In situ chemichromic studies of interactions between a lutetium bis-octaalkyl-substituted phthalocyanine and selected biological cofactors

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Spin-coated films, approximately 100 nm thick, of a newly synthesized bis[octakis(octyl)phthalocyaninato] lutetium(III) complex on ultrasonically cleaned glass substrates exhibit pronounced chemichromic behaviour with potential application in healthcare. *In situ* kinetic optical absorption spectroscopic measurements show that the phthalocyanine Q-band is red shifted by 60 nm upon oxidation arising from exposure to bromine vapour. Recovery to the original state is achieved by the treatment of the oxidized films with nicotinamide adenine dinucleotide and L-ascorbic acid (vitamin C) in an aqueous solution containing 1.5 M lithium perchlorate. The neutralization process is found to be governed by first-order kinetics. The linear increase of the reduction rate with increasing concentration of cofactors provides a basis for calibration of analyte concentrations ranging from 3.5 mM down to 0.03 mM.

Keywords: lutetium bisphthalocyanine; UV–visible absorption; biomolecules; chemichromic changes; rate of reduction

1. INTRODUCTION

Cofactors such as nicotinamide adenine dinucleotide (NAD⁺) and ascorbic acid (vitamin C) are catalytic agents for many important biological activities in the human body [1]. The reduced form of nicotinamide adenine dinucleotide (NADH) is the first enzyme of the mitochondrial electron transfer chain. In metabolism this compound acts as an electron exchanger in redox reactions and it is an important cofactor in mitochondrial respiration. The NAD⁺/NADH ratio in the range of 1–0.1 determines the normal metabolic state of the body and the increase in free NAD⁺/NADH ratio arising from calorie restriction leads to the reduction of several age-related diseases, such as cancer, neurodegenerative diseases and diabetes [2]. *In vitro* UV and nuclear magnetic resonance spectroscopic studies show that oxidation of NADH by the dopamine-derived quinones may induce mitochondrial dysfunction, such as mitochondrial swelling in the pathogenesis of Parkinson disease [3]. An unexpected elevation or lowering of

NADH may cause severe diseases like brain ischaemia and Leigh syndrome [4,5]. NADH/NAD⁺ redox imbalance is usually caused by elevated intracellular [Na⁺] during cardiac glycosides for heart failure treatment. The balance can be restored by an increase in mitochondrial [Ca²⁺] accumulation [6].

Vitamin C is an electron donor and helps metabolism by acting as a cofactor in enzymatic hydroxylation. It acts as a useful antioxidant and neurotransmitter, and also for developing bone structure and immune systems in the body. The daily dietary requirement of vitamin C is at least 75 mg for men and women but this is usually higher for smokers and pregnant women [7,8]. A deficiency of vitamin C may lead to scurvy, osteoporosis, bone homeostasis, high blood pressure and gallbladder disease [9]. Vitamin C-rich diets are clinically recommended for the healing of wounds and for the repair and maintenance of cartilage, bones and teeth [10]. It has been recently demonstrated *in vivo* that vitamin C is not only responsible as an antioxidant for suppressing osteoclast activity and number but also as a cofactor promotes osteoblast differentiation [11]. Evidence also exists for skeletal lesions associated with

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Figure 1. (a) Structural representation of bis[1,4,8,11,15,18,22,25-octakis(octyl)phthalocyaninato] lutetium(III), $R_{16}Pc_2Lu$, $R = C_8H_{17}$ (octyl) chains, (b) AFM image of $R_{16}Pc_2Lu$ film surface and (c) thickness scale. (Online version in colour.)

adult scurvy owing to lack of vitamin C [12]. A selection of vitamins used by postmenopausal women has been examined in a recent review for their effects on bone health, cardiovascular health, breast cancer, cognition and vasomotor symptoms. Low vitamin C intakes have been associated with a decline in bone mineral density specifically at the femoral neck and total hip. However, the role of vitamin C supplements appears to be limited at prevention of coronary heart disease and cancer incidence, including breast cancer [13]. A survey of hospitalized scurvy patients shows that the vitamin level in human serum can be reduced down to 0.006 mM which is six times lower than the minimum requirement of 0.036 mM in a healthy body [14].

Several spectroscopic, chromatographic and electrochemical studies have been proposed for rapid, cost-effective sensing of both NADH and vitamin C at varying concentrations in order to meet the requirements of pharmaceutical and food industries. Gold nanoparticles were electrodeposited on a carbon electrode and then modified with a layer of a conducting polymer (poly-5,20:50,200-terthiophene-30-carboxylic acid) to form a biosensor for determining the NADH in a cultured cell sample within a dynamic range of 1.0 μM and 5.0 mM. The detection limit was 0.5 μM [15]. Detection of NADH in an extended range of 20 μM –80 nM has been achieved using glassy carbon electrodes modified with room-temperature ionic liquid (*N*-butyl-*N*-methylpyrrolidinium bis(trifluoromethylsulphonyl) imide), carbon nanotubes and chlorpromazine in cyclic voltammetric measurements [16]. High performance liquid chromatography is commonly employed for fast electrochemical detection of vitamin C in human plasma samples [17] and it is reported that a detection limit as low as 1 μM was achieved by this method [18]. A vitamin C fluorescent sensor using a colloidal solution of lanthanide nanocrystals has recently been reported. When vitamin C was added to the colloidal solution, a concentration-dependent, linear increase of

fluorescence was observed [19]. The intensity of chemiluminescence for a system with a mixture of sodium hydrogen carbonate, hydrogen peroxide and CdSe/CdS quantum dots as the reagents was observed to be quenched, depending upon the concentration of vitamin C [20].

This article reports the results of *in situ* kinetic optical absorption measurements of novel thermotropic liquid-crystalline lutetium bisphthalocyanine sandwich complexes substituted with 16 octyl chains ($R_{16}Pc_2Lu$) to monitor their chemichromic changes owing to the presence of NADH and vitamin C (L-ascorbate) of varying concentrations in an aqueous medium of 1.5 M lithium perchlorate. The theme of this investigation stems from our recent work on exploitation of the electrochromic behaviour of bis[octakis(hexylthio)phthalocyaninato] dysprosium(III) spun films on indium tin oxide-coated glass substrates for *ex situ* detection of NADH having concentrations from 10^{-4} M to 5×10^{-3} M in phosphate buffer aqueous solutions [21]. *In situ* kinetic measurements have now provided us an additional insight into the mechanism of biorecognition of NADH and vitamin C by $R_{16}Pc_2Lu$ films peculiar to a redox state. The reduction rate of the oxidized $R_{16}Pc_2Lu$ is found to be linearly dependent upon the concentration of the cofactors. The method is reversible, offering detection limits in the region of 10^{-5} M for both NADH and vitamin C. It has been recently demonstrated that $R_{16}Pc_2Lu$ molecules are soluble in common volatile solvents and can, therefore, easily be solution-processed in thin film form on a solid substrate [22].

2. MATERIAL AND METHODS

The phthalocyanine compound $R_{16}Pc_2Lu$ used in this investigation, figure 1a, was prepared by reacting metal free 1,4,8,11,15,18,22,25-octakis(octyl)phthalocyanine with lutetium acetate in an octanol solution

using diaza(1,3)bicyclo[5.4.0]undecane as promoter. β -Nicotinamide adenine dinucleotide, reduced disodium salt (NADH of purity approx. 98%), L(+)-ascorbic acid (vitamin C) sodium salt (purity approx. 98%), lithium perchlorate (LiClO_4 ; purity approx. 95%) and bromine of reagent grade were procured from Sigma-Aldrich and their solutions were prepared in distilled water with resistivity of 18.2 M Ω cm to desired concentrations. The glass substrates were carefully cleaned in an ultrasonic bath (Ultrawave U50, UK) using distilled water, acetone and isopropanol in sequence, each for 15 min prior to the film deposition. The $\text{R}_{16}\text{Pc}_2\text{Lu}$ was dissolved in chloroform to produce a spreading solution having concentration of 10 mg ml $^{-1}$ for spin coating. A KW-4A spin coater from the Chemat Technology, Inc., USA, was employed to deposit the film by spinning 5 μl of the solution at 1000 r.p.m. for 30 s. The thickness of the resulting spun film on glass substrate was determined from an atomic force microscopic (AFM) image obtained using a Digital Instruments Dimension 3100 scanning probe microscope.

In order to investigate the interaction mechanism between the $\text{R}_{16}\text{Pc}_2\text{Lu}$ spun film and NADH, the as-deposited neutral $\text{R}_{16}\text{Pc}_2\text{Lu}$ spun films were oxidized by exposure to saturated bromine vapour for 2 min. The oxidized films were then reduced by NADH freshly dissolved in an aqueous solution of 1.5 M LiClO_4 in a 1 cm path length quartz cuvette. The kinetics of chemichromic modification of the $\text{R}_{16}\text{LuPc}_2$ spun films were monitored by recording spectral changes using a UV–visible spectrophotometer (Perkin Elmer LAMBDA 650) scanning at a rate of 654.92 nm min $^{-1}$ over the range 400–850 nm. Measurements were repeated at 19°C for NADH at different concentrations varying from 0.05 to 3 mM. The addition of LiClO_4 was to control the rate of reduction of $\text{R}_{16}\text{Pc}_2\text{Lu}$ film. Similar spectroscopic measurements were performed on oxidized $\text{R}_{16}\text{Pc}_2\text{Lu}$ spun films chemichromically modified by L(+)-ascorbic acid (vitamin C) of concentrations ranging from 0.03 mM to 3.5 mM.

3. RESULTS AND DISCUSSION

The AFM image in figure 1b shows that $\text{R}_{16}\text{Pc}_2\text{Lu}$ molecules formed a reasonably compact film for the spinning speed and the concentration of the spreading solution used. A similar non-uniform distribution of phthalocyanine molecules over the surface was observed for the as-deposited films of peripherally hexylthio-substituted liquid-crystalline lutetium bisphthalocyanine molecules [23]. Figure 1c shows the results of scanning the probe across a randomly selected area away from the film edge. Similar measurements were repeated at eight random areas and a value of 100 ± 14 nm was obtained for the mean thickness of the phthalocyanine film.

The Q-band arising from the phthalocyanine ligands appears at 718 nm in the absorption spectrum in figure 2a of the as-deposited neutral spun $\text{R}_{16}\text{Pc}_2\text{Lu}$ film. An extended Hückel molecular orbital model predicts the existence of doubly degenerate lowest unoccupied molecular orbital (LUMO) centred on the pyrrole and isoindole nitrogen. The interactions between

Figure 2. Electronic absorption spectra of $\text{R}_{16}\text{Pc}_2\text{Lu}$ as (a) neutral film, (b) Br_2 -oxidized film and (c) neutral species in solution. Inset: oxidation for 3 s (solid line) and oxidation for 7 min (dashed line).

the macrocyclic rings split the π highest occupied molecular orbital (HOMO) levels in the lanthanide sandwich complexes [24]. The relative energy distance between the bonding HOMO and LUMO levels is estimated to be 1.73 eV. The additional band at 653 nm is generally associated with the presence of a vibrational satellite [25]. The Q-band for the neutral $\text{R}_{16}\text{Pc}_2\text{Lu}$ molecules in chloroform solution in figure 2c is very sharp and blue shifted to 714 nm. This is due to the screening effect of the solvent preventing the aggregation of the molecules in solution [26]. This behaviour is consistent with the observations reported earlier for UV spectra of mesogenic octa-substituted lutetium(III) phthalocyanine derivatives. Small movements of the peak positions of one molecule relative to another were caused by different types of substituents. The red-shifted exciton for both molecules in the solid state is usually attributed to a staggered slipped stacking film structure involving inclined alignment of transition dipoles [27].

The spectrum in figure 2b shows a bathochromic shift of the principal Q-band to 778 nm after oxidation of the $\text{R}_{16}\text{Pc}_2\text{Lu}$ film with bromine. The following chemical reaction is believed to be responsible for oxidation [28]:

The broad band at 586 nm is indicative of a radical cation possibly owing to the presence of charge-transfer Br_2 – $\text{R}_{16}\text{Pc}_2\text{Lu}$ adducts [29]. Thus, the fully oxidized film contains $\text{R}_{16}\text{Pc}_2\text{Lu}$ as radical cations presumably balanced by Br^- anions trapped in the film. The inset shows the oxidation was nearly complete within 3 s. Exposure to Br_2 vapour for a period longer than 7 min did not produce additional oxidation states. A colour change from green to purple red was also visible on oxidation.

Figure 3. Plots of stability of Br₂-oxidized film of R₁₆Pc₂Lu after (a) 3 h, (b) 1 day, (c) 5 days, (d) 13 days of oxidation.

Figure 4. UV-visible absorption spectral changes as a function of time (indicated by arrows) during neutralization of R₁₆Pc₂Lu⁺ by water in the presence of a 1.5 M LiClO₄ aqueous solution. Data recorded after (a) 2, (b) 17, (c) 64, (d) 110, and (e) 198 min. Inset: plot of $\ln(A_t - A_\infty)$ versus time t in the inset shows an exponential time dependence of the instantaneous value A_t of absorbance at 778 nm in the form of

Figure 5. UV-visible absorption spectral changes as a function of time (indicated by arrows) of R₁₆Pc₂Lu⁺ in 3 mM NADH dissolved in a 1.5 M LiClO₄ aqueous solution. Data recorded after (a) 2, (b) 4, (c) 7, (d) 10 and (e) 90 min.

point observed at 763 nm in figure 4 indicates the charge-transfer band in the near infrared region associated with the free radical bisphthalocyanines [31]. The Q-band at 778 nm progressively decreased in intensity accompanied by an increase in the 718 nm band intensity. Similar electronic transitions were evident in photo-assisted reduction of lutetium bisphthalocyanines in the presence of thionyl chloride [32]. The linear plot of $\ln(A_t - A_\infty)$ versus time t in the inset shows an exponential time dependence of the instantaneous value A_t of absorbance at 778 nm in the form of

$$A_t = A_\infty + (A_0 - A_\infty) \exp(-k_{\text{obs}}t). \quad (3.1)$$

Taking the value of absorbance of the completely reduced film for A_∞ , the rate constant k_{obs} of reduction was estimated to be $2.8 \times 10^{-3} \text{ min}^{-1}$ from the slope of the graph, giving a value of 4 h for half-life of the oxidation.

R₁₆Pc₂Lu⁺ molecules became less stable in an aqueous solution with a smaller concentration of LiClO₄. Only 17 per cent of oxidized phthalocyanine was reduced in 1 h in the presence of 1.5 M LiClO₄ solution whereas reduction as large as 76 per cent took place in 0.7 M LiClO₄ over the same length of time. The oxidized film was reduced to a neutral state in water within 10 min.

3.1. Stability of oxidized film

It is important that the oxidation state of the R₁₆Pc₂Lu film is long lived for biorecognition of NADH and vitamin C. The degree of stability of the Br₂-oxidized R₁₆Pc₂Lu film was examined by monitoring the position of the Q-band for fully oxidized films stored in different environments. The film was kept in a closed container for about two weeks and UV-visible spectra in figure 3 recorded at different times exhibited no shift of the Q-band. This indicates that the neutralization process of the oxidized film is very slow in air. Similar slow reduction was observed for vacuum sublimed lutetium bisphthalocyanine films oxidized by bromine. It took at least two months for complete neutralization [30]. Figure 4 shows the spectral changes of the Br₂-oxidized film in 1.5 M lithium perchlorate (LiClO₄) aqueous solution over a period of more than 3 h. The isosbestic

3.2. In situ detection of nicotinamide adenine dinucleotide and vitamin C

In order to carry out controlled reduction reaction experiments for *in situ* detection of the cofactors, the aqueous solution containing 1.5 M LiClO₄ was chosen in preference to the aqueous solution alone as a medium for recording optical absorption in R₁₆Pc₂Lu⁺ film. Figure 5 shows the spectral changes of R₁₆Pc₂Lu⁺ film in the presence of 3 mM NADH in the 1.5 M LiClO₄ aqueous solution. The charge transfer is believed to have taken place between NADH and R₁₆Pc₂Lu⁺ in the following manner:

Figure 6. UV-visible absorption spectral changes as a function of time (indicated by arrows) of $R_{16}Pc_2Lu^+$ in 3.5 mM vitamin C in a 1.5 M $LiClO_4$ aqueous solution. Data recorded after (a) 1, (b) 3, (c) 5, (d) 7 and (e) 90 min.

and

Figure 6 shows the results of similar kinetic measurements of the oxidized film in the presence of 3.5 mM vitamin C in the 1.5 M $LiClO_4$ aqueous solution. As before, the presence of an isosbestic point at 763 nm was observed during reduction over 90 min for both cases.

Figure 7 shows the logarithm-linear plots of $(A_t - A_\infty)$ against t for 3 mM NADH and 3.5 mM vitamin C. The resulting graphs were found to be linear implying the validity of equation (3.1). Values of 4.3 and 4.8 min for the half-life were determined from the slope corresponding to neutralization by NADH and vitamin C, respectively. The reducing power of both redox agents can, therefore, be taken to be equal for practical purposes. Measurements were repeated for different lower concentrations of both NADH and vitamin C and the resulting plots in figure 8 of k_{obs} versus the concentration were found to be linear for both cofactors. The reduction process is, therefore, considered to be of first order [33]. The Lewis basicity of NADH and vitamin C became dominant in reducing the $R_{16}Pc_2Lu^+$ film to the neutral state over the ability of $LiClO_4$ to counteract the neutralizing effect of water molecules. The half-lives of the neutralization of the Br_2 -oxidized $R_{16}Pc_2Lu$ films in 1.5 M $LiClO_4$ became shorter, predictably showing the inverse dependence in the inset of figure 8 on the increasing concentrations of NADH and vitamin C. These observations are consistent with our Raman spectroscopic studies on the chemichromic changes in the redox state of drop cast films of $R_{16}Pc_2Lu$ using an excitation wavelength of 632.8 nm [34]. These films were oxidized by bromine vapour and then neutralized by 0.5 mM of NADH. The changes in relative intensities and positions of some bands are due to a change of electron distribution in the two macrocyclic rings.

It is also possible to use the dependence of the reduction rate on analyte concentration in figure 8 as a

Figure 7. Plot of $\ln(A_t - A_\infty)$ versus time for 3 mM NADH (dashed line) and 3.5 mM vitamin C (solid line) in a 1.5 M $LiClO_4$ aqueous solution.

Figure 8. Plot of k_{obs} versus concentration of NADH (dashed line) and vitamin C (solid line) in a 1.5 M $LiClO_4$ aqueous solution. Inset: dependence of half-life of the reaction ($t_{1/2}$) on concentration.

calibration curve for unknown concentrations of NADH and vitamin C. Using the same $R_{16}Pc_2Lu$ sample, the cycles of oxidation and reduction were repeated three times and the results were found to be reproducible. The detection limit of NADH and vitamin C was found to be 0.05 and 0.03 mM, respectively. This value for NADH is one order of magnitude smaller than one previously obtained with a spun film of dysprosium(III) derivative [21]. Vitamin C in the range 0.4–6.0 mM was recently detected using a probe of graphene nano-sheets immobilized on pyrolysed photoresist film. The detection limit was 0.12 mM [35]. Our results compare well with these published data. The detection requirement of plasma vitamin C in dialysis patients is reported to be high, up to 200 μM [36]. A biochemical sensor employing the $R_{16}Pc_2Lu$ films can easily be calibrated in terms of measurable half-life for dialysis applications in high level detection of vitamin C.

4. CONCLUSION

The solid thin film of electrochromic lutetium bisphthalocyanine $R_{16}Pc_2Lu$ was successfully employed for sensing the redox active biomolecules NADH and vitamin C. The study showed satisfactory response time, and linear concentration range up to two orders of magnitude: 0.05–3 mM for NADH and 0.03–3.48 mM for vitamin C. The addition of 1.5 M $LiClO_4$ was found to be satisfactory for supplying ClO_4^- ions in order to maintain a stable $R_{16}Pc_2Lu^+$ film in aqueous media for biorecognition. The reduction rate measured for the redox biomolecules was much faster than that of water in the presence of $LiClO_4$. The neutralization of the oxidized film in the presence of NADH and vitamin C was described in terms of first-order kinetics. The reducibility of both agents is believed to be similar. A reference plot of the rate constant versus concentration of NADH and ascorbic acid may be employed to determine an unknown concentration under similar ambient conditions. The method works well up to a concentration of 10^{-5} M and can be adapted for the development of practical biochemical devices to monitor NADH and vitamin C levels in plasma, serum, red cells, urine and other accessible tissues for biochemical and functional status.

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