Nonaqueous Diagnostic Polymers and Coatings

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SYNOPSIS

Water-borne enzymatic coating-based dry chemistries have been used for more than 20 years, especially by diabetics, for self-monitoring of blood glucose. Until now, it has been believed that enzymes work only in water and not in organic solvents. Synthesis of a hydroxylated acrylic polymer and the novel concept of dispersing enzymes have enabled us to design nonaqueous diagnostic coatings. These coating films gave excellent dose response and dynamic range. Additionally, these coatings can easily be ranged by a wide variety of antioxidants. In contrast, water-borne coatings are extremely difficult to range. Furthermore, nonaqueous coating films produce color signals that are independent of the reaction time with excellent thermal stability. Molecular forces and thermodynamical considerations have been used to explain the performance of the new dry chemistries. An organic reaction mechanism of ranging has also been proposed for the nonaqueous system. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

The wet chemistry method for analysis of body analytes, e.g., blood glucose or cholesterol, requires a laboratory, equipment, and trained analyst. Millions of people with diabetes are required to check their glucose and obtain results in a matter of a few minutes. Science has not yet invented an insulin formulation that will respond to the body's senses. 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 is essential for diabetes, and this has become possible for the past 20 years due to the advent of dry chemistries.[1-8] By regular and accurate monitoring of her blood glucose level by dry chemistry, an expectant 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 chemistries are not only useful to diabetics, but to patients with other medical problems. They are used in animal diagnosis, food, fermentation, and agriculture as well as in environmental and industrial monitoring.

In a typical glucose measuring dry reagent, glucose oxidase (GOD) and peroxidase (POD) enzymes, along with a suitable indicator, e.g., tetramethyl benzidene (TMB), are dissolved and/or dispersed in a latex or water-soluble polymer. This water-borne enzymatic coating is applied to a lightly TiO_2 pigmented plastic film and dried to a dry thin film. The coated plastic, cut to about 0.5×0.5 cm size, and mounted on a plastic strip is the dry reagent. The user applies a drop of blood and allows it to react with the dry reagent for about 60 s or less. The blood is wiped off and the developed color is then read by a meter or visually compared with a predesigned printed color block to precisely determine the glucose level in the blood. Thus, the dry chemistries are highly user friendly. Biochemical reactions in glucose determination are shown below[6]:

$$\text{D-glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase (GOD)}} \text{D-glucono-1,4-lactone} + H_2O_2$$

$$\text{D-glucono-1,4-lactone} + H_2O \rightarrow \text{Gluconic acid}$$

$$H_2O_2 + \text{indicator (reduced)} \xrightarrow{\text{Peroxidase (POD)}} \text{Indicator (oxidized)} + 2H_2O$$

For the past 20 years, dry reagent coatings have exclusively been water-borne because of the belief

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that enzymes function effectively only in a water medium. We have researched nonaqueous enzymatic coatings for dry chemistries to which red blood cells will not adhere in addition to giving quick endpoint reaction. These new coatings have superior thermostability as well. In this paper, we will describe synthesis of highly hydroxylated acrylic polymers, nonaqueous coating design, reaction stability, and a proposed stability mechanism. The new nonaqueous coatings can easily be ranged by a variety of antioxidants, whereas water-borne enzymatic coatings are very difficult to range. These differences are explained using organic reaction mechanisms.

EXPERIMENTAL

Synthesis of Highly Hydroxylated Acrylics

The nonaqueous diagnostic polymer must impart particular desirable properties to the resulting coating film. Most importantly, hydrophilicity and hydrogel character to the film must be imparted, thus allowing intimate contact with the aqueous whole blood sample. Furthermore, it may also provide some enzyme-stabilizing effects.

An acrylic resin comprising 65 wt % 2-hydroxyethyl methacrylate (HEMA), 33 wt % butyl methacrylate (BMA), and 2 wt % dimethylaminoethyl methacrylate (DMAEMA) was made by solution polymerization at 40% solid in xylene/1-methoxy-2-propanol (1/1). The polymerization was done at 90°C for 6 h using 1% azobisisobutyronitrile initiator. Another acrylic polymer, HEMA/BMA/*t*-butylaminoethyl methacrylate (65/34/1), was also made similarly. In fact, a very large number of polymers were made—the above compositions are typical among the best.

Enzyme Dispersion

The glass transition temperature (T_g) of glucose oxidase (GOD) and peroxidase (POD) is 50°C.[9] In organic solvents, these enzymes become extremely rigid and can be dispersed with ease. Dispersions were made by grinding GOD and POD in xylene/methoxy propanol with or without a surfactant using an Attritor mill (2–4 h) or a ball mill (24 h). Grinding was continued until dispersion of < 1 μm was obtained. Upon completion of dispersion, grinding media was strained and the dispersion stored at 4°C until its use. The composition of a typical dispersion is 1.876 g GOD, 4.298 g POD, 11.79 sodium dodecyl sulfate, 41.06 g xylene, and 41.06 g 1-methoxy-2-propanol.

Coating Compositions and Process Technology

A generalized method for preparation of the nonaqueous enzymatic coating involves adding polymer solution, TMB, mica, surface modifiers, and solvents to the enzyme dispersion.[10] Ranging compound can be postadded to the coating. Only slight mixing on a ball mill is required after ingredient addition to complete enzymatic coating preparation. The composition of a typical nonaqueous coating useful for low-range blood glucose detection is shown in Table I.

Many surfactants and surface modifiers were investigated; the four best combinations are presented in Table II.

To improve resolution in measurement of glucose levels in the high-range (220–800 mg/dL), many hindered phenol antioxidants were found useful. These antioxidants that function as ranging compounds are indicated in Table III.

Table I Composition of a Typical Nonaqueous Coating

	Wt %	Actual Batch (g)
HEMA/BMA/DMAEMA (65/33/2)[a]	33.29	1.68
TMB	2.38	0.12
GOD	1.17	0.0589
POD	2.68	0.1355
SDBS[b]	3.28	0.1656
Xylene	26.53	1.339
1-M-2-P[c]	26.53	1.339
Mica[d]	1.96	0.099

[a] 40% solid.
[b] Sodium dodecyl benzene sulfonate.
[c] 1-Methoxy-2-propanol.
[d] Cosmetic grade, C-4000, ultrafine.

Table II Surfactant and Surface Modifiers

Combination			
#1	#2	#3	#4
SDS[a] Igepal CO-530[b] Silane Z 6040[d]	SDS Flow tone 4[c]	SDBS	SDBS SDS
Features of reacted coating films			
Dull blue, no RBC[e] retention	Dull blue, very smooth surface, no RBC retention	Vibrant blue, no RBC retention	Good color, no RBC retention

[a] Sodium dodecyl sulfate.
[b] Nonionic (GAF).
[c] Sulfonated castor oil.
[d] Epoxidized (Dow Corning).
[e] Red blood cell.

Preparation of Coating Films

The coatings were applied on a lightly TiO_2 pigmented polycarbonate plastic film at a wet film thickness of 100 μm and dried in an air-forced oven at 50°C for 15 min. A laboratory coater was used for applying coating onto polycarbonate at 2 m/min rate.

Reactions of Coating Films

For dose response and other studies with whole blood, the coated films were incorporated into a touch and drain test device (Fig. 1).[6,11] In this device, the blood is caused to flow across the surface of the coating-film. The residence time can thus be regulated at will. The color signals of reacted coating films were monitored by diffuse reflectance spectrophotometry. Diffuse reflection occurs from within the layers of the dry chemistry coated onto white polycarbonate. The reflected light is distributed throughout an angle of 180°. There are several mathematical approaches available: The Kubelka and Munk equation is best known among several others that are used in relating analyte concentration to reflectance in dry chemistry:

$$C\alpha \frac{K}{S} = \frac{(1-R)^2}{R}$$

wherein C = analyte concentration; K = absorption coefficient of product; S = scattering coefficient of layer; and R = % reflectance/100.

In general, the relationship between reflectance and concentration is broadly similar to that of concentration and transmission (Beer–Lambert law). In actual practice, calibration of the result is made by a test-specific and lot-specific function curve contained in the magnetic code. Invariably, algorithms are also used frequently. A McBeth 1500 visible spectrometer was used for measuring color signals.

Table III Ranging Compounds

Polymer	Ranging Compound	Ranging Compound: TMB Molar Ratio (Optimum)
HEMA/BMA/DMAEMA (65/33/2)	APAC[a]	1 : 20
HEMA/BMA/TBAEMA (65/34/1)	APAC	1 : 15
HEMA/BMA/DMAEMA (65/33/2)	BHT[b]	1 : 2.5
	BHT/PG[c] (85/15)	1 : 3.0

[a] 3-Amino-9-(aminopropyl)–carbozole dihydrochloride.
[b] Butylated hydroxy toluene (2,6-di-*tert*-butyl-*p*-cresol).
[c] Propyl gallate.

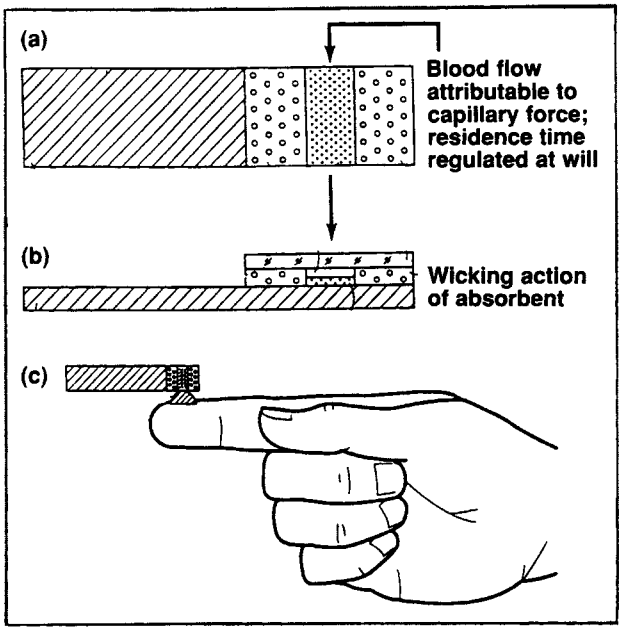


Figure 1 Touch and drain model: (a) dry-coated surface, (b) cross section of dry-coated surface, adhesive, and cover piece; (c) contact with blood drop results in blood filling the cavity. After desired reaction time, blood is drained off by touching end of cavity with absorbent material.

RESULTS AND DISCUSSION

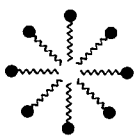
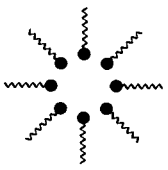
Comparison of Aqueous and Nonaqueous Diagnostic Coating Systems

The characteristics and performance of aqueous and nonaqueous diagnostic coatings differ. The composition and structural differences are indicated in Table IV.

Dose Response and Dynamic Range

Figure 2 shows the dose-response curves for six blood glucose samples from 30 to 231 mg/dL glucose for

Table IV Comparison of Aqueous and Nonaqueous Diagnostic Coatings

Feature	Aqueous	Nonaqueous
Continuous phase	Water	Organic solvents
Discontinuous phase		
Enzymes (GOD, POD)	Dissolved	Dispersed
Indicator (TMB)	Dispersed; adsorbed onto latex particles due to micellar forces	Dissolved; therefore coats enzyme dispersions very uniformly
Micelles (surfactant)		
Antioxidant	Dispersed; adsorbed onto latex particle due to micellar forces	Dissolved
Polymer binder	Latex polymer of very high molecular weight	Polymer solution of high molecular weight

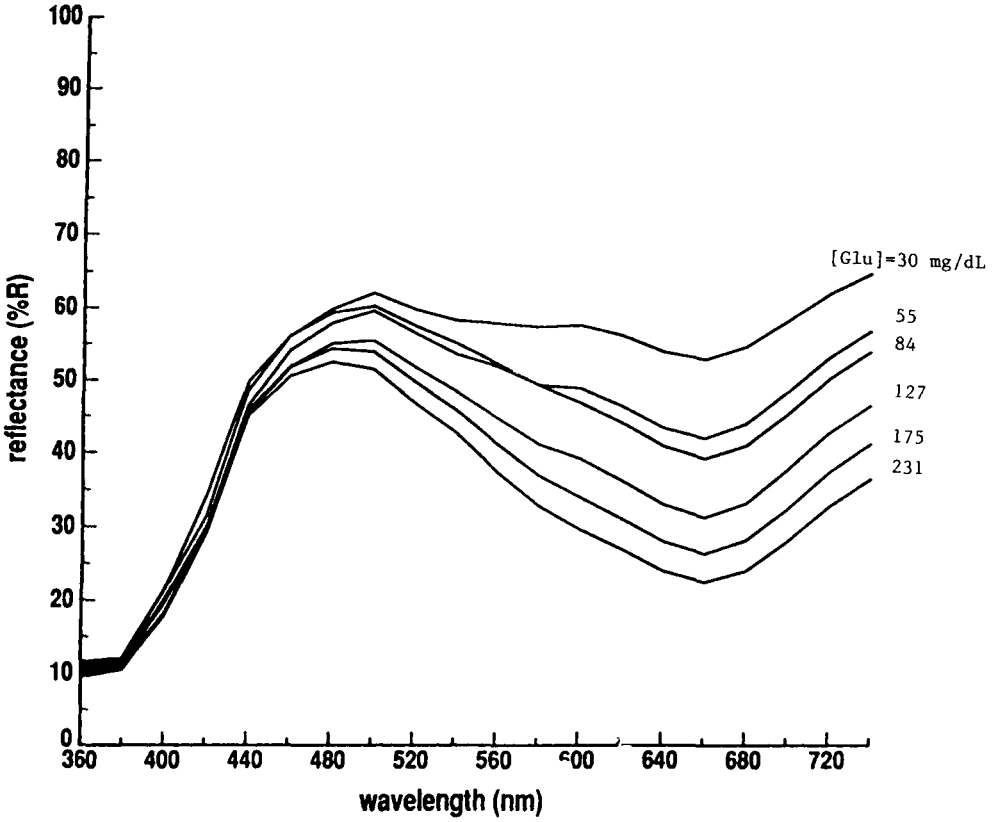


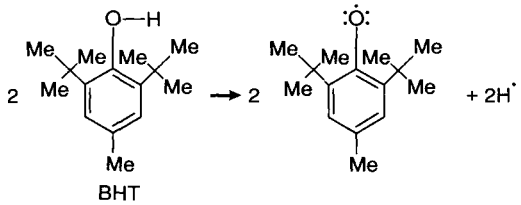
Figure 2 Low-range blood glucose dose response.

Table I coating-film. At 660 nm, there is a total change in reflectance (% R) of over 30, thus allowing accurate measurement of glucose at these levels. The dose response for the same coating-film in the high-range blood glucose (171–786 mg/dL) is shown in Figure 3. Note that the % R change is about 10 and therefore this will preclude an accurate measurement of glucose at these levels. Conventional aqueous diagnostic coatings also have limited dynamic range.

To improve dynamic range and resolution, a large number of antioxidants can be used (Table III) in nonaqueous coatings. A typical example is coating (Table I) to which BHT and PG have been added (Table III). The dose response of the ranged coating is illustrated in Figure 4. A difference of more than 30% R between lowest and highest blood glucose has been accomplished by the ranging compounds.

Mechanism of Ranging

The water-borne enzymatic coatings do not lend themselves to ranging easily. Most hindered phenols are ineffective. Only APAC is effective in some aqueous coatings. In contrast, almost all hindered phenols are effective in nonaqueous coatings. There are two main reasons for this disparity between the aqueous and nonaqueous coatings. The physical reason is due to the solubility of the hindered phenols in organic solvents. In nonaqueous coatings, the ranging compounds uniformly coat the enzyme particles. In a similar vein, the indicator TMB is uniformly coated onto enzyme particles. H_2O_2 generated due to the reaction of blood glucose in the coating-film is thus readily available to the antioxidant. In aqueous coatings, the antioxidant will be found discontinuously as aggregate, large insoluble particles and adsorbed onto emulsion particles. Much of the generated H_2O_2 does not find the antioxidant. The antioxidants, e.g., BHT, are radical scavengers:



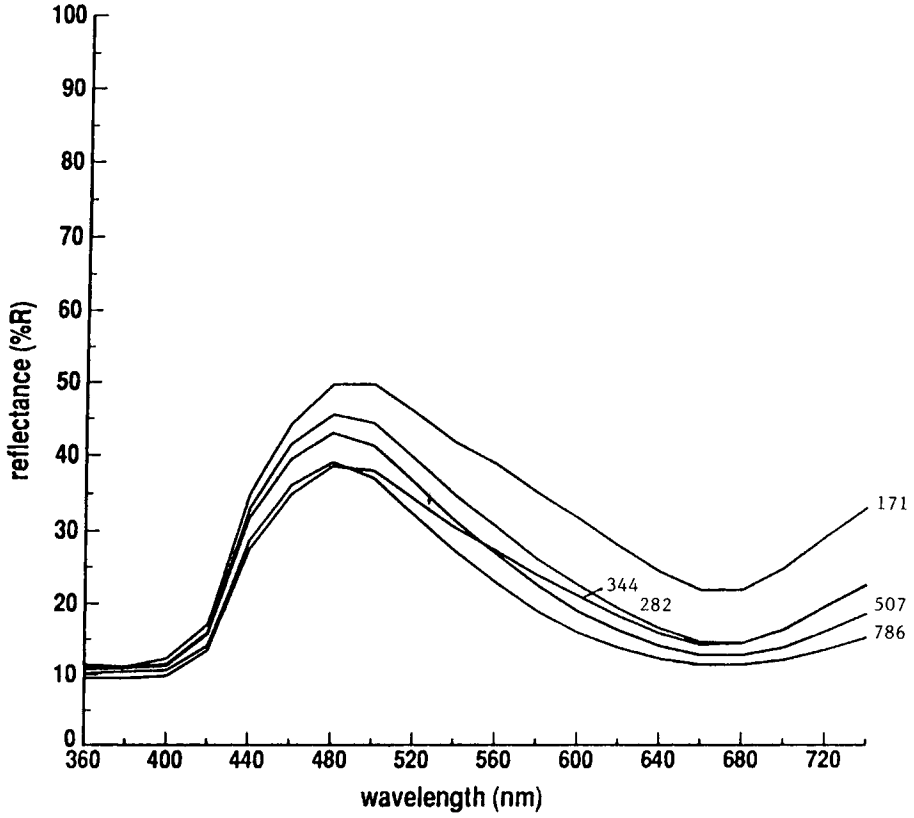


Figure 3 High-range blood glucose dose response.

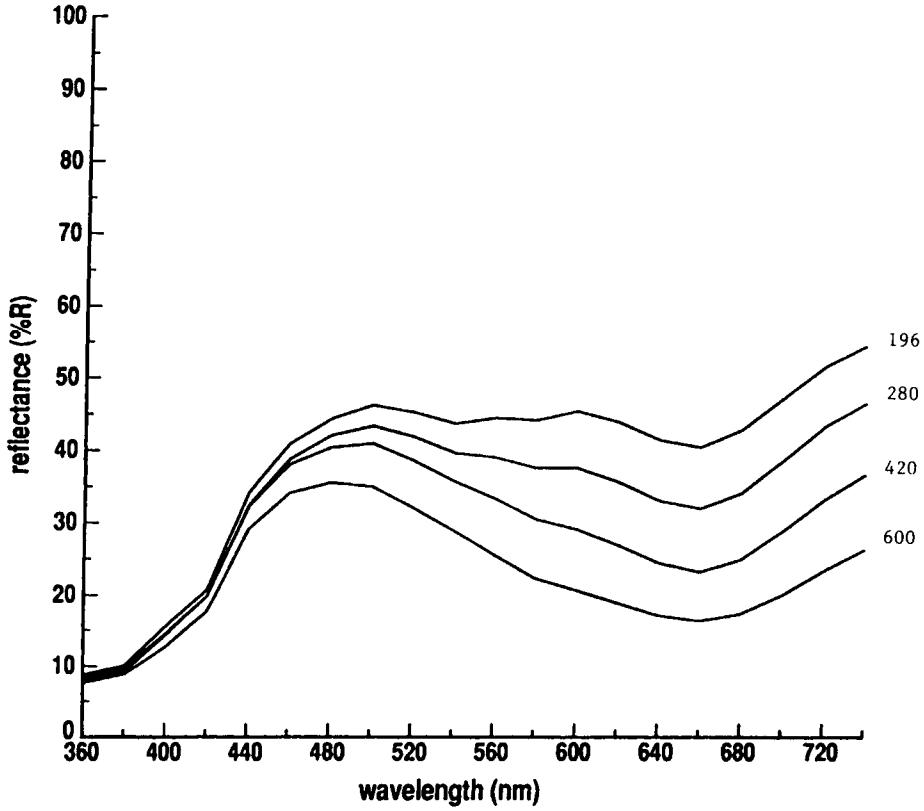
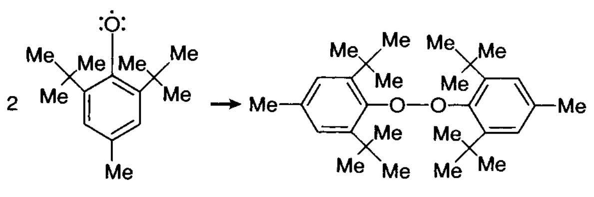


Figure 4 High-range blood glucose dose response. Coating-film contains antioxidants.

The generated RO$^{\bullet}$ radical is very stable and persistent due to resonance and inductive effects. It therefore helps consume generated H_2O_2:

$$HO^{\bullet}{}^{\bullet}OH + 2H^{\bullet} \rightarrow 2H_2O$$



In aqueous coatings H_2O_2 is regenerated as shown below:

$$OH^{\bullet} + H_2O \rightarrow HO^{\bullet}{}^{\bullet}OH + H^{\bullet}$$

Thus, nonaqueous coatings lend themselves to ranging, whereas aqueous coatings do not respond to ranging.

Characteristics of the New Chemistry

In dry chemistries, endpoint reactions are preferred over kinetic reactions. The new chemistry has not only quick endpoint but several other desirable characteristics.

The chemistry is extremely fast-reacting and the generated color signal is independent of the blood residence time. In Figure 5, color signals generated as a function of reaction time from 5 to 60 s have been shown. Thus, we find that the reaction is independent of the residence time.

The diagnostic polymers used in the nonaqueous coatings are "hydrogel" in nature and are fully wetted by blood almost instantaneously. Microscopic dye penetration experiments have shown that the liquid penetrates the polymer as well as the coatings in less than 2 s. The generated colors remain stable immediately after reaction and also for an extended time.

In general, uric acid is known to produce interference by lowering color signals (increasing % R) in dry chemistries. Blood glucose containing a high level of uric acid (30 mg/dL) produced a lowering of the color signal in nonaqueous coating films. This interference was, however, prevented by buffering the coatings to pH 5.5 (Fig. 6).

Long-term stability of nonaqueous coating-films under elevated temperature (up to 65°C) and moderate humidity (up to 50% HR) was very good. Stability of these coating-films were much better than

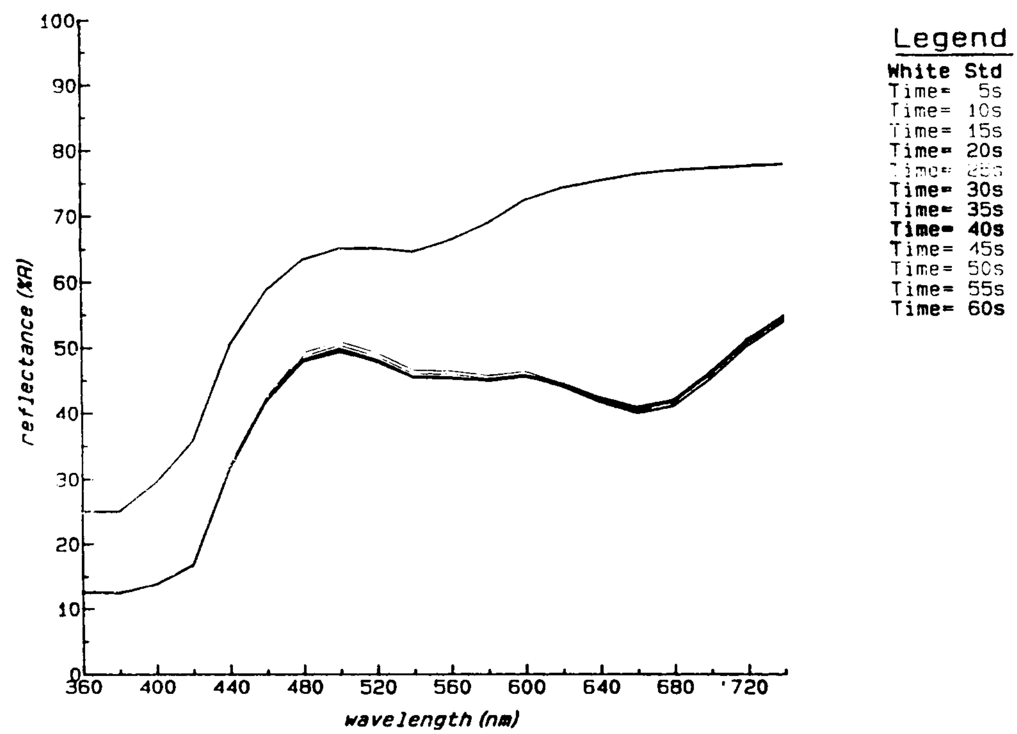


Figure 5 Color as a function of reaction time in a coating with APAC : TMB at 1 : 20 molar ratio.

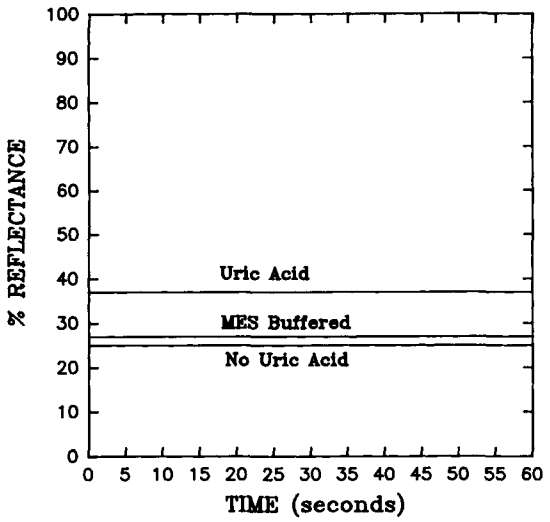


Figure 6 Uric acid interference and correction by buffering at pH 5.5.

that of comparable aqueous films. For a discussion of stability, see Ref. 12.

The color resolution and sensitivity of reacted coating-films were found to be very good. The % R dose response data for a high-range high-solid coating were fitted to the best nonlinear function using equation $y = Ae^{-bx} + CX + D$, where y is % R; X, the concentration of glucose in blood in mg/dL; A, 66.93; b, −0.087; C, −0.09; and D, 4.28 (Fig. 7). Coating films that deviate from such fittings lack accuracy and precision.

Stability of the New Chemistry

The conformation of the enzyme molecule in solutions is determined by a complicated network of both hydrogen bonds and electrostatic as well as hydrophobic interactions. To sustain native conformation, the enzyme molecule must have both surface and interior water. Introduction of organic solvents in the dispersions and coatings did not strip or distort these waters since the catalytic activity remained intact. In fact, the exterior surface water acts as a "lubricant or flexibilizer" in nonaqueous medium, thus allowing the catalytic reactions to occur.

Steric stabilization produces added thermal stability in nonaqueous systems. The GOD molecule is about 50 Å in size, whereas a typical diagnostic emulsion polymer is 200–500× in size. The emulsion particles therefore cannot compress the protein. If compression were to take place, there will be a change in the free energy, given by the Gibbs–Helmholtz equation[13,14]:

$$\Delta F_R = \Delta H_R - T\Delta S_R$$

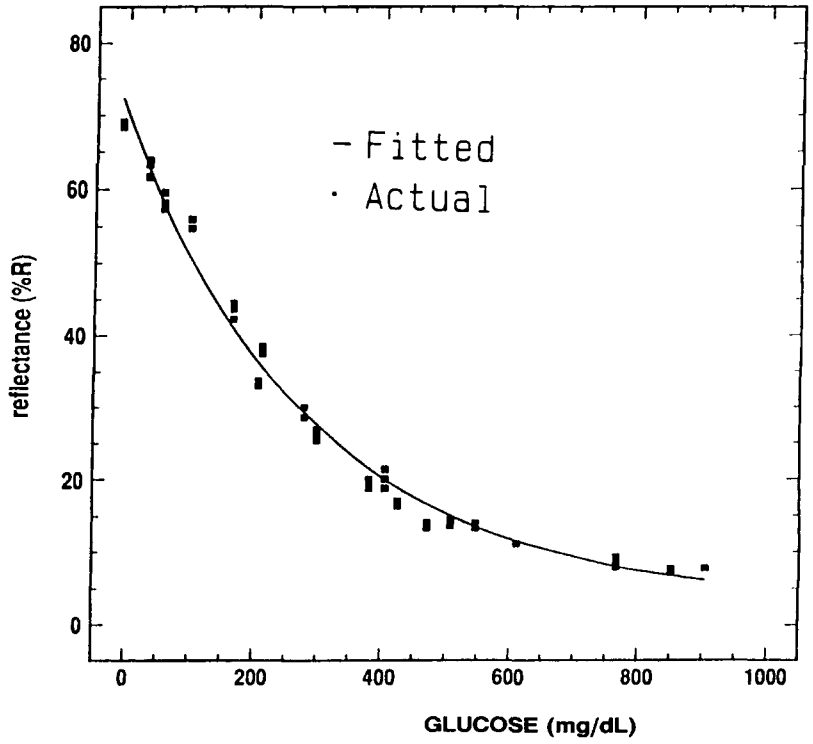


Figure 7 Nonaqueous coating film fitted % R model.

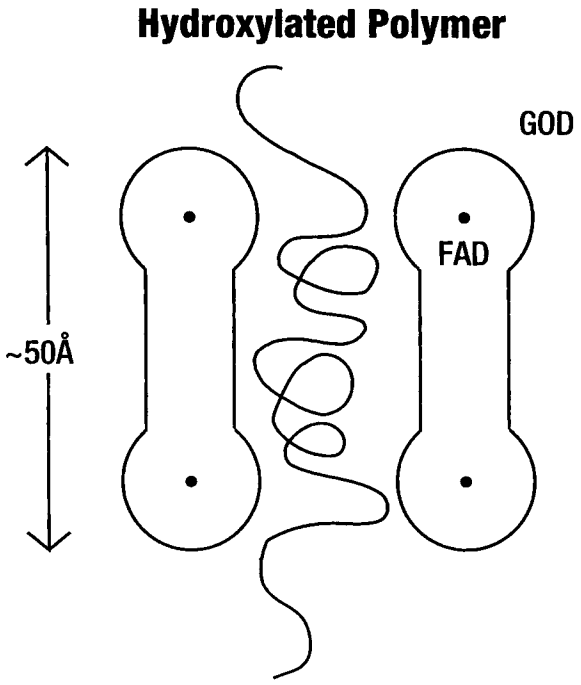


Figure 8 Compression of enzyme by polymer produces stability.

There are three possibilities regarding the change of free energy ΔF_R. Case I: $\Delta F_R = 0$; if no hydroxylated polymer is present. Case II: $\Delta F_R = +$; hydroxylated polymer will stabilize the enzyme dispersion particles and will not sensitize them. Case III: $\Delta F = -$; polymer sensitizes and destabilizes them. Thus, a positive ΔF_R is important for coating and coating-film stability. In nonaqueous systems, molecular and micellar forces can produce added stability. The coils of the hydroxylated acrylic polymer surround the protein, pack well, and compress the protein with a positive ΔF_R change, thus improving thermal stability (Fig. 8).

CONCLUSIONS

A series of highly hydroxylated acrylics was synthesized and found useful in nonaqueous dry chemistries due to their hydrophilic and hydrogel nature. In organic solvents, GOD and POD enzymes become very rigid and could easily be dispersed. The nonaqueous coatings designed from the enzyme dispersions and polymer solution gave coating films to which red blood cells will not adhere in a capillary gap-type device. These coatings could be easily ranged with antioxidants. Thus, the new nonaqueous chemistry has an excellent dynamic range and good dose response with blood glucose in the 20–800 mg/dL glucose range.

A mechanism of ranging has also been presented. The unusual thermal stability has been explained in light of intermolecular forces and Gibbs–Helmholtz free energy.

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