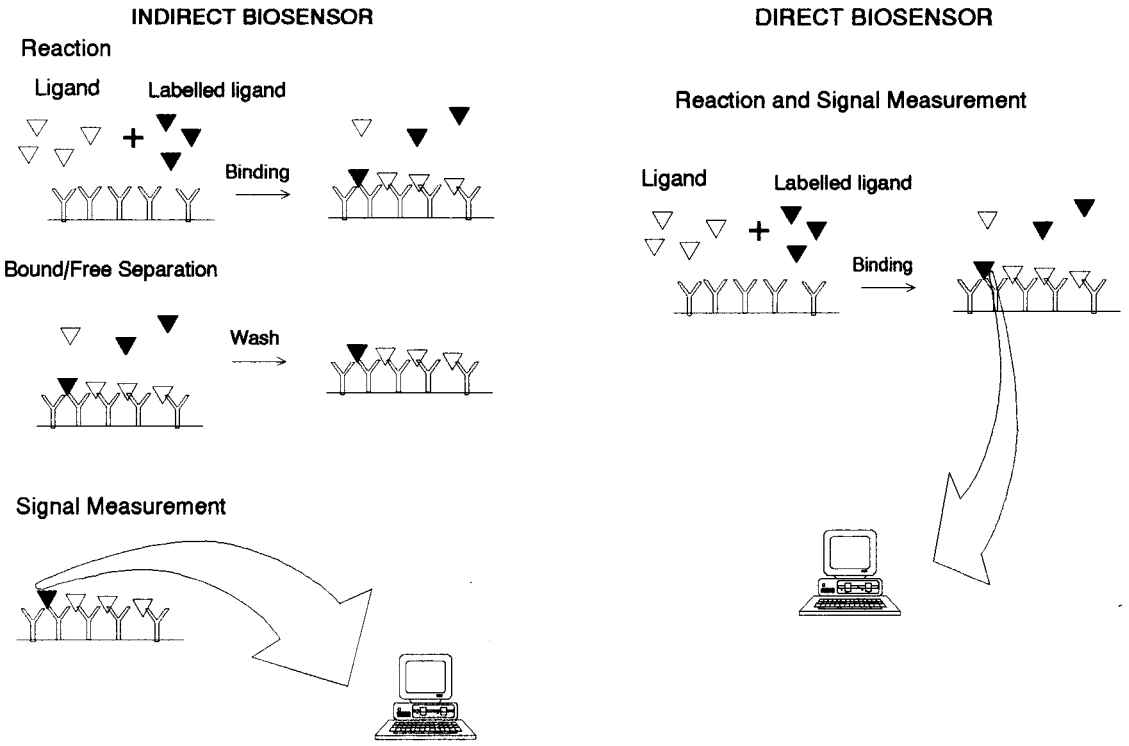


Fig. 1 Sketch emphasizing the difference between indirect (heterogeneous) and direct (homogeneous) biosensing systems.

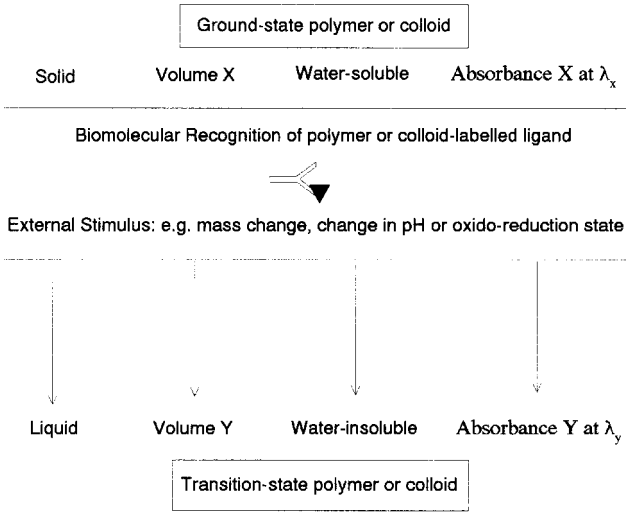


Fig. 2 Schematic diagram illustrating the concept of applying 'intelligent' synthetic materials in direct biosensing systems for biomolecular recognition.

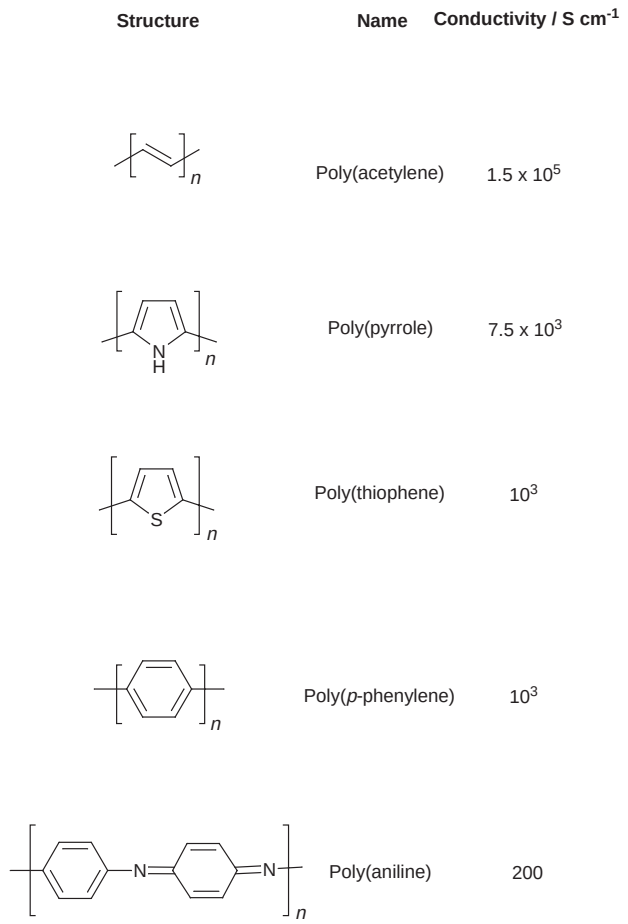


Fig. 3 Structures and conductivities of the most extensively studied conductive polymers.

E_g of conductive polymers is usually above 1.5 eV. In the case of poly(pyrrole), it levels at 3.2 eV and by increasing p-doping it decreases progressively to 2.7 and 1.0 eV, respectively.[20]

When compared to other cyclic conductive polymers, poly(aniline) presents some unique structural features which allow for the control of its conductive and optical properties.[23] This control results from the possibilities that exist to fine-tune the level of oxidation and protonation of poly(aniline). These possibilities are the consequence of the many structural variations due to the alternation in the polymer backbone of *p*-phenylene rings with the nitrogen atoms. The degree of oxidation depends on the fraction of these latter which is imine or amine. This is exemplified by the two extreme structures in Fig. 5A and B, with respectively a 0 and 100% degree of oxidation. In between these two extreme states, the degree of oxidation may be varied in order to afford numerous

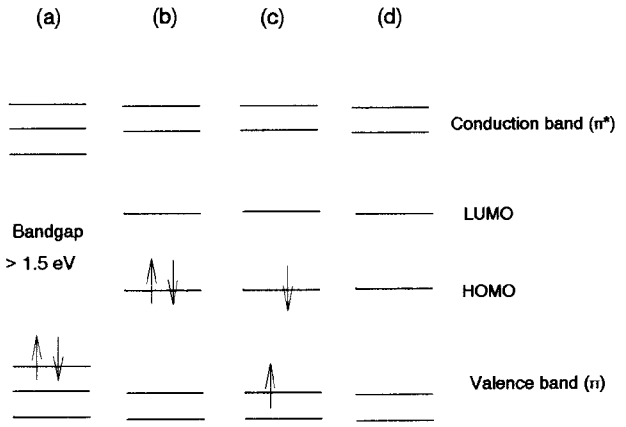


Fig. 4 Schematic illustration of the energy level of one electron organic molecule in its electronic configuration adopting the equilibrium geometry of respectively (a) its ground state, (b) its first ionized state, (c) its polaron and (d) bipolaron states.

intermediate structures each displaying different conductivities and electronic absorption spectra upon external protonation. When an anionic substituent capable of protonation is introduced on the *p*-phenylene ring, the polymer structure may be in a partial conductive form when the substituent is protonated (Fig. 5C). This latter structure transforms into the fully conductive form upon self-doping (Fig. 5D) resulting from the loss of the proton by the substituent.[24] The E_g and chromic transitions of poly(aniline) upon n-doping are illustrated by the electronic absorption spectra in Fig. 6.

Another class of conjugated polymers worth mentioning is the substituted poly(diacetylenes). As illustrated in Fig. 7, the structure of these polymers is made of ene–yne alternating bonds. The molecules of this polymer class are particularly flexible and they undergo important color changes when subjected to planar–nonplanar conformational transitions.[25]

Up to the mid eighties, all the conductive polymers available were soluble only in organic solvents,[26–28] and their use in aqueous biological systems was consequently restricted to that of electronic transfer films incorporated in amperometric sensors.[29–31] These water-insoluble polymers can only be doped by the addition of oxidative dopant counter-ions which diffuse in and out of the polymer backbone to balance the charges created.[32] This process is called external doping and is illustrated in Scheme 1a with poly(thiophene) as an example. In the late eighties, it was observed that the introduction of an alkanesulfonate sidechain in cyclic or heterocyclic monomers yielded water-soluble conductive polymers.[33,34] Moreover, because the counter-ion is attached to the polymer through a covalent bond, these water-soluble polymers can lose a proton upon oxidation, simultaneously with the loss of an electron to

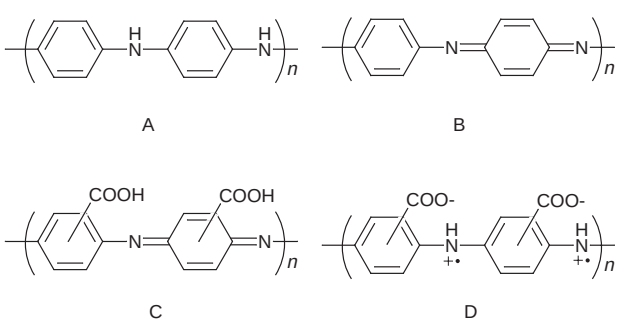


Fig. 5 Extreme structures of poly(aniline) with an oxidation degree of 0 and 100% (respectively A and B). The introduction of a carboxylic substituent on the *p*-phenylene ring transforms the polymer into a partially conductive form (C), capable of affording the fully conductive form (D) upon self-doping.

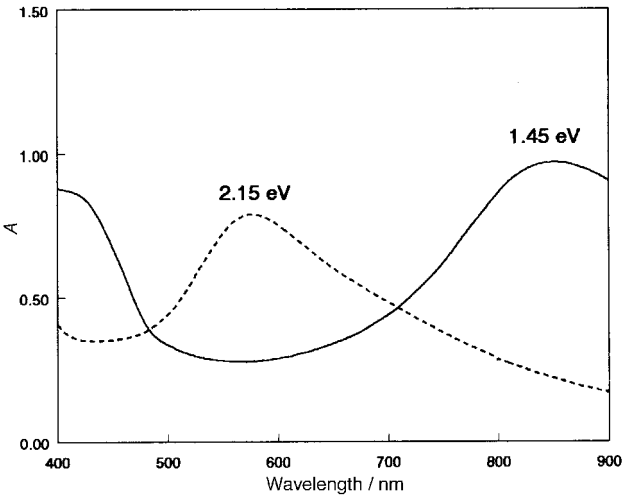


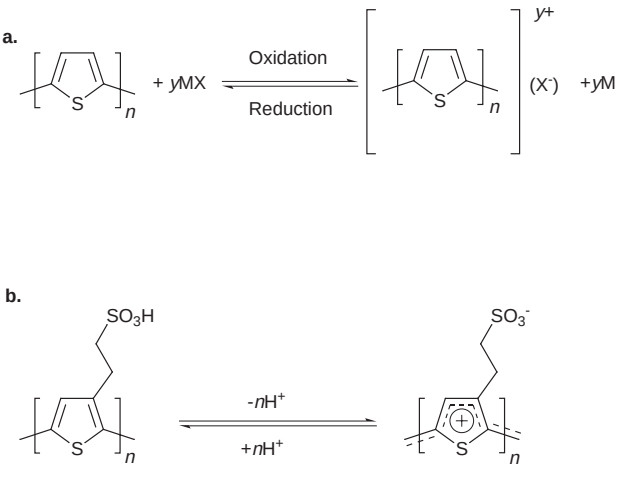
Fig. 6 Electronic absorption spectra of colloidal poly(aniline) respectively in its oxidized (dotted line, E_g 2.15 eV) and n-doped state (solid line, E_g 1.45 eV).

produce self-doped polymers. The process of self-doping is illustrated in Scheme 1b with poly(thiophene-3-ethanesulfonate) as the example. The advent of water-soluble and self-doped conductive polymers allowed for their direct use in biochemical reactions, particularly as electron transfer means in enzyme sensors.[30,35]

Synthesis

Whether soluble or insoluble in water, most conductive polymers are synthesized by electrochemical or chemical means. These synthetic procedures have been extensively reviewed, in particular for poly(thiophene).[36] Whether chemical or electrochemical, the most commonly used synthetic processes occur through the oxidation of the monomers. The most commonly used chemical oxidants are iron(III) chloride and ammonium persulfate.

When substituted heterocyclic monomers are used as starting blocks, oxidative polymerization presents the drawback of a lack of control of the structure of the oligomers or polymers formed. As shown in Scheme 2, the resulting substituted polymers are structurally inhomogeneous.[37] This may be of importance for the application in biomolecular recognition because there is experimental evidence that the regiochemistry of substituted poly(heterocycles) controls their conformational features, which in turn, governs the degree of π–π conjugation between adjacent rings.[36] This control has consequently a



Scheme 1

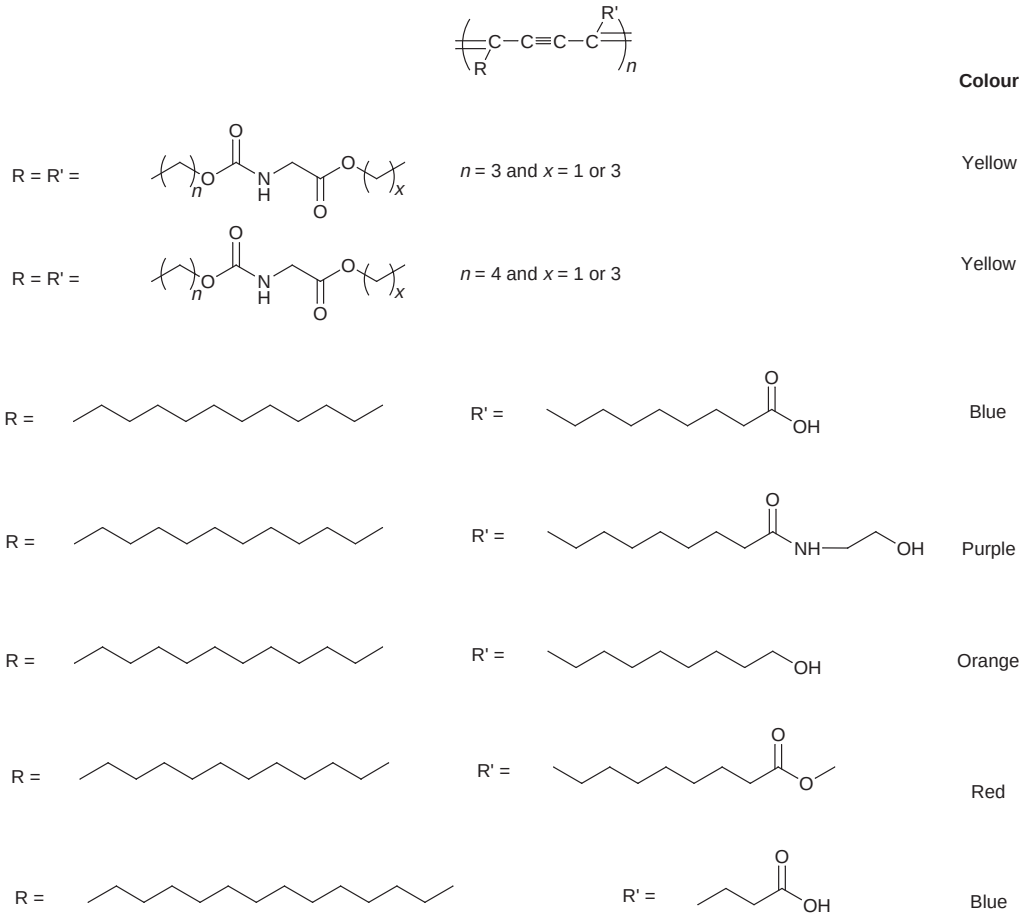
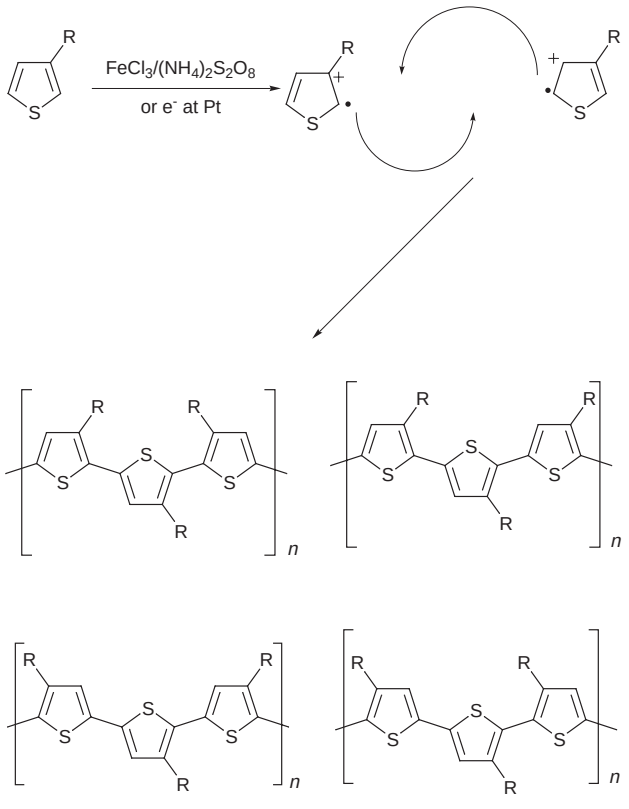


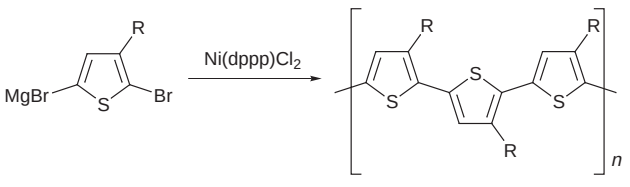
Fig. 7 Examples of substituted poly(diacetylene) structures and their corresponding colour in the coplanar conformation.



Scheme 2

tremendous influence on the capacity of the polymer to translate a departure from coplanarity by a chromic transition.[38] Poly(thiophene) derivatives are in this respect of particular interest because of their specific photochemical properties.[11,12] As discussed below, such departure from coplanarity is an important mechanism of transducing biomolecular recognition. As shown in Scheme 3, control of the polymer architecture can be achieved by coupling 2-bromo-3-alkyl-5-thienylmagnesium bromide in the presence of a nickel catalyst.[39] Other strategies using the cross coupling of 2-bromo-3-alkylthiophene with 2-(trimethylstannyl)-3-alkylthiophene monomers have also been reported to provide a similar advantage.[40]

It has been claimed that water-soluble poly(anilinesulfonate) could not be synthesized by chemical or electrochemical oxidation from *o*-aminobenzene- or *m*-aminobenzene-sulfonic acid because the substitution of a hydrogen atom on the phenyl ring by electron withdrawing substituents like $-SO_3H$ led to an increase of the steric hindrance and to a strongly deactivating influence likely to limit the polymerization process. Until recently, this problem was addressed by sulfonating the already polymerized aniline with fuming sulfuric acid.[24]



Scheme 3

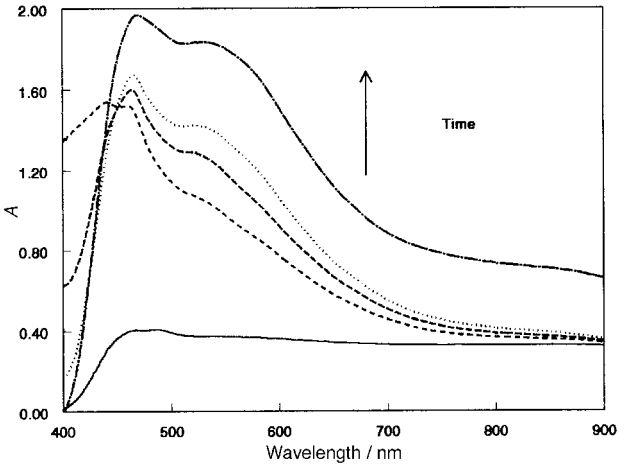


Fig. 8 Difference electronic spectra recorded at various times (3, 5, 120, 192 and 288 h; arrow) during the polymerization of *o*-anthranilic acid in aqueous phase using ferric chloride as the oxidant.

This strategy leads however to ill defined and partially sulfonated polymers. The question is still under debate and is quite controversial. In a recent paper,[41] the synthesis of fully sulfonated poly(aniline) from *o*- and *m*-aminobenzenesulfonic acid by chemical oxidation in the liquid phase under high pressure has been reported. Poly(aniline) substituted with a carboxylic acid group has been obtained by oxidative polymerization of *o*-anthranilic acid with ammonium persulfate, affording a water-soluble polymer.[42] However, the UV–vis spectra of the polymerized material showed no absorption in the visible region and the authors concluded that the carboxylic acid substituent restricted the π-conjugation along the polymer chain. However, the successful electropolymerization of substituted poly(anilines) from *o*- and *m*-aminobenzoic acid as well as from *m*-aminobenzenesulfonic acid has also been reported and the polymers obtained were shown to be self-doped and electrochemically active in aqueous solutions.[43] In a more recent publication,[44] we have shown that conjugated and water-soluble poly(*o*-anthranilic acid) could be obtained by chemical oxidation with ferric chloride. Progressive π–π* transitions at lower energy levels (475 nm, 2.6 eV and 530 nm, 2.3 eV) occur during the polymerization process as shown in the difference spectra in Fig. 8, which are indicative of the progressive conjugation along the polymer backbone during synthesis. The same approach allowed us to polymerize also various substituted poly(thiophenes).[44,45]

Conductive polymers have also been synthesized in colloidal form, particularly poly(aniline) and poly(pyrrole).[46–48] These materials can only be externally doped since they do not contain any counter ion covalently linked to the polymer backbone. One exception to this is the colloidal copolymers of poly(pyrrole) and poly(styrenesulfonate), the sulfonate counter-ions being incorporated into the poly(pyrrole) during the polymerization providing cation-exchange sites.[49] This latter copolymerization approach could generate in the near future new composite materials with promising properties for application in the biomolecular recognition field.

Finally, substituted poly(diacetylenes) on the one hand can be produced by 1,4 addition of the substituted diacetylenic monomers initiated by either γ[25] or UV[50] irradiation. On the other hand, soluble derivatives of poly(acetylene) have been obtained by ring-opening metathesis polymerization of monosubstituted cyclooctatetraenes.[51]

Chromic transition of water-soluble conductive polymers upon oxido-reduction

As a consequence of their self-doped nature, water-soluble conductive polymers are highly sensitive to the presence of protons and hence to pH. A decrease in pH enhances doping and conversely, pH increases progressively undope the polymer. The doping–undoping process induced by pH changes is translated by important modifications in the absorption at the wavelength corresponding to the π–π* transition of the polymer. The pH effect on a buffered solution of poly(*o*-anthranilic acid) is illustrated in Fig. 9. It is worth noting that when the conductive polymers most effective at translating a biomolecular recognition process by chromic transition are considered, the highest pH effect is observed around physiological pH values, such as in the example of Fig. 9. In other cases, the range of pH sensitivity may be broad and this property can be applied to the fabrication of reversible optical sensors for pH measurement such as recently reported with poly(pyrrole).[52]

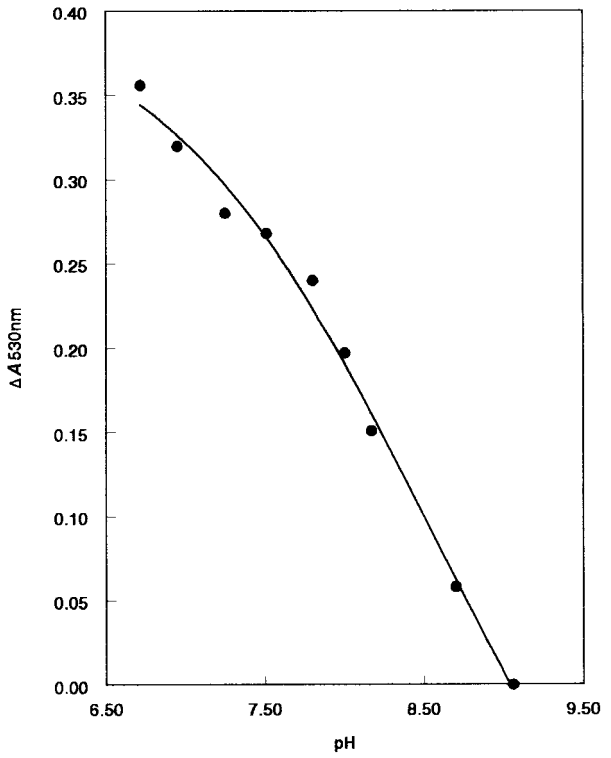


Fig. 9 Evolution of the $A_{530\ nm}$ of a diluted buffered solution of poly(*o*-anthranilic acid) as a function of pH between pH 6.5 and 9.

Similarly, the respective addition of oxidants or of reductants to the polymers at a fixed pH exerts a profound influence on the absorbance (A) at the π–π* transition wavelength. This effect is illustrated with poly(*o*-anthranilic acid) in Fig. 10. The addition of increasing millimolar concentrations of ammonium persulfate to the polymer solution buffered at pH 9 increases the $A_{530\ nm}$ and conversely, the addition of the same increasing concentrations of sodium borohydride to the polymer solution buffered at pH 7 decreases the A at the same wavelength in a corresponding way. This capacity of conductive polymers to respond spectrophotometrically to oxido-reduction has been applied to the quantitative detection of ascorbic acid using poly(thiophene) films.[5] We have adapted the technique in an automated clinical chemistry analyzer using poly(*o*-aminobenzenesulfonic acid) and poly(*o*-anthranilic acid) in aqueous solution. In these conditions, the polymer reduction by ascorbic acid monitored by photometry occurs in less than 10 min.

The chromic sensitivity of conductive polymers to both pH and oxido-reduction could lead in the near future to the availability of cheap analytical reagents or instruments, finding application in clinical chemistry, pharmacology, biotechnology or the food industry.

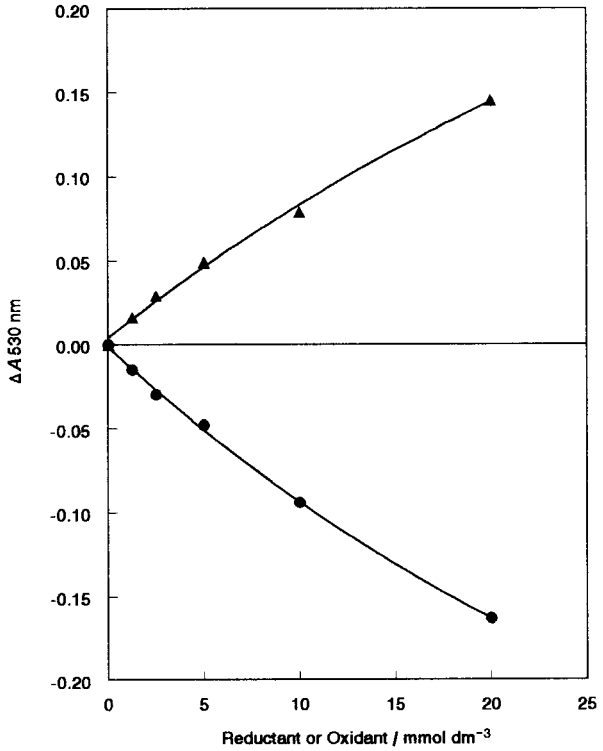


Fig. 10 Effect of increasing concentrations of either ammonium persulfate (triangles) or sodium borohydride (circles) on the *A* at 530 nm of poly(*o*-anthranilic acid) diluted in 50 mM phosphate buffer adjusted respectively at pH 9 or 7.

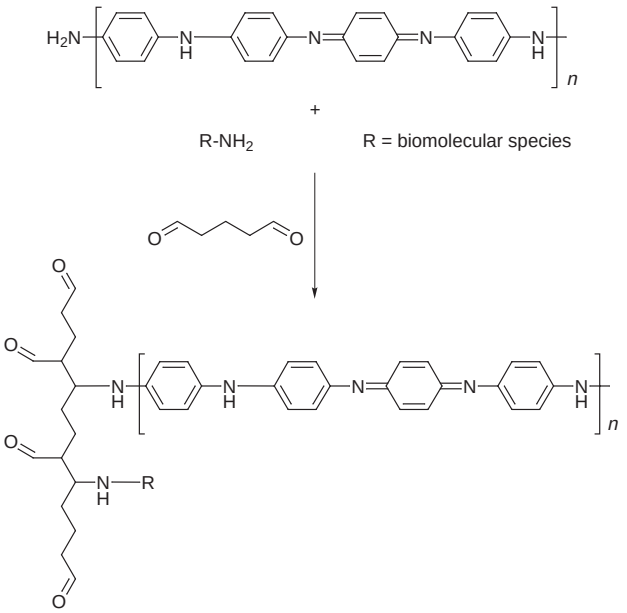
Covalent coupling of water-soluble conductive polymers to biomolecules

The covalent linkage between one of the biomolecular reactants and the water-soluble conductive polymer label is of prime importance because it is supposed to secure the close proximity of the label to the biomolecular recognition site. Consequently, zero-length linkages or small bridges are usually preferable to long connecting arms. This prerequisite is of less importance for water-insoluble polymers since the mechanism of transducing the biomolecular recognition event is likely to proceed from different principles which are discussed in the next section.

Most molecules used in biomolecular recognition are proteins, peptides or sugars which contain amine, hydroxy, mercapto or carboxylic groups available for coupling to conductive polymer labels. When the molecule to be labelled is a small ligand such as a steroid or a drug which does not contain vacant functionalities, it must be chemically modified prior to coupling.

As shown in Scheme 4, poly(aniline) contains terminal amines which can be used in a cross-linking reaction to corresponding amines in proteins or peptides through glutaraldehyde. Except for poly(aniline), most conductive polymers do not have corresponding groups available for coupling. Consequently, substituting the polymer backbone is useful not only for granting self-doping capacity and water solubility, but also for providing vacant functionalities for their covalent coupling to ligands and receptors. For instance, the substitution of poly(thiophene) with carboxylic acid groups allows for its covalent linkage in aqueous solutions to amine residues (*i.e.* ε-amino groups of lysine in proteins) in presence of a water-soluble carbodiimide such as 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide. The carbodiimide forms a reactive carboxylic anhydride intermediate (Scheme 5) which further reacts with the amine to form an amide bond.[53]

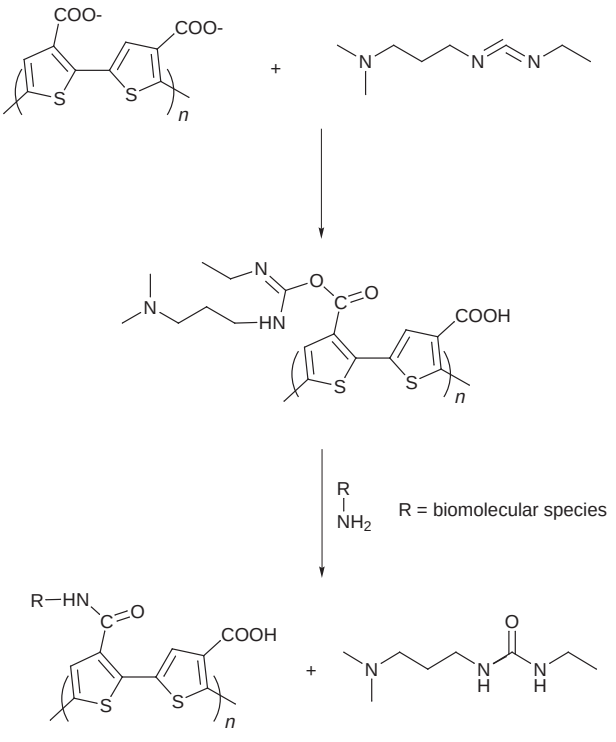
Alternatively (Scheme 6), conductive polymers which contain a reactive heterocycle such as poly(pyrrole) can be either *N*- (Scheme 6a) or *C*- (Scheme 6b) acylated after polymerization with bromoacetyl bromide, depending on the synthetic conditions. These derivatives can then further be either carboxylated (Scheme 6c) or aminated (Scheme 6d) by being reacted with either mercaptoacetic acid or triethylenetetramine, respectively.[54] The derivatized polymer can then be linked covalently to ligands, receptors or antibodies.



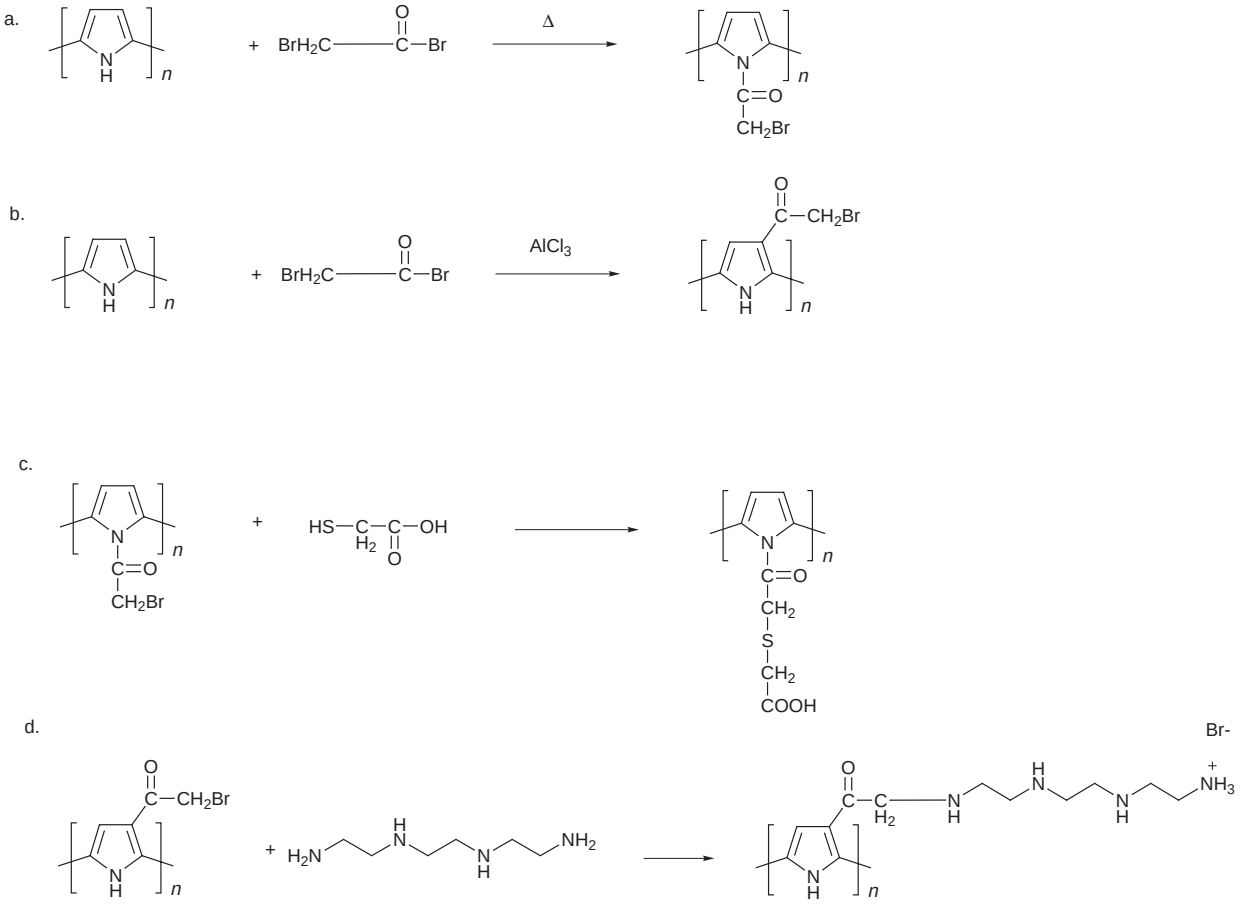
Scheme 4

Chromic transition of conductive polymers upon biomolecular recognition

Colloidal conductive polymers have been used as early as in 1992 by Tarcha's group at Abbott Laboratories as reporter reagents in solid-phase heterogeneous immunoassays.[55] In this particular application, advantage was taken of the deep colour of the colloidal particles so as to detect either by densitometry or visually any binding of antibody coated particles to the



Scheme 5



Scheme 6

antigen previously captured by the solid phase. The mechanism of indicating the biomolecular recognition in this kind of application does not depart from the use of a coloured poly(styrene) latex and is consequently beyond the scope of the present discussion.

For the sake of clarity, I will arrange the next discussion around the regioregularity of the conductive polymers considered. The mechanism of transducing biomolecular recognition by chromic transition is indeed likely to differ depending on the degree of regioregularity of the polymeric structures.

Substituted poly(diacetylene) derivatives are regioregular and undergo dramatic color changes upon planar–nonplanar conformational transitions induced by either a change in temperature (thermochromism), solvent composition (solvatochromism) or mechanical stress (mechanochromism).[25,50] As further shown in Fig. 7, the side-chain structure plays a critical role in determining the original colour of the polymer in the planar conformation of the ene-yne backbone, and hence on the type of chromic transition occurring upon departure from coplanarity.[25,50] Similar effects have been observed with regioregular substituted poly(acetylenes)[51] and poly(thiophenes)[38,56] which can undergo severe thermochromic, solvatochromic and ionochromic transitions. In poly(thiophenes), the departure from a coplanar conformation decreases the conductivity of the polymer[57] and can be counteracted by the rigidification of the π-conjugated system.[58] Such chromic transition resulting from a decrease in the effective conjugation length of the polymer backbone has also been observed when the weight stress due to a biomolecular species binding its counterpart is applied on a given polymeric structure. Such application of molecular mechanochromism has been reported[59] with the use of a Langmuir–Blodgett film made of a polymerized diacetylenic lipid matrix functionalized with a sialic acid ligand specific for the influenza virus hemagglutinin. When the hemagglutinin receptor binds the ligand, the film changes colour from blue to red. The red colour intensity is directly proportional to the quantity of virus reacted with the film. The system consequently constitutes a direct colorimetric biosensor. In another article,[60] the authors reported the colorimetric detection of the molecular recognition between the Cholera toxin and Gm1 ganglioside incorporated in liposomes made of a substituted poly(diacetylene). In this latter system, the intensity of the color change was directly proportional to the dose of toxin interacting with the ligand and the least detectable dose was in the mg l^{-1} range. Other examples of application have been reported with substituted poly(diacetylenes) derivatized with toxin- or virus-specific ligands, which undergo a blue–red chromic transition upon ligand recognition by the pathogen.[61,62] A departure from coplanarity has also been observed when a biotin-functionalized regioregular substituted poly(thiophene) reacts with the biotin receptor (avidin) in aqueous solution. This effect results in the polymer solution turning from violet to yellow.[63]

The transduction of biomolecular recognition by chromic transition resulting from specific changes in the conjugated polymer backbone conformation is possible with well organized regioregular polymers. The mechanism of chromic transition by random polymers in the same circumstances is less clear. We have reported the synthesis of several water-soluble conductive random polymers or oligomers and their use as transducing reagents acting by chromic transition in homogeneous competitive immunoassays for antigens and haptens.[44,45] Currently, these assays allow detection of ligands in the nanomolar range.[45] Fig. 11 illustrates the performance of these reagents by showing the kinetics of the chromic transition occurring when theophylline labelled with poly(thiophene-3-carboxylic acid) associates with a specific antibody and its eventual dissociation from the complex by an excess

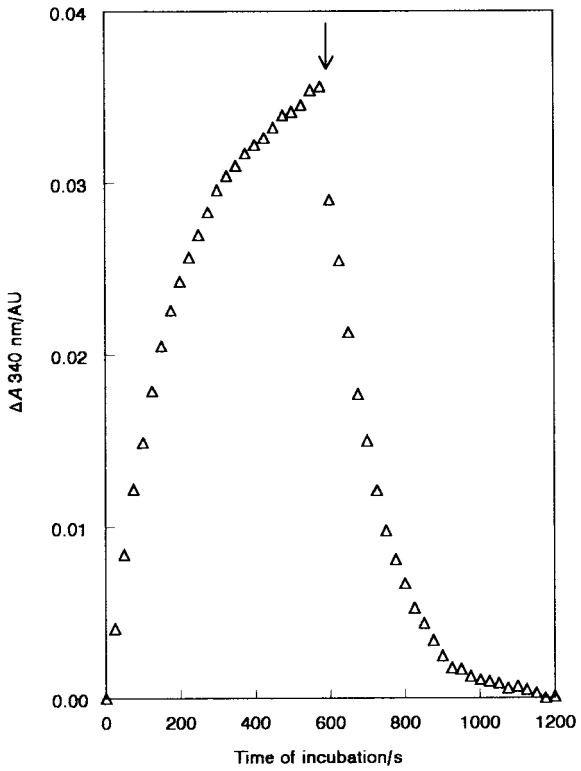


Fig. 11 Kinetics of association and dissociation between theophylline labelled with poly(thiophene-3-carboxylic acid) and a specific anti-theophylline antibody transduced by the chromic transition of the label as measured in a clinical chemistry automated analyzer at 340 nm. The dissociation (arrow) is induced by the addition of a 100 fold excess of unlabelled theophylline.

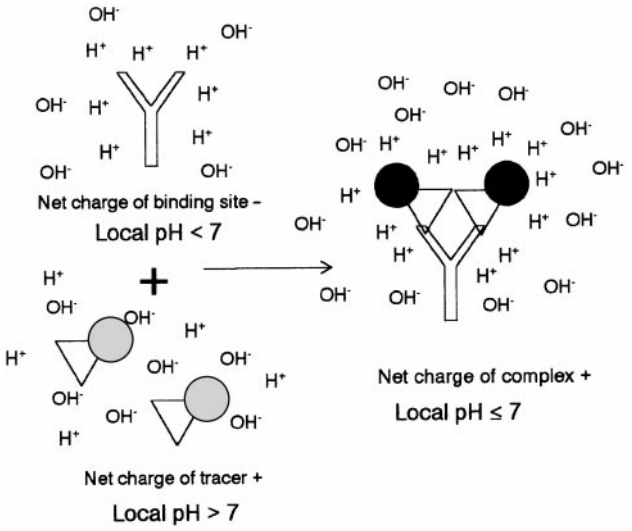


Fig. 12 Schematic drawing illustrating the capacity of water-soluble conductive polymers to translate directly a biomolecular recognition event by a change in their visible absorption spectrum under the influence of the local pH existing around the binding pocket. The conductive polymer is represented by the circles, respectively in its ground-state colour (hatching) and transition-state colour (black). The ligand and receptor are respectively represented by a triangle and Y.

of unlabelled theophylline. The rapidity of the kinetics makes it possible to measure the signal in a random access clinical chemistry analyzer. These instruments, which are widely used in clinical laboratories, are walk-away instruments capable of mixing and incubating reagents and samples in individual cuvettes according to programmed conditions. They are equipped with a flash lamp photometer allowing monitoring of subtle changes of A in each cuvette at a given wavelength and at short regular intervals (usually 25 s).[64] The potential application of conductive polymers to homogeneous immunoassays that can be run in such automated instruments constitutes an interesting and quite revolutionary challenge for the next few years.

Because of the lack of regularity in the polymeric chains used in such applications, the molecular mechanism inducing the chromic transition cannot be assigned only to a departure from coplanarity. As discussed previously, these water-soluble polymers are easily doped or undoped by subtle pH changes, and we have suggested that the chromic transition depends on the local pH existing around the binding pocket of the receptor or the antibody, which is known to change upon ligand recognition.[65] This mechanism is illustrated by the schematic drawing in Fig. 12. Such an explanation is further supported by the fact that we were unable to apply the technique to sandwich assays using labelled antibodies, most probably because the conductive polymer label attached to an antibody molecule is situated too far from the binding pocket to sense and transduce the pH change occurring upon ligand recognition.

Colloidal gold

Nature and properties

Gold hydrosols are hydrophilic colloids made of monodisperse, finely divided particles (5–100 nm diameter) of metallic gold suspended in water. Under normal circumstances, the metallic gold is surrounded by a predominantly anionic double layer. The sol is very stable due to the repulsive forces and shows a deep red color. However, due to induced changes in the outer charges, the particles are agglutinated and coagulated by the addition of millimolar concentrations of various salts and this irreversible process changes the colour from red to blue.[66] Colloidal gold hydrosol can be protected from agglutination by salts by coating the particles with a protein layer.

Gold hydrosols are characterized by a discrete band in the visible electronic spectrum which is called the surface plasmon resonance (SPR). It is a quantized plasma oscillation occurring at the surface of the gold particles, resulting in a characteristic peak location and width at maximal A.[67] The SPR wavelength and width depend on the refractive index of the particles and hence on their average diameter. Any increase in average particle diameter in the sol induces a red shift of the SPR wavelength along with a decrease in maximal A. This is illustrated in Fig. 13 which shows the change occurring in the visible electronic spectrum of colloidal gold when the particles are coated with a small layer of human serum albumin. We have applied this characteristic of colloidal gold in quantitative colorimetric assays for proteins.[68,69]

Synthesis

Isodisperse colloidal gold hydrosols are synthesized by the controlled reduction of an aqueous solution of tetrachloroauric acid. The reduction of Au^{3+} ions produces a supersaturated molecular Au^0 solution and as the Au^0 concentration increases the gold atoms cluster and form seeds of nuclei. Particle growth occurs by further deposition of metallic gold upon the nuclei.[70]

Strong reductants like white phosphorus or sodium borohydride produce a great number of nuclei and hence rather small particles with average diameter of 2 to 10 nm depending on the synthetic conditions. Bigger particles (20 to 100 nm average diameter) can be obtained by using a weaker reductant like sodium citrate advocated by Frens.[71] The size of the particles obtained by this method depends on the citrate : tetrachloroauric acid ratio. The lower the ratio, the greater the diameter of the particles.

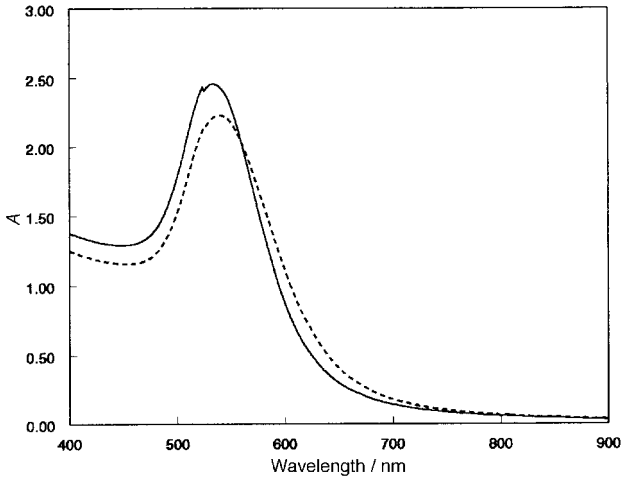


Fig. 13 Visible electronic spectrum of a colloidal gold hydrosol (solid line) compared to that of the same sol in which the particles are coated with a small layer of human serum albumin (dashed line). The change in refractive index due to the protein coating induces a red shift and a decrease in A at the SPR wavelength.

Coating colloidal gold particles with proteins

The coating of colloidal gold nanoparticles with proteins is performed by electrostatic charge adsorption. Colloidal gold nanoparticles remain negatively charged over a wide range of pH. When a partially protonated protein is contacted with the particles at a suitable pH, the positive charges on the protein are attracted by the anionic layer surrounding the particles with which it forms ionic bonds. The stability granted to the hydrosol by the protein coating process is usually verified by the absence of coagulation upon addition of a concentrated salt solution.[72]

When colloidal gold is coated with proteins capable of biomolecular recognition such as receptors or antibodies, the selection of the pH for coating is most critical. This parameter allows control of the part of the protein which is positively charged and hence determines the correct orientation of the external binding site of the particle for proper ligand recognition.[73,74] This point is illustrated in Fig. 14 with the stability and reactivity of colloidal gold particles coated by an antibody at various pHs. The decrease in $A_{600\ nm}$ after salt addition indicates that the sol is stabilized for a large range of pH (6.5 to 8), although the peak of ligand recognition by the gold probes is much narrower and lies at coating pHs around 7–7.5. Whilst the optimal pH for coating usually lies around the isoelectric point of the protein, there are no general rules and adsorption interaction isotherms such as that shown in Fig. 14 should be conducted in each case. However, once the optimal conditions are identified, the coating process is usually reproducible and easy to scale-up.

Mechanism of chromic transition upon biomolecular recognition

When colloidal gold probes coated with an antibody bind the specific ligand, a red shift occurs in the SPR wavelength of the gold sol along with a decrease in A at the maximal wavelength. When the antibody used for coating recognizes various molecular determinants on the ligand, the gold probes progressively form a lattice of agglutinated particles around ligand molecules and the SPR shift reflects the apparent increase in diameter of the individual particles in the solution which turns blue.[75] The remarkable deep red colour and capacity of colloidal gold to undergo a chromic transition upon agglutination have made possible its use for many years as a reporter reagent in either heterogeneous enzyme-like immunoassays[76–78] or in homogeneous agglutination immunoassays,[77,79–81] respectively. The same principle of chromic transition resulting from the selective agglutination of the particles has been also applied to the detection of complementary polynucleotide strands.[75,82]

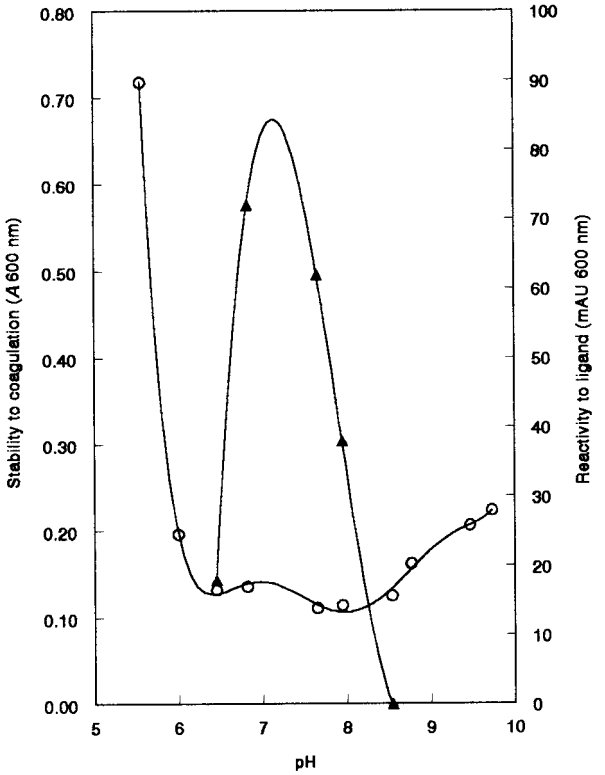


Fig. 14 Effect of coating pH on the capacity of the antibody-coated gold probes to recognize the specific ligand. The decrease in A at 600 nm after salt addition to the coated particles (open circles, left axis) shows stabilization of the colloid between pH 6.5 and 8. The capacity of the particles to bind a small amount of the ligand, as measured by the increase of A at 600 nm after incubation which is representative of the refractive index change (solid triangles, right axis) peaks at a narrower coating pH range of 7–7.5, indicating proper antibody orientation at this pH.

Recently,[83] I have shown that a lattice formation was not an absolute requisite for observing a measurable SPR shift and that such a shift could be observed when colloidal gold particles coated with a monoclonal antibody specific for a single epitope bind the protein ligand. This is illustrated by the artist's view on the cover of this issue. In this case, the shift in SPR wavelength reflects very small changes in the refractive index at the particle surface which are the direct result of mass changes in the approximate medium induced by the biomolecular recognition event. The SPR wavelength shift phenomenon is further dose-related as illustrated by the visible spectra shown in Fig. 15. These spectra were recorded after the respective incubation of monoclonal antibody-coated colloidal gold particles with increasing concentrations of the specific ligand, human chorionic gonadotropin (a pregnancy protein). The SPR wavelength shift of colloidal gold is used as transducer of the biomolecular recognition event of protein ligands in an automated clinical chemistry analyzer, the signal being measured at wavelengths in the red part of the spectrum (600 nm), where both the wavelength shift and the increase in peak width resulting from changes in the refractive index of the particles enhance the A in a dose-dependent manner. Fig. 16 shows the typical dose–response curve of a homogeneous immunoassay for human ferritin performed in a clinical chemistry analyzer. This new application of the chromic transition of colloidal gold upon biomolecular recognition is likely to have a tremendous potential, not only as a bioanalytical tool, but also as a real-time biosensing system providing kinetic[83–85] and even conformational[86] information

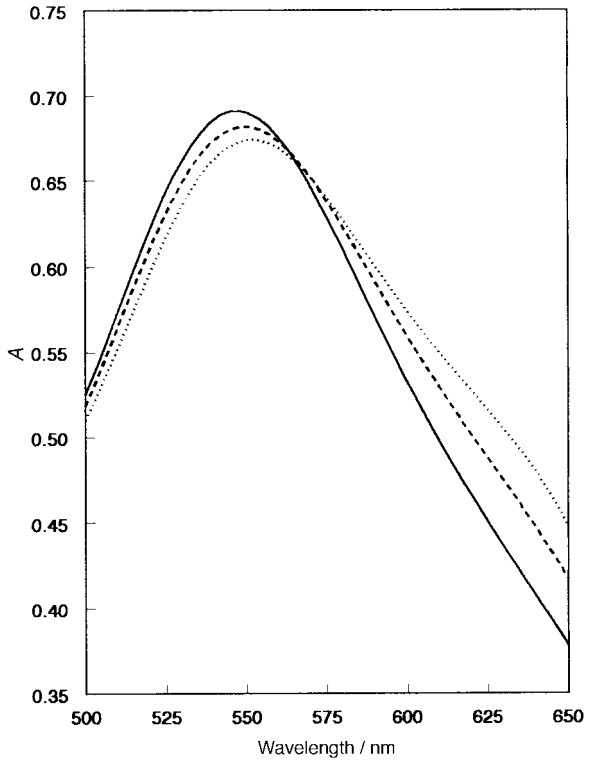


Fig. 15 Visible electronic spectra recorded after incubation of mixtures containing a fixed amount of colloidal gold particles coated with a specific monoclonal antibody and increasing concentrations (respectively no ligand added, solid spectrum; 33 pmol dm^{-3}, dashed spectrum; 60 pmol dm^{-3}, dotted spectrum) of the specific ligand protein, human chorionic gonadotropin.

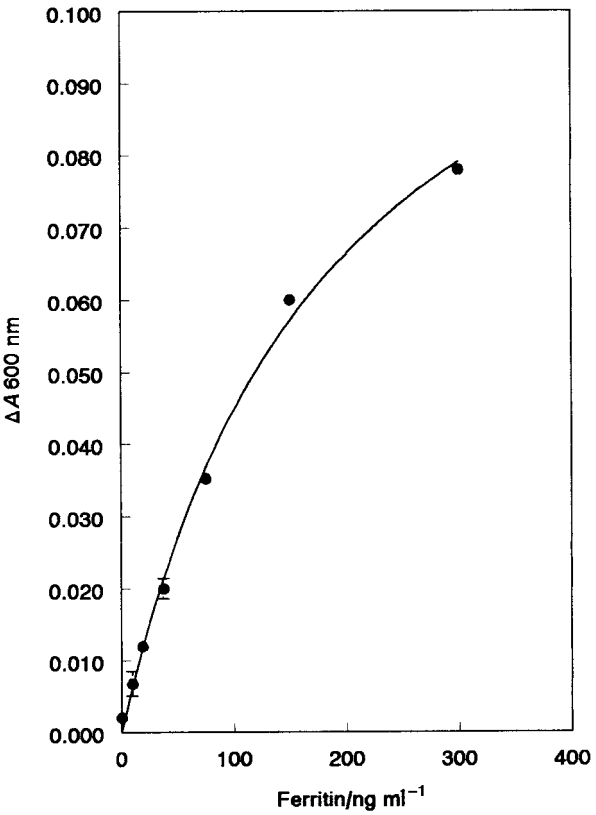


Fig. 16 Dose–response curve of an homogeneous immunoassay for human ferritin using antibodies labelled with colloidal gold. The assay is run in an automated clinical chemistry analyzer (Cobas-Mira) with an incubation time of 4 minutes. Data are the average ±sd of duplicate measurements.

on the biomolecular interaction such as those obtained in more sophisticated systems like the BIAcore instrument. This new applicability of the technique confirms its interesting potential in the new drug discovery process, particularly for either the selection of the highest affinity ligands[87] or the detection of natural ligands and of orphan receptors,[88] respectively.

Colloidal gold has also been incorporated into a classical SPR biosensing system,[89] comprising the interaction between an antibody immobilized on a gold film and the antigen coated on colloidal gold particles. The presence of the colloid increased tremendously the SPR sensitivity to protein–protein interactions.

Future prospects

One of the problems usually pointed out in the use of colloidal gold as a chromic transition reagent is the lack of possible covalent linkage of the biomolecular species to the probes. When they are stored in an insufficiently stabilizing medium, the particles coated by charge adsorption are prone to the progressive release of the adsorbed protein in the surrounding medium, which decreases their reactivity. This problem can be circumvented by the use of thiol-derivatized gold nanoparticles, the synthesis of which has been reported in two-phase[90] as well as single-phase[91] liquid systems. The attachment of a monolayer of bifunctional organic thiol molecules to the gold nanoparticles provides a vacant functionality for the covalent linkage of proteins.[91] In a similar way, gold colloids have been stabilized by molecules containing amine functional groups,[92] including poly(amidoamine) dendrimers.[93] This latter process allows for the amine groups to be available at the external surface of the particles.

The quite recent discovery and progressive understanding of the redox chemical character of alkanethiolate monolayer protected colloidal gold nanoparticles, which exhibit HOMO–LUMO E_g of 0.4 to 0.9 eV[94] open new prospects for application in biosensors. In the light of these new perspectives, I have attempted the template polymerization of substituted poly(anilines) on colloidal nanoparticles according to a scheme similar to that already reported for poly(diacetylene).[95] As shown in Fig. 17, a suitable selection of the conductive polymer with a π–π^* transition absorption wavelength matching that of colloidal gold SPR affords a composite colloidal material displaying a tremendously enhanced absorption at the SPR wavelength. The use of such a material, which combines the capacities for chromic transition of both gold and water-soluble conductive polymers, could allow for a further

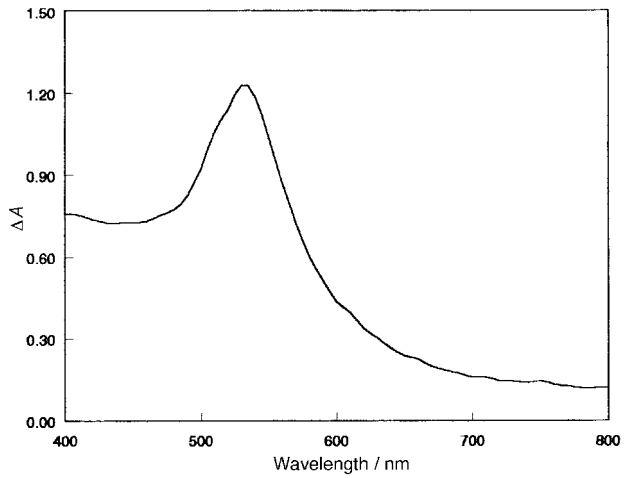


Fig. 17 Difference visible spectrum of *o*-anthranilic acid polymerized on colloidal gold nanoparticle templates *versus* that of the underivatised gold colloid.

improvement in the level of analytical sensitivity attained by the current homogeneous liquid-phase biosensors based on these independent chromic transducers.

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