Kirk-Othmer Encyclopedia of Chemical Technology.

MEDICAL DIAGNOSTIC REAGENTS

Purified enzymes are widely used in medical diagnostic reagents in the measurement of analytes in urine, plasma, serum, or whole blood. Enzymes are very specific catalysts that can be derived from plants and animals, although microbial fermentation (qv) is the most popular production method. Enzymes are used extensively in diagnostics, immunodiagnostics, and biosensors (qv) to measure or amplify signals of many specific metabolites. Purified enzymes are expensive. This is the main reason for the increasing utilization of reusable immobilized enzymes in clinical analyses (see Enzyme applications; Immunoassay).

The main development in medical diagnostic reagents since the 1960s has been the steady growth of dry (solid-phase) chemistry systems. Dry chemistry systems have made substantial gains over wet clinical analysis in the number of tests performed in hospitals, laboratories, and homes because of ease, reliability, and accuracy.

Wet chemistry methods for analysis of body analytes, eg, blood glucose or cholesterol, require equipment and trained analysts (see Automated instrumentation). In contrast, dry chemistry systems can be used at home. Millions of people with diabetes check their blood glucose levels and are able to obtain results in a matter of a few minutes. An insulin delivery system that can respond to changes in the blood glucose level is not available. Injected insulin does not automatically adjust, and therefore the dose required to mimic the body's response must be adjusted daily or even hourly depending on diet and physical activity. Self-monitoring of blood glucose levels, essential for diabetics, has become possible owing to the advent of dry chemistry systems (1–8) (see Insulin and other antidiabetic drugs). By regular and accurate monitoring of her blood glucose level by dry chemistry, an expectant diabetic mother can have a normal pregnancy and give birth to a healthy child. Athletes with diabetes can self-test their blood glucose to avoid significant problems. Dry chemistry systems are useful not only to diabetics, but also to patients having other medical problems. These systems are also used in animal diagnosis, food, fermentation, agriculture, and environmental and industrial monitoring.

The principles and biochemical reactions involved in diagnostic reagents are described herein. Construction of dry chemistry systems and advances are also addressed, as are biosensors.

1. Enzyme-Catalyzed Reactions in Solution

1.1. Measurement Considerations

A prototype enzyme-catalyzed reaction where one substrate (S) produces only one product (P) may be described by

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P}$$

where E is enzyme, ES is the enzyme–substrate complex, and k_i represents the reaction rate constants. The reaction can be followed by monitoring the loss of substrate or the formation of product. A graph of the concentration of substrate, or product, vs time gives an exponential curve. In the equilibrium method (end point)

used for S determination, data are collected when the concentration of S or P are time-independent. Methods where data are obtained from the early linear part of the curve are known as kinetic methods (see Kinetic measurements). The reaction rate of the enzyme-catalyzed reaction shown is given by the Michaelis-Menten equation:

$$v = V[S]/(K_m + [S])$$

where v represents the rate of reaction; $V = k_2[E_0]$, the maximum rate of the reaction when $[S]$ is the initial substrate concentration and $[E_0]$ is the initial enzyme concentration; and K_m is the Michaelis constant for the enzyme and this particular substrate.

For measurement of a substrate by a kinetic method, the substrate concentration should be rate-limiting and should not be much higher than the enzyme's K_m. On the other hand, when measuring enzyme activity, the enzyme concentration should be rate-limiting, and consequently high substrate concentrations are used (see Catalysis).

Glucose [50-99-7], urea [57-13-6] (qv), and cholesterol [57-88-5] (see Steroids) are the substrates most frequently measured, although there are many more substrates or metabolites that are determined in clinical laboratories using enzymes. Co-enzymes such as adenosine triphosphate [56-65-5] (ATP) and nicotinamide adenine dinucleotide [53-84-9] in its oxidized (NAD^+) or reduced (NADH) [58-68-4] form can be considered substrates. Enzymatic analysis is covered in detail elsewhere (9).

Assays using equilibrium (end point) methods are easy to do but the time required to reach the end point must be considered. Substrate(s) to be measured reacts with co-enzyme or co-reactant (C) to produce products (P and Q) in an enzyme-catalyzed reaction. The greater the consumption of S, the more accurate the results. The consumption of S depends on the initial concentration of C relative to S and the equilibrium constant of the reaction. A change in absorbance is usually monitored. Changes in pH and temperature may alter the equilibrium constant but no serious errors are introduced unless the equilibrium constant is small. In order to complete an assay in a reasonable time, for example several minutes, the amount and therefore the cost of the enzyme and co-factor may be relatively high. Sophisticated equipment is not required, however.

1.2. Indicators

There are certain compounds that are suitable as indicators for sensitive and specific clinical analysis. Nicotinamide adenine dinucleotide (NAD) occurs in oxidized (NAD^+) and reduced (NADH) forms. Nicotinamide adenine dinucleotide phosphate (NADP) also has two states, $NADP^+$ and NADPH. NADH has a very high uv–vis absorption at 339 nm, extinction coefficient $= 6300\ (M{\cdot}cm)^{-1}$, but NAD^+ does not. Similarly, NADPH absorbs light very strongly whereas $NADP^+$ does not.

An example of the application of these compounds as indicators is in the determination of pyruvate.

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{L}-\text{lactate} + NAD^+$$

The absorbance change (ΔA) at 340 nm can be used to determine the amount of pyruvate remaining. The lactate dehydrogenase [9001-60-9] catalyzed reaction can also be used in the reverse direction to measure lactate. The reaction takes place in a buffer of pH 9–10 that neutralizes liberated H^+.

Two or more linked enzyme reactions can lead to a change in the concentration of NADH or NADPH that is equivalent to the concentration of the original analyte. The reference glucose measurement using hexokinase [9001-51-8] and glucose-6-phosphate dehydrogenase [9001-40-5] is an example:

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose}-6-\text{phosphate} + \text{ADP}$$

$$\text{glucose}-6-\text{phosphate} + \text{NADP}^{+} \xrightarrow[\text{dehydrogenase}]{\text{glucose}-6-\text{phosphate}} 6-\text{phosphogluconate} + \text{H}^{-} + \text{NADPH}$$

The second enzymatic reaction converts $NADP^+$ to NADPH and H^+, and the appearance of NADPH is measured at 340 nm.

In the enzymatic assays of cholesterol, glucose, and urea, oxygen is used and H_2O_2 is formed. The reaction for uric acid [69-93-2] is

$$\text{uric acid} + O_2 + 2H_2O \longrightarrow \text{allantoin} + CO_2 + H_2O_2$$

The H_2O_2 generated reacts with a chromogen in the presence of the enzyme peroxidase [9001-05-02] to produce a color change. A frequently used reaction is the peroxidase-catalyzed coupling of H_2O_2 and 4-aminoantipyrine [83-07-8] and phenol [108-95-2] to produce a quinoneimine dye. Methods using peroxidase are prone to interference by compounds such as ascorbic acid, uric acid, and acetylsalicylic acid.

1.3. Measurement of Analytes

Biochemical reactions used in the measurement of selected analytes are commercially available as prepackaged kits of reagents. Measurement of the reactions given plus many other analytes can be made (10, 11).

1.3.1. Cholesterol

The end point for the cholesterol reaction can be determined by following dye formation. Additionally, the amount of oxygen consumed can be measured amperometrically by an oxygen-sensing electrode (see Electroanalytical techniques). The H_2O_2 produced by cholesterol oxidase requires phenol to produce dye.

$$\text{cholesterol esters} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{fatty acids}$$

$$\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest}-4-\text{en}-3-\text{one} + H_2O_2$$

$$H_2O_2 + \text{chromogen} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{dye} + H_2O$$

A popular alternative to the step utilizing chromogen is to substitute *p*-hydroxybenzenesulfonate for phenol in the reaction with the pyridine nucleotide:

$$H_2O_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} + 2\ H_2O$$

$$\text{acetaldehyde} + \text{NADP}^{+} \xrightarrow{\text{aldehyde dehydrogenase}} \text{acetate} + \text{H}^{+} + \text{NADPH}$$

Free cholesterol can also be determined, if cholesterol esterase is omitted.

1.3.2. Citrate

The citrate reaction is followed by monitoring the decrease in the concentration of NADH. Oxaloacetate instantly decarboxylates to pyruvate.

$$\text{citrate} \xrightarrow{\text{citrate lyase}} \text{oxaloacetate} + \text{acetate}$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{malate dehydrogenase}} \text{L}-\text{malate} + \text{NAD}^+$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{L}-\text{lactate} + \text{NAD}^+$$

1.3.3. Creatinine

The most widely used creatinine methods are based on reaction between creatinine and picrate ions formed in an alkaline medium.

$$\text{creatinine} + \text{H}_2\text{O} \xrightarrow{\text{creatinase}} \text{creatine}$$

$$\text{creatine} + \text{ATP} \xrightarrow{\text{creatine kinase}} \text{creatine phosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{L}-\text{lactate} + \text{NAD}^+$$

The loss of NADH is followed for determination of the enzyme creatine kinase.

1.3.4. Galactose

$$\text{galactose} \xrightarrow{\text{galactose oxidase}} \text{galactolactone} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 + \text{chromogen} \xrightarrow{\text{peroxidase}} \text{dye} + \text{H}_2\text{O}$$

1.3.5. Glucose

$$\text{glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{H}_2\text{O}_2$$

$$H_2O_2 + \text{chromogen} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{dye} + H_2O$$

The reaction can be followed by measurement of dye formation. The rate of oxygen depletion can be measured using an oxygen electrode. Additional reagents can be added to prevent the formation of oxygen from the generated H_2O_2. The reactions are as follows:

$$H_2O_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} + 2\ H_2O$$

$$H_2O_2 + 2H^+ + 2\ I^- \xrightarrow{\text{molybdate}} I_2 + 2\ H_2O$$

1.3.6. Lactate

The formation of NADH is followed by measuring the absorbance at 340 nm.

$$\text{L}-\text{lactate} + NAD^+ \xrightarrow{\text{lactate dehydrogenase}} \text{pyruvate} + NADH + H^+$$

1.3.7. Triglycerides

The loss of NADH is followed at 340 nm. This is one of over 20 variations of the method.

$$\text{triglyceride} + 3\ H_2O \xrightarrow{\text{lipase}} \text{glycerol} + 3\ \text{fatty acids}$$

$$\text{glycerol} + ATP \xrightarrow{\text{glycerol kinase}} \text{glycerol}-3-\text{phosphate} + ADP$$

$$ADP + \text{phosphoenolpyruvate} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + ATP$$

$$\text{pyruvate} + NADH + H^+ \xrightarrow{\text{lactate dehydrogenase}} \text{L}-\text{lactate} + NAD^-$$

1.3.8. Blood Urea Nitrogen

$$\text{urea} + H_2O \xrightarrow{\text{urease}} 2\ NH_3 + CO_2$$

$$NH_3 + \alpha-\text{ketoglutarate} + NADPH + H^+ \xrightarrow{\text{glutamate dehydrogenase}} NADP^+ + H_2O + \text{glutamate}$$

1.4. Assay of Enzymes

In body fluids, enzyme levels are measured to help in diagnosis and for monitoring treatment of disease. Some enzymes or isoenzymes are predominant only in a particular tissue. When such tissues are damaged because of a disease, these enzymes or isoenzymes are liberated and there is an increase in the level of the enzyme in the serum. Enzyme levels are determined by the kinetic methods described, ie, the assays are set up so that the enzyme concentration is rate-limiting. The continuous flow analyzers, introduced in the early 1960s, solved the problem of the high workload of clinical laboratories. In this method, reaction velocity is measured rapidly; the change in absorbance may be very small, but within the capability of advanced kinetic analyzers.

Enzymes, measured in clinical laboratories, for which kits are available include γ-glutamyl transferase (GGT), alanine transferase [9000-86-6] (ALT), aldolase, α-amylase [9000-90-2], aspartate aminotransferase [9000-97-9], creatine kinase and its isoenzymes, galactose-1-phosphate uridyl transferase, lipase, malate dehydrogenase [9001-64-3], 5′-nucleotidase, phosphohexose isomerase, and pyruvate kinase [9001-59-6]. One example is the measurement of aspartate aminotransferase, where the reaction is followed by monitoring the loss of NADH:

$$\text{L}-\text{aspartate}+\alpha-\text{ketoglutarate} \xrightarrow{\text{aspartate aminotransferase}} \text{oxaloacetate}+\text{L}-\text{glutamate}$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{malate dehydrogenase}} \text{L}-\text{malate} + \text{NAD}^+$$

A second is the measurement of creatine kinase:

$$\text{creatine phosphate} + \text{ADP} \xrightarrow{\text{creatine kinase}} \text{creatine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose}-6-\text{phosphate} + \text{ADP}$$

$$\text{glucose}-6-\text{phosphate} + \text{NADP}^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose}-6-\text{phosphate}} 6-\text{phosphogluconate} + \text{NADPH} + \text{H}^+$$

This reaction is followed by monitoring NADPH formation.

1.5. Immobilized Enzymes in Diagnostic Reagents

The use of immobilized, instead of soluble, enzymes for measurement of analytes has received considerable attention, especially for clinical analyses (12–17). Use of immobilized enzymes offers the advantages of greater accuracy, stability, and convenience. Only a few methods utilizing immobilized enzymes have become commercially available, although these methods may not have achieved full potential in clinical chemistry. Dry chemistry has outpaced all other methods combined in clinical chemistry. Biosensors are gaining momentum, however, and are expected to continue to increase in usage as a result of advances in redox polymers.

2. Dry Chemistry

Diagnostic medicine is placing demands on technology for newer materials and application techniques, and polymers are finding ever-increasing use in diagnostic medical reagents (7).

Dry or solid-phase chemistry has origins that reach back to the ancient Greeks. Some 2000 years ago, copper sulfate was an important ingredient in tanning and preservation of leather (qv). Dishonest traders were adulterating valuable copper sulfate with iron salts. The first recorded use of a dry chemistry system was described by Pliny. The method for detection of iron involved soaking reeds of papyrus in a plant gall infusion or a solution of gallic acid. The papyrus would turn black in the presence of iron. In 1830, a filter paper impregnated with silver carbonate was used to detect uric acid qualitatively.

Self-testing, eg, measuring one's own self, is likewise not a new concept. Around the turn of the century diabetics were encouraged to monitor their glucose level by testing their urine with Benedict's qualitative test (18). When insulin became available in the early 1920s, self-testing of urine became necessary. In the mid-1940s, a dry tablet was compounded consisting of sodium hydroxide, citric acid, sodium carbonate, and cupric sulfate (19). Adding this tablet to a small urine sample resulted in boiling of the solution and reduction of the blue cupric sulfate to a yellow or orange color if glucose was present. Glucose urine strips, impregnated and based on enzymatic reactions using glucose oxidase, peroxidase, and an indicator were introduced around 1956 (20). Dry chemistry blood glucose test strips coated with a semipermeable membrane to which whole blood could be applied and wiped off were introduced in 1964. As of the mid-1990s, low cost, lightweight plastic-housed reflectance meters having advanced data management capabilities are available and extensively used.

Impregnated dry reagents have coarse texture, high porosity, and uneven large pore size, resulting in nonuniform color development in the reacted strip. In the early 1970s, a coating-film type of dry reagent was developed by applying an enzymatic coating onto a plastic support. This gave the surface a smooth, fine texture, resulting in uniform color development (21). Nonwipe dry chemistry systems, wherein the devices handle the excess blood by absorption or capillary action, appeared in the marketplace in the late 1980s.

Discrete multilayered coatings, developed by the photographic industry (see Photography), were adapted in the late 1970s to coat dry reagent chemistry formats for clinical testing (22, 23). Each zone of a multilayered coating provides a unique environment for sequential chemical and physical reactions. These devices consist of a spreading layer, separation membrane, reagent zone, and reflective zone, coated onto the base support. The spreading layer wicks the sample and applies it uniformly to the next layer. The separation layer can hold back certain sample components, eg, red blood cells, allowing only the desired metabolites to pass through to the reagent zone that contains all the necessary reagent components. Other layers may be incorporated for filtering, reflecting, or eliminating interfering substances.

2.1. Background

Enzymes are essential in user-friendly diagnostic dry chemistry systems. In a typical glucose-measuring dry reagent, glucose oxidase (GOD) and peroxidase (POD), along with a suitable indicator eg, 3,3′,5,5′-tetramethylbenzidine (TMB), are dissolved or dispersed in a latex or water-soluble polymer. This coating is applied to a lightly pigmented plastic film and dried to a thin film. The coated plastic can be cut to a suitable size, eg, $0.5\ \text{cm} \times 0.5\ \text{cm}$, and mounted on a plastic handle. The user applies a drop of blood on the dry reagent pad, allowing contact for 60 s or less. The blood can be wiped off manually with a swab or by the device itself. The developed color is then read by a meter or visually compared with the predesignated printed color blocks to determine the precise glucose level in the blood.

Dry chemistry test kits are available in thin strips that are usually disposable. They may be either film-coated or impregnated. The most basic diagnostic strip consists of a paper or plastic base, polymeric binder, and reactive chemistry components consisting of enzymes, surfactants, buffers, and indicators. Diagnostic coatings or impregnation must incorporate all reagents necessary for the reaction. The coating can be either single or

multilayer in design. A list of analytes, enzymes, drugs, and electrolytes assayed by dry chemistry diagnostic test kits follows (24):

Analytes	lactate dehydrogenase (LDH)
glucose	creatine kinase
urea	MB isoenzyme (CK–MB)
urate	lipase
cholesterol (total)	amylase (total)
triglycerides	
bilirubin (total)	
ammonium ions	*Drugs*
creatinine	phenobarbitone
calcium	phenytoin
hemoglobin	theophylline
HDL cholesterol	carbamazepine
magnesium(II)	
phosphate (inorganic)	*Electrolytes*
albumin	sodium ion
protein in cerebrospinal fluid	chloride
	carbon dioxide
Enzymes	
alkaline phosphate (ALP)	

Dry chemistry systems are widely used in physician's offices and hospital laboratories, and by millions of patients in their own homes worldwide. These systems are used for routine urinalysis, blood chemistry determinations, and immunological and microbiological testing. The main advantage of this technology is elimination of the need for reagent preparation and many other manual steps common to liquid reagent systems. This yields greater consistency and reliability of test results. Furthermore, dry chemistry systems have longer shelf stability and hence there is a reduced waste of reagents. Each test unit contains all the reagents and reactants necessary to perform assays.

Dry chemistry tests are used for the assay of metabolites by concentration or by activity in a biological matrix. In general, reactive components are present in amounts in excess of the analyte being determined to make sure that the reactions go to completion quickly. Other enzymes or reagents are used to drive the reactions in the desired direction (25). Glucose and cholesterol are the analytes most commonly measured.

2.2. Components of Dry Chemistry Systems

The basic components of typical dry chemistry systems that utilize reflectance measurement are a base support material, a reflective layer, and a reagent layer which can be either single- or multilayered. The base layer serves as a building base for the system and usually is a thin, rigid thermoplastic film. The reflective layer, usually a white, pigment-filled plastic film, coating, foam, or paper, reflects whatever light is not absorbed as a result of the chemistry, to the detector. The reagent layer contains the integrated reagents for a specific set of reactions. Typical materials include paper or fiber matrix and coating film as well as various combinations.

A test system having a single-layer coating reagent that effectively excludes red blood cells (RBC) has an emulsion-based coating containing all the reagents for a specific chemistry. The emulsion is coated onto a lightly filled thermoplastic film and dried. For glucose measurement, the coating should contain GOD, POD, and TMB. It may also contain a buffer for pH adjustment, minor amounts of ether–alcohol type organic coalescing agent, and traces of a hindered phenol-type antioxidant to serve as a color signal ranging compound. Whole blood is applied and allowed to remain in contact for 60 s or less. Excess blood is wiped off and the developed color is

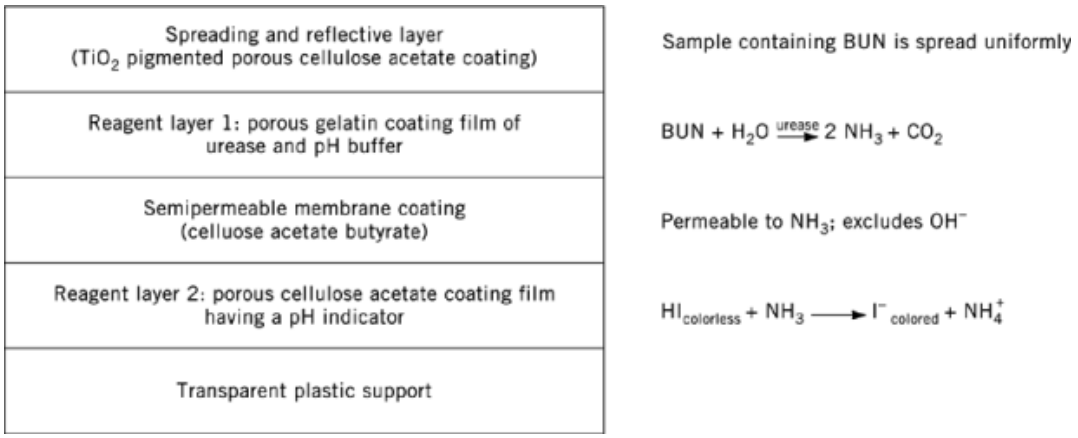

Fig. 1. A multilayer coating dry chemistry test for blood urea nitrogen (BUN) where HI and I^- represent the acid base forms of a pH indicator, respectively (24). See text.(Courtesy of the American Chemical Society.)

read visually or by meter. Another type of dry chemistry system consists of a reagent-impregnated paper in between a film membrane and a reflective support layer.

The schematic of a typical multilayer coating dry test system, in this case a test for blood urea nitrogen (BUN) is shown in Figure 1. Sample containing BUN is spread uniformly by the top layer, a spreading and reflective layer. Immediately underneath, the first reagent layer is a porous coating film containing the enzyme urease [9002-13-15] and buffer (pH 8.0). A semipermeable membrane coating allows the NH_3 formed in the first reagent layer to permeate to the second reagent layer excluding OH^-. The second reagent layer is composed of a porous coating film containing a pH indicator. The indicator color develops when NH_3 reaches the semipermeable coating film. Typically, such dry reagents are slides $(2.8\text{-cm} \times 2.4\text{-cm})$ having an application area of 0.8 cm^2, and the spreading layer is about 100 μ m thick. Touch-and-drain dry chemistry construction is shown in Figure 2 (6). This construction is useful in evaluation of diagnostic coating films and may also find acceptance by users.

2.3. Polymers and Coatings

Advances in polymer chemistry have resulted in many successful medical devices, including diagnostic assays (26). Polymers (qv), which can be manufactured in a wide range of compositions, are used to enhance speed, sensitivity, and versatility of both biosensors and dry chemistry systems to measure vital analytes. Their properties can be regulated by composition variations and modifications. Furthermore, polymers can be configured into simple to complex shapes.

2.3.1. Polymers

In most dry chemistry systems, polymers account for more than 95% of the strips. Polymer chemistry has been linked to biochemistry in order to develop improved dry chemistries (6, 24). The polymer binder incorporates the system's chemistry components in the form of either a coating or impregnation. The reagent matrix must be carefully selected to mitigate or eliminate nonuniformity in the concentrations of reagents resulting from improper mixing, settling, or nonuniform coating thickness. Aqueous-based emulsion polymers and water-soluble polymers (qv) are extensively used. A list of commonly used matrix binders follows (27, 28).

Polymers must be carefully screened and selected to avoid interfering with the analyte chemistry. The polymer properties, eg, composition, solubility, viscosity, solid content, surfactants (qv), residual initiators,

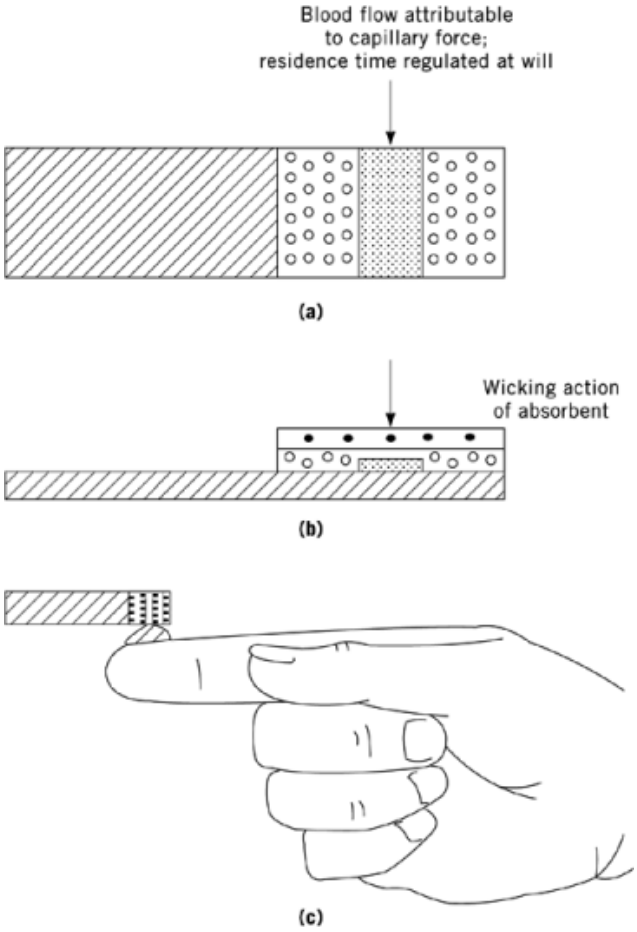

Fig. 2. Touch-and-drain dry chemistry construction: (**a**) dry coated surface; (**b**) cross-section of dry coated surface, adhesive, and cover piece; (**c**) contact with blood drop results in blood filling the cavity. After desired reaction time, blood is drained off by touching end of cavity with absorbent material (6).(Courtesy of the American Chemical Society.)

film-forming temperature, and particle size are all important to the dry chemistry system (29). In general, the polymer should be a good film former and have good adhesion to the support substrate. Furthermore, it should have no or minimal tack for handling purposes during manufacturing of the strip. The coated matrix or impregnation must have the desired pore size and porosity to allow penetration of the analyte being measured, as well as the desired gloss, swelling characteristics, and surface energetics. Swelling of the polymer binder owing to the absorption of the liquid sample may or may not be advantageous, depending on the system. Emulsion polymers have a distinct advantage over water-soluble ones because of high molecular weight, superior mechanical properties, and potential for adsorbing enzymes and indicators by micellar forces. Polymeric binders used in multilayered coatings include various emulsion polymers; gelatin (qv); polysaccharides such as agarose; water-soluble polymers such as polyacrylamide, polyvinylpyrrolidinone, poly(vinyl alcohol), and copolymers of vinylpyrrolidinone and acrylamide; and hydrophilic cellulose derivatives, eg, hydroxyethylcellulose and methylcellulose.

Emulsion polymers	Water-soluble polymers
acrylics	poly(vinyl alcohol)
poly(vinyl acetate):	polyvinylpyrrolidinone
homo and copolymers	highly hydroxylated acrylic
styrene acrylics	poly(vinylethylene glycol acrylate)
polyvinyl propionate:	polyacrylamide
homo and copolymers	hydroxyethyl cellulose
ethylene vinyl acetate	other hydrophilic cellulosics
lightly cross-linkable acrylics	various copolymers
polyurethanes	

2.3.2. Water-Borne Coatings

The most important coating film is that of waterborne coatings because enzymes are water soluble. During the late 1980s a tough coating film which would allow whole blood to flow over the film was developed (7). This system is shown in Figure 3. Soak-through technology is no longer utilized. Red blood cells (RBC) must roll over and not stick to the coating film. Investigations involving almost all types of emulsion and water-soluble polymers showed a styrene–acrylate emulsion polymer to be most suitable. A coating containing 51 g styrene–acrylate emulsion in water (50% solids), 10 g linear alkylbenzene sulfonate (15 wt % in water), 0.1 g GOD, 0.23 g POD, 0.74 g TMB, 10.0 g hexanol, and 13.2 g 1-methoxy-2-propanol gave a good dose response for measurement of blood glucose, as seen in Figure 4. This coating film gave a linear dose response, in transmission mode, up to 1500 mg/dL glucose. The coating was slightly tacky and the measurements were found to be highly dependent on the blood residence time.

To mitigate or eliminate tack, ultrafine mica was found to be effective (see Mica). This made rolling and unrolling of coated plastic for automated strip construction possible. In devices wherein blood residence time is regulated, the residence time-dependent coating is not a problem. Dependence of results on blood residence time creates a serious problem where residence time cannot be regulated by the strip. To make the coating film residence time-independent, continuous leaching of color from films during exposure time is essential. Enzymes and indicator can leach rapidly near the blood/coating film interface and develop color that can be drained off. The color formed on the coating film is then independent of the exposure time. It was found that this gave a coating film having minimum time dependency. A low molecular weight polyvinylpyrrolidinone, eg, PVP K-15 from GAF or PVP K-12 from BASF, gave coatings having no residence time dependency. Such a coating consists of 204 g styrene–acrylate emulsion (50% solids), 40 g linear alkylbenzene sulfonate (15% solution in water), 0.41 g GOD (193 unit/mg), 1.02 g POD (162 unit/mg), 2.96 g TMB, 7.4 g PVP K-12, 32 g micromica C-4000, 20 g hexanol, and 6 g Igepal CO-530 (a surfactant). The nonionic surfactant serves as a surface modifier to eliminate RBC retention.

Excellent correlation was found when results at 660 nm and 749 nm were compared using a reference hexokinase glucose method (27). The dose response was excellent up to 300 mg/dL glucose. In general, water-borne coatings do not lend themselves to ranging by antioxidants (qv).

2.3.3. Nonaqueous Coatings

Since the 1970s, dry reagent coatings have been exclusively water-borne because of the belief that enzymes function only in aqueous medium. Nonaqueous enzymatic coatings for dry chemistries have been researched, developed, and refined, however (24, 30); red blood cells do not adhere to such coatings. Additionally, quick end points are obtained. These coatings give superior thermostability. Furthermore, these coatings can be easily ranged by antioxidants, whereas water-borne coatings are difficult to range. Nonaqueous hydroxylated acrylic polymers have been synthesized which have good hydrophilicity and hydrogel character. The enzymes GOD and POD are insoluble in organic solvents but become extremely rigid and can be dispersed with ease.

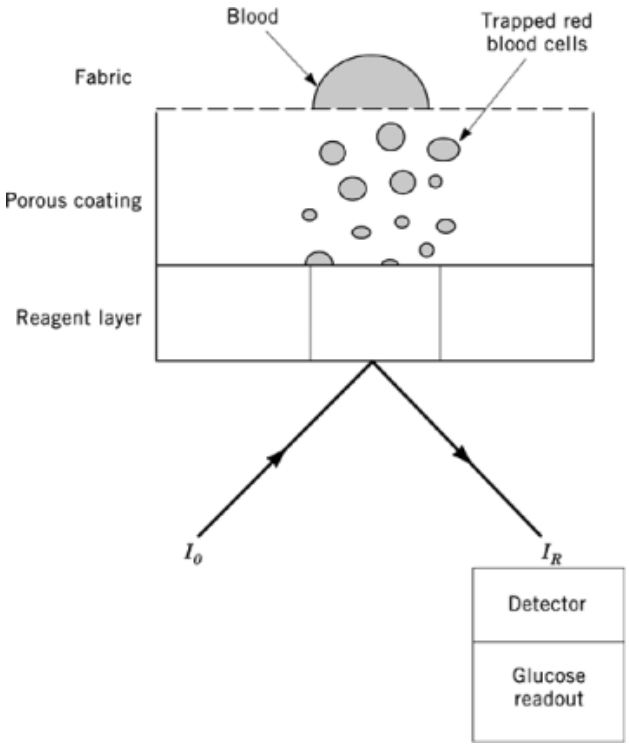

Fig. 3. Design of topover diagnostic coatings, where I_0 and I_R represent the initial and reflected light intensities, respectively.

Dispersions of less than 1 μm were made using an Attritor mill or a ball mill. To prepare nonaqueous coatings, polymer solution, TMB, mica, surface modifiers, and solvents were added to the enzyme dispersion and slightly mixed on a ball mill. Ranging compound can be post-added. The composition of a typical nonaqueous coating useful for low range blood glucose measurement, gram basis, is 33.29 hydroxyethyl methacrylate–butyl methacrylate–dimethylaminoethyl methacrylate (65:33:2) polymer (40% solid), 2.38 TMB, 1.17 GOD, 2.68 POD, 3.28 sodium dodecyl benzene sulfonate [25155-30-0], 26.53 xylene, 26.53 1-methoxypropanol, and 1.96 cosmetic-grade C-4000 ultrafine mica. Many surfactants and surface modifiers that eliminated RBC retention were investigated. Antioxidants that function as ranging compounds in these nonaqueous systems include 3-amino-9-(aminopropyl)-carbazole dihydrochloride, butylated hydroxy toluene [128-37-0] (BHT), and a combination of BHT–propyl gallate. These ranging compounds are effective in a ranging compound-to-TMB indicator molar ratio of 1:2.5–1:20.

The long-term stability of the nonaqueous coating films under elevated temperature and moderate humidity is reported to be better than aqueous coatings (30). Furthermore, color resolution and sensitivity of reacted nonaqueous coating films are excellent.

2.4. Molded Dry Chemistry

In general, most enzymes are very fragile and sensitive to pH, solvent, and elevated temperatures. The catalytic activity of most enzymes is reduced dramatically as the temperature is increased. Typical properties of diagnostic enzymes are given in Table 1. Common enzymes used in diagnostics, eg, GOD and POD, are

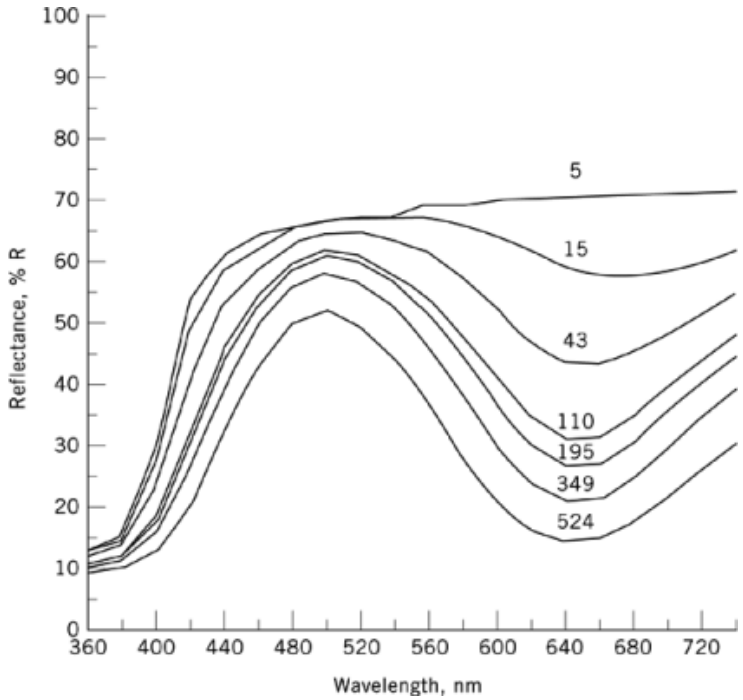

Fig. 4. Dose response for blood glucose measurement using a dry chemistry system having a water-borne, tough coating film. The numbers represent glucose concentrations in mg/dL. A 120-s blood residence time was used.

almost completely deactivated around 65°C in solid form or in aqueous solution. Thermal analysis work on these biopolymers was reported in 1991 (31). The differential scanning calorimetry (dsc) results indicating glass-transition temperature (T_g), melting temperature (T_m), and decomposition temperature (T_d) are shown in Table 2. BelowT_g, the enzymes are in a glassy state and should be thermally stable. Around T_g, onset of the rubbery state begins, and the enzyme becomes prone to thermal instability. When the enzymes melt around T_m, all the tertiary structures are destroyed, thus making the enzyme completely inactive. The presence of ionic salts and other chemicals can considerably influence enzyme stability. The redox center, flavin adenine dinucleotide (FAD), in GOD can conduct electrons and is catalytically relevant. To keep or sustain enzymatic activity, the redox centers must remain intact. The bulk of the enzyme, polymeric in composition, is an insulator, thus altering it does not reduce the enzyme's catalytic activity. An enzymatic compound containing GOD, POD, TMB, a linear alkylbenzene sulfonate, and polyhydyroxyethyl methacrylate (PHEMA) compression molded between 105–150°C has given a response to glucose (32). Molding at 200°C resulted in enzyme deactivation. A mechanism has been proposed where the enzymes are protected by the tight PHEMA coils. It has been suggested that molding of strips using reaction injection molding (RIM) may lead to useful chemistries, including biosensors, in the future.

2.5. Application of Diagnostic Technology in Monitoring Diabetes

Very frequent measurements of blood glucose to manage diabetes are one of the most important applications of diagnostic reagents. It has been estimated that there are about 15×10^6 diabetics in the United States, although only half that number have been diagnosed with the disease. More than 1.5 million diabetics are

treated with injected insulin. The rest are treated with weight loss, diet, and oral antidiabetic drugs, eg, the sulfonylureas Tolbutamide, Tolazamide, Chloropropamide, Glipizide, and Glyburide.

Table 1. Properties of Diagnostic Enzymes

Parameter	Cholesterol oxidase (CO)	Cholesterol esterase (CE)	Glucose oxidase (GOD)	Peroxidase (POD)
source	*Streptomyces*	*Pseudomonas*	*Aspergillus*	horseradish
EC	1.1.3.6	1.1.13	1.1.3.4	1.11.1.7
CAS Registry Number	[9028-76-6]	[9026-00-0]	[9001-37-0]	[9001-05-02]
molecular weight	34,000	300,000	153,000	40,000
isoelectric point	$5.1 \pm 0.1, 5.4 \pm 0.1$	5.95 ± 0.05	4.2 ± 0.1	
Michaelis constant, *M*	4.3×10^{-5}	2.3×10^{-5}	3.3×10^{-2}	
inhibitor	Hg^{2+}, Ag^{+}	Hg^{2+}, Ag^{+}	Hg^{2+}, Ag^{+}, Cu^{2+}	CN^{-}, S^{2-}
pH, optimum	6.5–7.0	7.0–9.0	5.0	6.0–6.5
temperature, optimum, °C	45–50	40	30–40	45
pH stability[a]	5.0–10.0	5.0–9.0	4.0–6.0[b]	5.0–10.0

[a] At 25°C for 20 h, unless otherwise indicated.
[b] At 40°C for 1 h.

Table 2. DSC Analysis of Diagnostic Enzymes

Enzyme	Source	T_g, °C	T_m, °C	T_d, °C
cholesterol oxidase	*Nocardia*	50	98	210
cholesterol oxidase	*Steptomyces*	51	102	250
cholesterol esterase	*Pseudomonas*	43	88	162
glucose oxidase	*Aspergillus*	50	105	220
peroxidase	horseradish	50	100	225

The U.S. market for drugs to control blood glucose totals about $\$1 \times 10^9$, equally divided between insulin and all other antidiabetic drugs (33). Insulin sales are expected to grow by about 10% annually, whereas the antidiabetic drug market as a whole is expected to shrink by about 3%. The blood glucose monitoring market totals about $\$7.5 \times 10^8$ in the United States and is expected to grow at a rate of 10% annually.

3. Biosensors

Biosensors (qv) and DNA probes are relatively new to the field of diagnostic reagents. Additionally, a near-infrared (nir) monitoring method (see Infrared technology and raman spectroscopy), a reagentless, noninvasive system, is under investigation. However, prospects for a nir detection method for glucose and other analytes are uncertain.

In the early 1960s, a promising approach to glucose monitoring was developed in the form of an enzyme electrode that used oxidation of glucose by the enzyme GOD (34). This approach has been incorporated into a few clinical analyzers for blood glucose determination. Three detection methods used in the glucose enzyme electrode are shown in Figure 5. Oxygen consumption, measured in the earliest method (Fig. 5**a**), requires a reference, nonenzymatic electrode to provide an amperometric signal. The second approach (Fig. 5**b**) detects H_2O_2 but requires an applied potential of about 650 mV and an inside permselective membrane. The third-generation biosensor (Fig. 5**c**) takes advantage of the fact that the enzymatic reaction happens in two steps. The GOD enzyme is reduced by glucose, and then the reduced enzyme is oxidized by an electron acceptor,

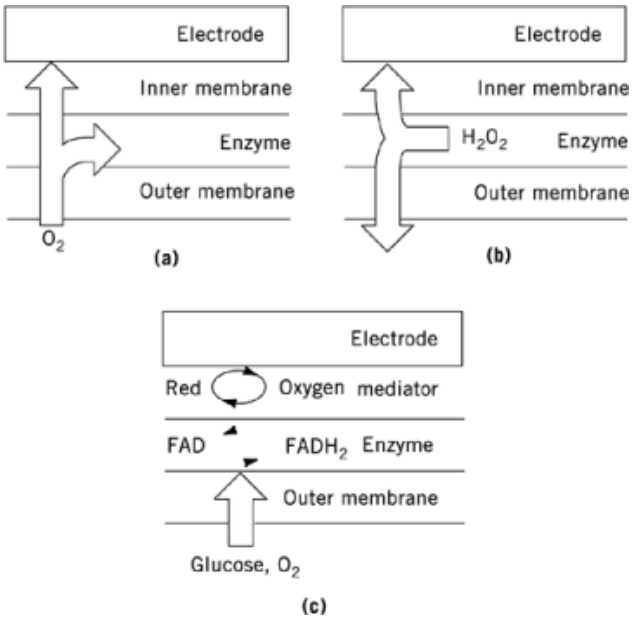

Fig. 5. Detection methods for glucose enzyme electrode based on (**a**) oxygen, (**b**) hydrogen peroxide, and (**c**) a mediator. See text.

ie, a mediator, specifically, a redox polymer. Direct electron transfer between GOD and the electrode occurs extremely slowly; therefore an electron acceptor mediator is required to make the process rapid and effective (35).

Much work has been done on exploration and development of redox polymers that can rapidly and efficiently shuttle electrons. In several instances an enzyme has been attached to the electrode using a long-chain polymer having a dense array of electron relays. The polymer which penetrates and binds the enzyme is also bound to the electrode.

Extensive work has been done on osmium-containing polymers . Large numbers of such polymers have been made and evaluated (36). The most stable and reproducible redox polymer of this kind is a poly(4-vinyl pyridine) (PVP) to which $Os(bpy)_2Cl_2$, where bpy = $2,2'-$bipyridine, has been attached to 1/16th of the pendant pyridine groups. The resultant redox polymer is water insoluble and biologically compatible by partial quaternization of the remaining pyridine groups using 2-bromoethyl amine. The newly introduced quaternized amine groups can react with a water-soluble epoxy, eg, polyethylene glycol diglycidyl ether, and GOD to produce a cross-linked biosensor coating film. Such coating films produce high current densities and a linear response to glucose up to 600 mg/dL. The synthesis and application of osmium polymers have been refined (37). Osmium monomers that can also shuttle electrons much as the polymer does have also been made (38). A schematic depiction of these polymer–GOD hydrogel films is shown in Figure 6 (38).

Flexible polymer chains have also been used for relays (39, 40) to provide communication between GOD's redox centers and the electrode. These ferrocene-modified siloxane polymers are stable and nondiffusing. Biosensors based on these redox polymers gave good response and superior stability. Commercial electrochemical microbiosensors, eg, Exactech (Medisense) and a silicon-based 6+ system (*i*-Stat) have appeared in the marketplace. These newer technologies should certainly impact rapid blood chemistry determinations by the year 2000. A typical important example is that of blood glucose determination using very small ($<5-\mu$L) blood volumes obtained by finger-stick. These detection instruments can be designed more compactly than

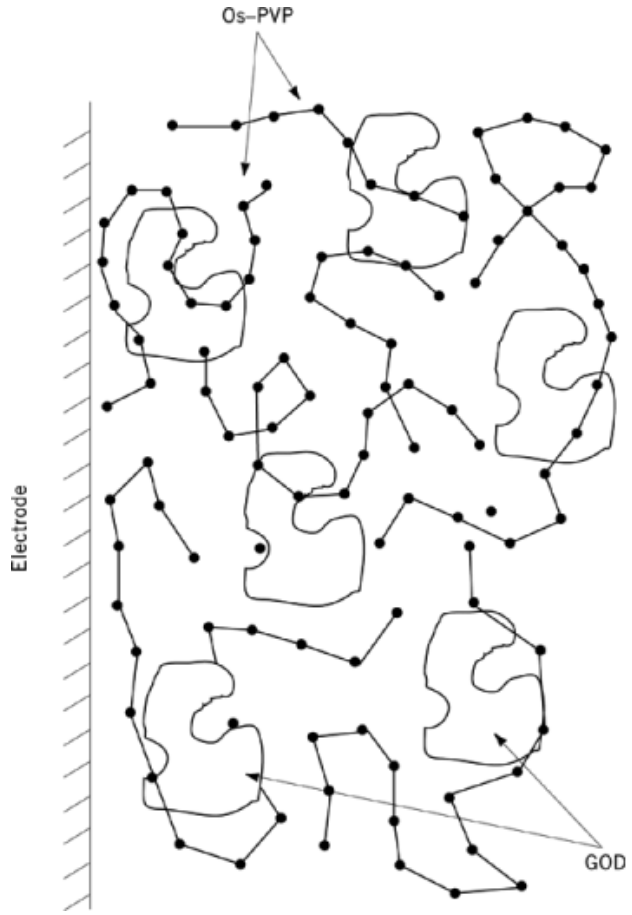

Fig. 6. Depiction of (–•––•––•–) Os–PVP polymer–GOD hydrogel film on an electrode.

optoelectronic systems. There is certainly a market for small, disposable electrochemical tests to be used in the emergency room and in surgical and critical care units, as well as at home.

A compound which is a good choice for an artificial electron relay is one which can reach the reduced $FADH_2$ active site, undergo fast electron transfer, and then transport the electrons to the electrodes as rapidly as possible. Electron-transport rate studies have been done for an enzyme electrode for glucose (G) using interdigitated array electrodes (41). The following mechanism for redox reactions in osmium polymer–GOD biosensor films has been proposed.

$$\mathrm{GOD(FAD) + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} GOD(FAD)\cdot G \xrightarrow{k_2} GOD(FADH_2) + glucolactone}$$

$$\mathrm{GOD(FADH_2) + 2\ Os(III) \xrightarrow{k_3} GOD(FAD) + 2\ Os(II) + 2\ H^+}$$

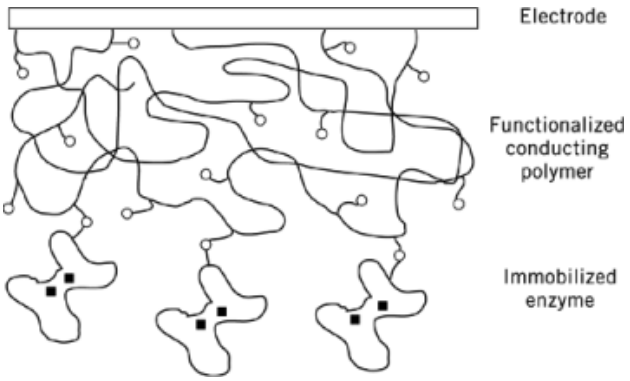

Fig. 7. Schematic representation of enzyme covalently bound to a functionalized conductive polymer where (◦) represents the functional group on the polymer and (■) the active site on the enzyme (42). Courtesy of the American Chemical Society.

$$Os_1(II) + Os_2(III) \xrightarrow{k_e} Os_1(III) + Os_2(II)$$

$$Os_2(II) \xrightarrow{\text{fast}} Os_2(III) + e^-$$

The next generation of amperometric enzyme electrodes may well be based on immobilization techniques that are compatible with microelectronic mass-production processes and are easy to miniaturize (42). Integration of enzymes and mediators simultaneously should improve the electron-transfer pathway from the active site of the enzyme to the electrode.

Functionalized conducting monomers can be deposited on electrode surfaces aiming for covalent attachment or entrapment of sensor components. Electrically conductive polymers (qv), eg, polypyrrole, polyaniline [25233-30-1], and polythiophene[25233-34-5], can be formed at the anode by electrochemical polymerization. For integration of bioselective compounds or redox polymers into conductive polymers, functionalization of conductive polymer films, whether before or after polymerization, is essential. In Figure 7, a schematic representation of an amperometric biosensor where the enzyme is covalently bound to a functionalized conductive polymer, eg, β-amino(polypyrrole) or poly[*N*-(4-aminophenyl)-2,2′-dithienyl]pyrrole, is shown. Entrapment of ferrocene-modified GOD within polypyrrole is shown in Figure 7.

There is a pressing need for an implantable glucose sensor for optimal control of blood glucose concentration in diabetics. A biosensor providing continuous readings of blood glucose would be most useful at the onset of hyper- or hypoglycemia, enabling a patient to take corrective measures. Furthermore, incorporating such a biosensor into a closed-loop system having a microprocessor and an insulin infusion pump could provide automatic regulation of the patient's blood glucose. Two novel technologies have been used in the fabrication of a miniature electroenzyme glucose sensor for implantation in the subcutaneous tissues of humans with diabetes (43). An electrodeposition technique has been developed to electrically attract GOD and albumin onto the surface of the working electrode. The resultant enzyme–albumin layer was cross-linked by glutaraldehyde [111-30-8]. A biocompatible polyethylene glycol–polyurethane copolymer has also been developed to serve as the outer membrane of the sensor to provide differential permeability of oxygen relative to glucose, in order to avoid oxygen deficit encountered in physiologic tissues.

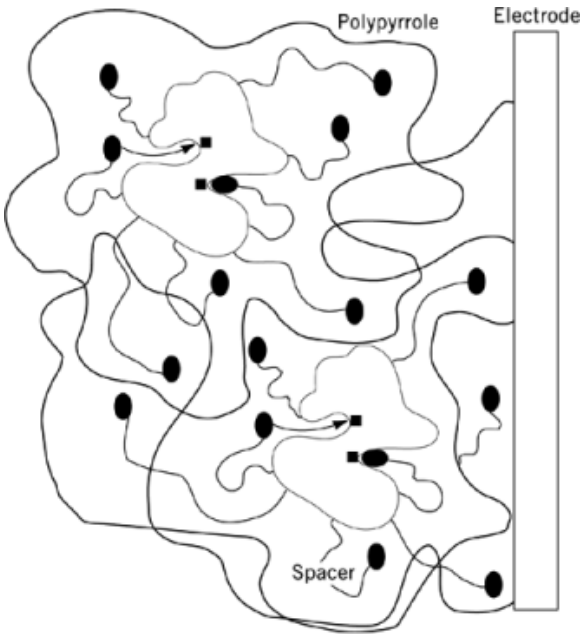

Fig. 8. Entrapment of mediator-modified enzymes within a conductive polymer film where (⬮) represents the mediator ferrocene and (■) the active site of the enzyme glucose oxidase (GOD) (42).

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