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Optical Determination of pH on Surfaces Using Immobilized Fluorescent Dyes

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The attachment of pH-sensitive Fluorescent dyes on to hydroxylapatite for measuring the pH of small volumes is described. Fluorescein and acridine were used, both activated with reactive functions, such as isothiocyanate or succinimidyl ester groups, to enhance the possibility of a covalent linkage to the surface. First investigations were carried out on synthetic hydroxylapatite as the surface material. After the preparation of a particular surface with the fluorescent dye, steady-state and time-resolved fluorescence spectroscopies were employed for estimating the pH value of a solution applied to the surface. In this paper we present the results of our investigations done with both methods. Fluorescein shows significant variation in excitation spectra with pH, whereas in the case of acridine, the fluorescence lifetimes are very sensitive to pH.

KEY WORDS: pH measurement on surfaces; immobilization; fluorescence spectroscopy.

INTRODUCTION

The determination of the pH very close to surfaces is important in applications in the food industry and dental research. In recent research, the status of healthy teeth is considered as a dynamic equilibrium between demineralization and remineralization of surface enamel. At a neutral pH saliva is oversaturated in calcium and phosphate (with respect to dental apatite), favoring remineralization. In an acidic environment, e.g., after the consumption of cariogenic food, associated with bacterial acid production, demineralization is the consequence. So far, knowledge on the chemical mechanisms underlying these processes is only fragmentary. This is due mainly to problems in determining—favorably, *in vivo*—the relevant parameters directly on the surface of teeth. Surface measurements of pH here are an important aspect. Glass electrodes integrated into dental appliances and telemetric signal transfer [1] are the present techniques for pH measurements in the oral cavity. Even when miniaturized, the size of the electrodes is not optimum for surface measurements. Additional interferences are uncontrolled liquid junction potentials between saliva and the reference electrodes and hydrodynamic aspects. Saliva, with widely varying physical and chemical properties, is far from a thermodynamic standard solution so that these pH determinations are no more than a rough estimate. Our approach to the problem is pH-sensitive fluorescent dyes on tooth surfaces. Signal transfer via fiber optics is anticipated.

Fluorescence measurements on solid surfaces are described in a survey by Hurtubise [2,3]. He examined different substrates such as filter paper, silica gel, and aluminium oxide, on which he absorbed different fluorescent dyes. General problems in solid-surface measurements are that both the source and the luminescence radiation is scattered by the surface and that it is difficult to get a homogeneous distribution of the dye immobilized on the surface. To overcome these specific problems caused by the surface, dyes have to be found showing

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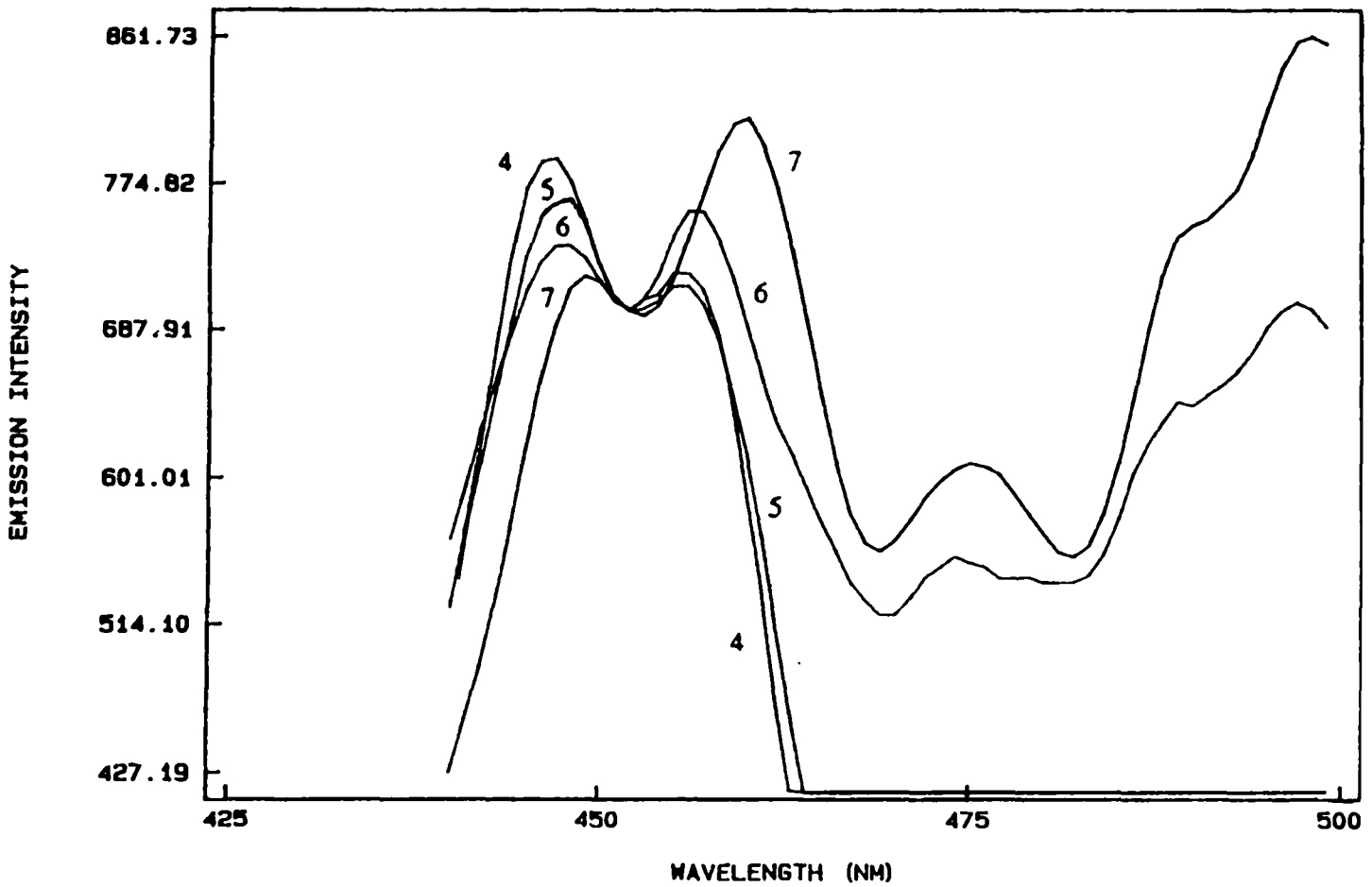

Fig. 1. pH dependence of excitation spectra of fluorescein immobilized on apatite.

a strong dependence on pH but not fluorescence intensity. This paper describes two courses that can be followed. Fluorescein immobilized on phosphorapatite surfaces shows a characteristic intensity ratio as a function of pH, therefore the absolute intensity value is not necessary for pH detection. Principally intensity ratio measurements such as that are performanced in great numbers [4,5]. In a second experiment it is shown how the detection of the fluorescence lifetime of fluorescent dyes can be used for characterization of the pH close to surfaces. The detection of pH based on fluorescence lifetimes in another context was recently shown by Thompson and Lakowicz [6].

EXPERIMENTAL

Hydroxylapatite as surface material was prepared by the following procedures. (a) Hydroxylapatite powder was pressed for 10 s at 20 kN/cm^2. The thickness of the resulting pills was 3 mm and the diameter was 12 mm. A pill was added to a solution of 1 mg fluorescein isothiocyanate dissolved in 4 ml acetone for 10 h. The pill was washed with water until the solution showed no significant fluorescence. The pill was dried for 30 min at 80°C.

(b) For time-resolved studies, a cross-linker was used for covalent binding between surface and fluorescent dye. Two grams hydroxylapatite powder was added to a solution of 15 ml toluene and 2 ml 3-aminopropyltrimethoxysilane and refluxed for 4 h. The solution was allowed to cool and filtered. The residue was washed with toluene and dried at 80°C for 1 h. With this material a pill was pressed as described above. It was added to a solution of 1 mg succinimidylacridine-9-carboxylate in 5 ml acetone. When using acridine as the fluorescent dye, the solutions have to be kept strictly in the dark, because of photoinduced reactions of acridine. After

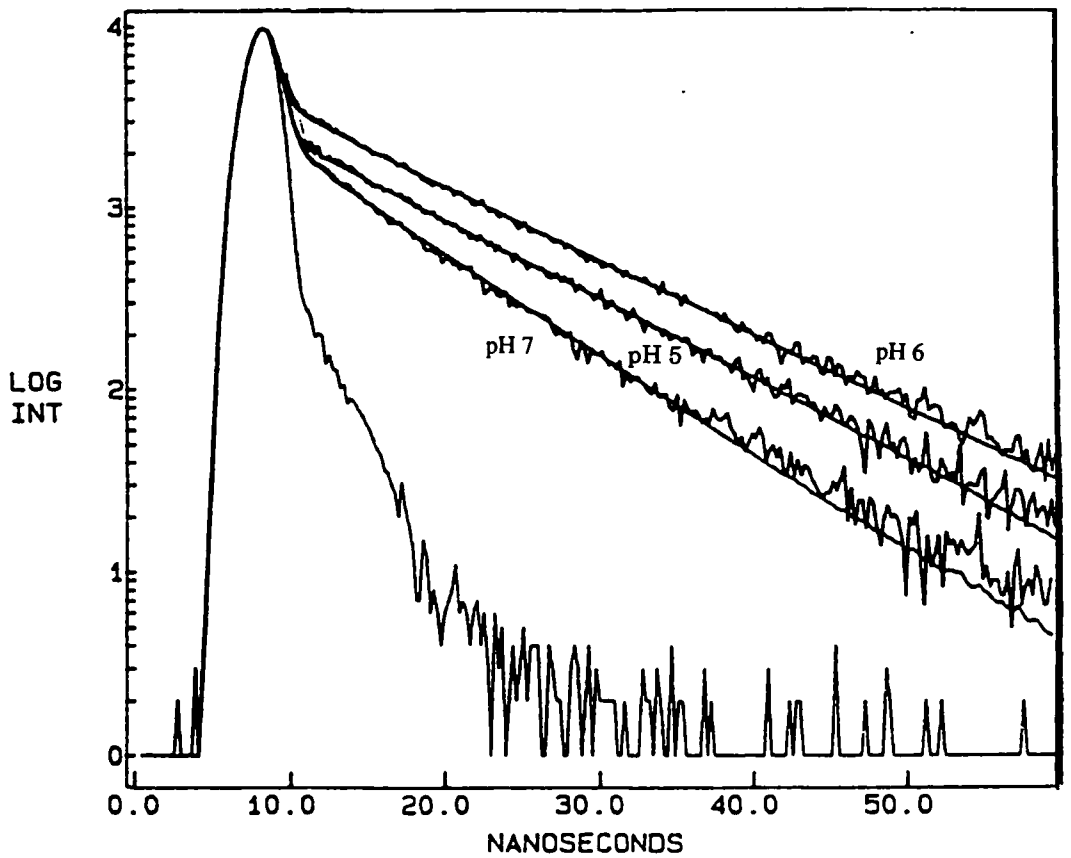

Fig. 2. pH dependence of fluorescence lifetimes of acridine immobilized on apatite.

Table I. pH Dependence of Fluorescence Lifetimes of Acridine Immobilized on Apatite

	pH		
	7	6	5
Fluorescence lifetime τ_{av} (ns)	4.3	7.5	5.7
Error χ^2	1.839	1.632	1.622

Table II. pH Dependence of Fluorescence Lifetimes of Acridine in Solution

	pH		
	4	5	6
τ_1	10.041	18.001	14.878
F_1	0.81	0.763	0.342
τ_2	14.679	7.875	7.964
F_2	0.19	0.237	0.658
τ_{av}	10.9	15.6	10.3

washing the pills with acetone and water, they were dried as described above.

Corrected spectra were recorded on an LS-100 spectrofluorimeter from PTI (Wedel, Germany). The pills were fixed at an angle of 45° concerning both the incident light and the fluorescence.

For time-resolved studies, the weak fluorescence decay curves were registered by the time-correlating single-photon counting technique using the LS-100 spectrometer (PTI). The excitation source was a hydrogen-filled flash lamp with a full width at half-maximum pulse of 1.7 ns.

RESULTS

The absorption spectra and the fluorescence excitation spectra of fluorescein in solution are pH sensitive

as described in the literature [7]. It has long been known that the strong fluorescence of this dye appears mainly in alkaline solutions; a decrease in the pH reduces the fluorescence intensity. In this context the excitation spectra for fluoresceinisothiocyanate absorbed on hydroxylapatite prepared by method a were taken. The emission wavelength was set at 520 nm; the excitation scanned from 400 to 500 nm. Each pill was treated with 20 μl of the respective buffer solution by a micropipette just before the spectra were taken. Peaks at 447, 457, 475, and 497 nm were observed, which were pH sensitive. So the pH at the surface could be determined by calculating the ratio of the intensity of the fluorescence emission at two wavelengths. The spectra are similar to those in solution, but two additional peaks, at 447 and 457 nm, were observed, which are obviously due to interactions of the dye molecules with the surface. These spectra are very sensitive to the dye concentration on the surface, so that in some cases concentration-dependent self-quenching could be observed. Due to the characteristic intensity ratio, the absolute value of the fluorescence intensity does not have to be known.

For time-resolved studies, acridine was found to be suitable as the fluorescent dye, because of its long fluorescence lifetimes and excellent pH dependence in solution. Many investigations of the fluorescence of acridine are found in the literature [8,9]. The fluorescence lifetimes of succinimidylacridine-9-carboxylate immobilized with 3-aminopropylsilane as cross-linker on apatite as described in method b were estimated as described above concerning the application of buffer solution and probe positioning. The decay curves were expressed by a biexponential fit with $t_{av} = t_1F_1 + t_2F_2$, where F_1 and F_2 are the relative amplitudes of the components. The decay curves for pH 6, 5, and 7 are listed in Table I. The excitation wavelength was 350 nm; the emission wavelength was set at 450 nm. The lifetimes were shorter than the pH-dependent lifetimes of acridine measured in solution (Table II). The measurements in solution and on the surface showed no systematic drop in lifetime corresponding to the decreasing pH value, but the lifetimes changed irregularly. The combination of τ_1 and τ_2 could be used for estimating the pH. Additionally a sharp bend could be seen at the beginning of the decay curves of all surfaces prepared, indicating an interaction between fluorescent dye and surface. This effect was observed in a similar context by Kemnitz *et al.* [10].

DISCUSSION

A general aim of our efforts was to develop a calibration-free measurement of pH on surfaces. For this purpose, both the excitation ratio of fluorescein peaks and the pH-dependent fluorescence lifetimes of acridine are suitable, as shown above. These dyes were immobilized on phosphorapatite either by covalent linkage or by absorption based upon ion-exchange effects. For covalent linkage, the dyes were derivatized with aminoreactive functions such as isothiocyanate or succinimidylester groups. For further assessment of the success of the immobilization procedure, surface analytical techniques such as ESCA are necessary. Concerning the interpretation of the fluorescence spectra on the surface, it is obvious that different dye concentrations have an important influence on the spectra, such as shifting of emission maxima or, in time-resolved studies, shortening of fluorescence lifetimes due to dimer effects. In the case of acridine, several aspects have to be considered, for example, the excited-state proton transfer reactions, which are described in detail in the literature [9,10]. Probably, a biexponential fit is not the best choice, because in our experiments a mixture of species and interactions is expected: acridine base, acridinium cation, fluorescent dimers built by photochemical reactions, and two-dimensional Förster energy transfer processes. Hence, further investigations are necessary for using acridine as a pH detector molecule at surfaces.

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